

Study on heavy metal resistant fecal Coliforms isolated from industrial, urban wastewater in Arak, Iran

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ABSTRACT: In this essay, the heavy metal resistance patterns of bacterial strains isolated from industrial wastewater, domestic wastewater and various parts of the wastewater treatment system of Arak city have been studied. 28 intestinal bacterial strains were screened and identified as *Klebsiella*, *Escherichia coli*, *Citrobacter* and *Enterobacter* species biochemical methods. The minimum inhibitory concentration (MIC) against the various heavy metals including cadmium chloride, nickel sulfate, mercuric chloride, potassium chromate and potassium tellurite were determined using the agar dilution method and some antibiotics by disk method. In this study, for first time, we have found high MIC for *Klebsiella* sp., 22 mM (4032 µg/ml) for cadmium, 20 mM (3884 µg/ml) for chromium, 20 mM (6157 µg/ml) for telluride, 10 mM (2628 µg/ml) for nickel and 1mM (271.5 µg/ml) for mercury. Furthermore, the bacterial strain *Escherichia coli* DH5α was transformed with plasmid isolated from Cd²⁺ resistant *Klebsiella* sp. The same size of the plasmid was isolated from transformed *E.coli* DH5α and separated on 0.8% agarose gel electrophoresis. The curing was carried out by exposing cell culture overnight to Sodium Dodecyl Sulphate, which resulted in losing their resistance to cadmium, and therefore, the ability of *Klebsiella* sp. resistant to cadmium is plasmid mediated.

Key words: Intestinal Coliforms, Heavy metal resistant, Antibiotic resistance, Bioremediation, Plasmid curing, Plasmid transfer.

INTRODUCTION

Environmental pollution with toxic heavy metals is spreading throughout the world along with industrial progress. The important toxic metals cadmium, nickel find its way to the water bodies through wastewater (Ajmal *et al.*, 1998). Hazardous characteristics of the pollutants cause renal dysfunction, bone degeneration, liver, lungs and blood damage (Ebdon *et al.*, 2001). Heavy metals are metals with densities higher than 5 g/cm³ (Nies, 2000). Heavy metals in wastewater come from industries and municipal sewage, and they are one of the main causes of water and soil pollution. Cadmium is the most dangerous metal ion for human health due to its hazardous characteristics and non-biodegradability. It has been reported that cadmium damages the cells by a broad spectrum of effects on cell metabolism. It is known to bind with essential

respiratory enzymes (Nies., 2003). Cause oxidative stress (Banjerdikij *et al.*, 2005). And inhibits DNA repair (Jin *et al.*, 2003). Nickel is the most abundant heavy metal contaminants of the environment due to its release during mining and smelting practices (Prasad *et al.*, 2000). Nickel is required as an essential co-factor in several bacterial enzymes, which carry out metabolic functions (Mulrooney *et al.*, 2003). Chromium (VI) is the toxic form of chromium released during many industrial processes; including electroplating, leather tanning and pigment manufacture (Prasenjit *et al.*, 2005). Chromium (VI) is extremely toxic, mutagenic and carcinogenic which effects on biological systems. Toxic oxyanions of tellurite and chromate are abundantly found in industry wastes of tannery, electroplating, textile and the like. With high solubility, they penetrate the environment and underground waters, and have destructive effects on DNA and thiol

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groups of enzymes. In addition, they have harmful effects on health through liver necrosis, cancer (Groudeva *et al.*, 2001). The role of microorganisms in bioremediation is important because of their ability to degrade hazardous compounds into harmless ones. Coliform bacteria are often associated with enteric pathogenic organisms and have been shown to be useful indicators of the presence of fecal contamination. Coliform bacteria occur normally in the intestines of humans and other warm-blooded animals and are discharged in great numbers in human and animal waste. In polluted water, coliform bacteria are found in densities roughly proportional to the degree of fecal pollution. When members of the coliform group are present, other kinds of microorganisms capable of causing disease also may be present. The coliform group includes all aerobic and facultatively anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria which ferment lactose with gas production in prescribed culture media within 48 hours at 35 °C.

Coliform bacteria include *Escherichia coli*, *Citrobacter*, *Enterobacter*, and *Klebsiella* species. Bacterial resistance to metal ions is frequently determined by plasmids, which in many cases encode resistance to antibiotics (Edward *et al.*, 2009). This resistance could also be chromosomal. Some heavy metal resistance determinants move from plasmid to chromosome (or in the reverse direction). This makes plasmid encoding heavy metal resistance an important aspect of environmental research. The plasmid can be the source of resistance genes for cloning purpose which have potential use in biotechnology such as the manufacture of biosensors and bioremediation processes (Collard *et al.*, 1994). This study aimed to isolate and identify heavy metal resistant fecal coliforms bacteria from wastewater treatment plant in Arak city one of industrial cities also, we identified plasmid or chromosome mediated determinants of *Klebsiella pneumoniae* confer resistance to cadmium heavy metal and ampicillin antibiotic.

MATERIALS & METHODS

The sampling area was the wastewater treatment plant in Arak city, Iran. Seven samples collected from industrial wastewater, domestic wastewater, entry of treatment plant, anaerobic pond, facultative pond 1 and facultative pond 2.

The output of treatment plant where biological treatment of wastewater had taken place, samples were collected in sterile glassy bottles from the surface of them. Total of seven samples were selected for the study. Fecal coliforms isolated from wastewater with Ec broth media and incubated at 37 °C for 24-48 h.

After the incubation period the plates were evaluated for any kind of growth on the media. The isolated and distinct colonies on these selective media were subculture repeatedly on the same media for purification. The pure culture was identified on the basis of their morphology and biochemical characters.

The isolated bacteria have been grown on EMB agar (Merck) and MacConkey agar (Merck). The shape and color of colonies were observed and bacterial cells were examined under the microscope after Gram staining. The isolates were identified by biochemical methods including oxidase, catalase, urease, H₂S and Indole production, utilization of glucose and sucrose and MRVP, citrate and motility tests. These tests were performed to identify and characterize bacterial isolates according to procedures described in (Riazul *et al.*, 1999).

Maximum resistance of the isolated bacteria against heavy metals were determined by gradually increasing the concentration of Cd²⁺, Hg²⁺, Ni²⁺, CrO₄²⁻ and TeO₃²⁻ in BHI agar plates until the strains were unable to give colonies on the plates. The initial concentration used was 0.5 up to 22 mM. The culture growing on the last concentration was transferred to the higher concentration by streaking on the plates. The minimum inhibitory concentration (MIC) was noted when the isolates failed to grow on plates even after ten days of incubation.

The various bacterial isolates were tested for antibiotic sensitivity by growing these bacteria on Muller Hinton agar (Merck) plates with antibiotic discs (Himedia) of Amikacin (30 ì g/Disc), Ciprofloxacin (5 ì g/Disc), Gentamycin (10 ì g/Disc), Tobramycin (10 ì g/Disc), Sulfamethoxazol, Tetracycline (30 ì g/Disc), Ampicillin (10 ì g/Disc), Chloramphenicol (30 ì g/Disc), and Cefixime (5 ì g /Disc) at 37 °C for 24 h. Formation of an inhibition zone around the discs was taken as the indicator of bacterial sensitivity to antibiotics.

Plasmids were isolated according to (Holmes *et al.*, 1984). The size of the plasmids was determined by agarose gel electrophoresis.

The plasmids were used to transform *E. coli* DH5 α for propagating the plasmid. Transformation procedure was performed as described by Sambrook (Sambrook *et al.*, 2007). Bacteria has been transformed were screened on selective medium plates containing 200 ì gCd/ml.

The curing of the plasmids was conducted by using SDS solution (10% w/v, pH 7.4) which added to BHI broth (double strength) and the final volume was made up to 10 mL in bottles with distilled H₂O to give 5, 4, 3, 2, 1 and 0.5% SDS in the BHI (normal strength).

An inoculum of 100 µl (1.2832 × 10⁹ cells/mL) of the wild-type was then used to seed the BHI broth containing SDS and the cultures were incubated overnight with shaking at 27 °C (Mansi *et al.*, 2000).

Samples were taken out of the medium, diluted with BHI broth and spread on plates to get well-separated colonies. Isolated colonies were transferred simultaneously onto grids made on BHI agar medium plates with and without 100 µg Cd/ml. The colonies appearing on BHI agar plates without Cd were checked for their antibiotic resistance to ascertain curing of the plasmids and transformation.

RESULTS & DISCUSSION

In this study, intestinal bacterial strains including *Escherichia coli*, *Klebsiella*, *Citrobacter* and *Enterobacter* species were isolated from effluent samples above and the minimum inhibitory concentration (MIC) were determined against the various heavy metals including cadmium chloride, nickel sulfate, mercuric chloride, potassium chromate and potassium tellurite using the agar dilution method and antimicrobial resistance patterns by disk method. (Table 1 and 2)

In this study, isolated coliforms from industrial waste water with high MIC 22mM (4032 µg/ml) than cadmium about *K. pneumonia* that it is 5.5 fold higher than MIC reported by Filali and et al (733 µg/ml & 4 mM) (Filali *et al.*, 2000) and 20 fold higher than MIC reported by Karbasizaed and et al (200 µg/ml) (Karbasizaed *et al.*, 2003). And 1.5 folds higher than MIC reported by Sharma and et al (15 mM) (Sharma *et al.*, 2000). also 20 mM MIC (3884 µg/ml) than chromium,

in *Klebsiella* sp., *E. coli*, *Citrobacter* sp. that are respectively 26, 19, 17.5 fold higher than MIC reported by Rajbanshi and et al (Rajbanshi *et al.*, 2008) and 20 mM (6157 µg/ml) than telluride, 10 mM (2628 µg/ml) than nickel and 1mM (271.5 µg/ml) than mercury, were reported for *Klebsiella* sp.

K. pneumonia revealed he most resistant species to all of the metals tested, followed by the *E. coli*, *Citrobacter* sp. and *Enterobacter* sp.

The microorganisms resistant to antibiotics and tolerant to heavy metals might be result in exposure to metal contaminated environment that cause co-incident selection for resistance factors for heavy metals and antibiotics. *Klebsiella* species showed that resistance to a variety of toxic, heavy metals and antibiotics. Hence they have generated a high degree of interest in the area of environmental bioremediation (Bruins *et al.*, 2003).

Most resistance patterns are related to bacteria in industrial wastewater that it dues to the permanent contact these bacteria with heavy metals. High levels of metals in industrial wastewater treatment causes microorganisms among fecal coliforms making resistance mechanisms that leading to selection of resistant strains with high ability for tolerance of metal toxicity, on the other hand resistant bacteria can with the transfer of genetic elements to other strains increase the number of resistant bacteria (Sabry *et al.*, 1997) (Fig. 1).

Although, the number of coliforms in domestic waste are more than industrial wastewater, but they

Table 1. Antimicrobial resistance patterns of coli forms isolates from the industrial wastewater

Bacteria strain	<i>Klebsiella</i>	<i>Escherichia coli</i>	<i>Citrobacter</i>	<i>Enterobacter</i>
Amikacin (30 µg /Disc)	Sensitive	Sensitive	Sensitive	Sensitive
Ciprofloxacin (5 µg /Disc)	Sensitive	Sensitive	Sensitive	Sensitive
Gentamycin (10 µg /Disc)	Sensitive	Sensitive	Sensitive	Sensitive
Tobramycin (10 µg /Disc)	Sensitive	Sensitive	Sensitive	Sensitive
Sulfamethoxazol	Resistance	Resistance	Sensitive	Sensitive
Tetracycline (30 µg /Disc)	Resistance	Sensitive	Resistance	Resistance
Ampicilin (10 µg /Disc)	Resistance	Resistance	Resistance	Resistance
Chloramphenicol (30 µg /Disc)	Sensitive	Sensitive	Sensitive	Resistance
Cefixime (5 µg /Disc)	Sensitive	Resistance	Resistance	Resistance

Table 2. Antimicrobial resistance patterns of coliforms isolates from the domestic wastewater

Bacteria strain	<i>Klebsiella</i>	<i>Escherichia coli</i>	<i>Citrobacter</i>	<i>Enterobacter</i>
Amikacin (30 µg /Disc)	Sensitive	Sensitive	Sensitive	Sensitive
Ciprofloxacin (5 µg /Disc)	Sensitive	Sensitive	Sensitive	Sensitive
Gentamycin (10 µg /Disc)	Sensitive	Sensitive	Sensitive	Sensitive
Tobramycin (10 µg /Disc)	Sensitive	Sensitive	Sensitive	Sensitive
Sulfamethoxazol	Resistance	Resistance	Sensitive	Sensitive
Tetracycline (30 µg /Disc)	Resistance	Sensitive	Resistance	Sensitive
Ampicilin (10 µg /Disc)	Resistance	Resistance	Resistance	Resistance
Chloramphenicol (30 µg /Disc)	Sensitive	Sensitive	Sensitive	Resistance
Cefixime (5 µg /Disc)	Sensitive	Resistance	Resistance	Resistance

Heavy Metal Resistant Coliforms

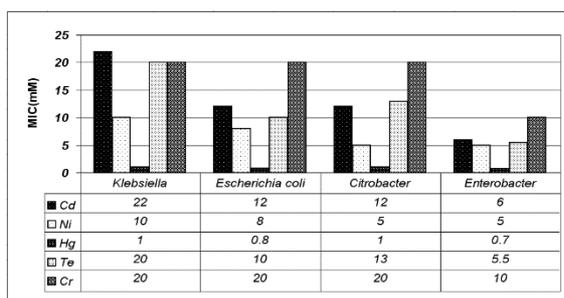


Fig. 1. Minimum inhibitory concentration (MIC), heavy metals for coliforms in industrial wastewater. Cd: cadmium chloride, Ni: nickel sulfate, Hg: mercuric chloride, Te: tellurite potassium, Cr: potassium chromate

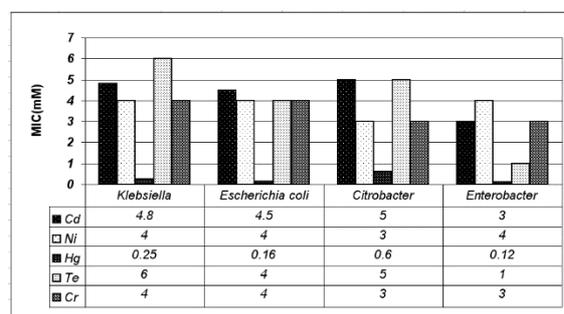


Fig. 2. Minimum inhibitory concentration (MIC), heavy metals for coliforms in domestic waste

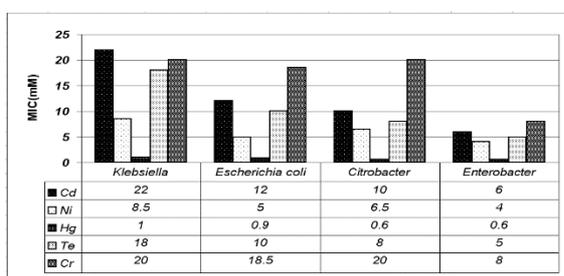


Fig. 3. Minimum inhibitory concentration (MIC), heavy metals for coliforms in entry of wastewater treatment plant

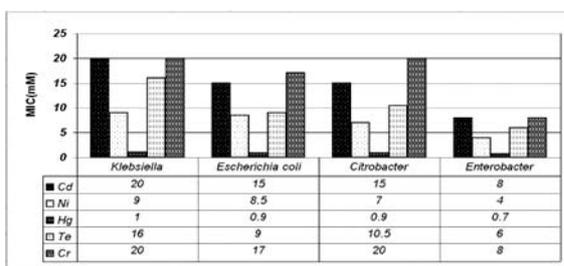


Fig. 4. Minimum inhibitory concentration (MIC), heavy metals for Coliforms in anaerobic pond

have been shown less resistance to heavy metals because in wastewater bacteria have been less in contact with heavy metals (Fig. 2). Bacteria isolated from entry of treatment plant showed high resistance pattern to heavy metals because in this place, domestic

and industrial wastewater have been mixed together (Fig. 3). The Results of MIC for other places of treatment plant are presented in figs. 4, 5, 6 and 7. The results of measurement the amount of heavy metals in Arak wastewater treatment plant showed that

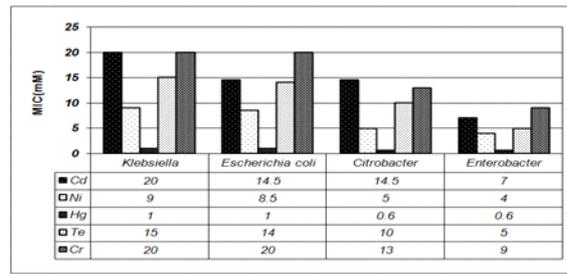


Fig. 5. Minimum inhibitory concentration (MIC), heavy metals for Coliforms in facultative pond 1

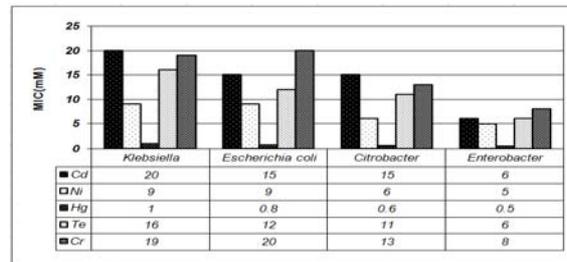


Fig. 6. Minimum inhibitory concentration (MIC), heavy metals for coliforms in facultative pond 2

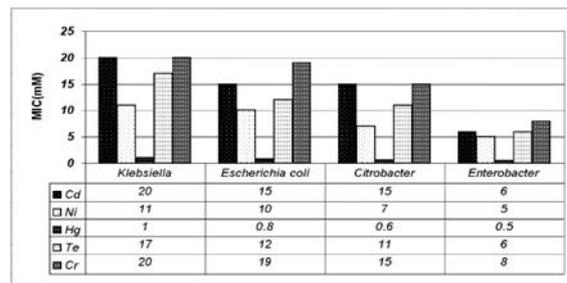


Fig. 7. Minimum inhibitory concentration (MIC), heavy metals for coliforms in wastewater treatment plant output

Table 3. Standard of quality in the case of heavy metals for Iran and America EPA about the effluent discharged to surface waters

Contaminants	Unit	Iran standard	EPA1
Cadmium	mg/l	0.05	0.05
Nickel	mg/l	2	2.38
Chromium	mg/l	2	1.71
Mercury	mg/l	<0.01	<0.01

Table 4. Concentration of heavy metals in entry and output wastewater of Arak city wastewater treatment plant

Contaminants	Unit	Entry of treatment plant	Output of treatment plant
Cadmium	mg/l	0.02	<0.01
Nickel	mg/l	0.25	0.1
Chromium	mg/l	0.1	0.06
Mercury	mg/l	>0.01	<0.01

the amount of them in output waste water for cadmium, nickel, chromate, mercuric are <0.01, 0.1, 0.06, <0.01 mg/lit, respectively, and the concentration of these heavy metals in entry of treatment plant are 0.02, 0.25, 0.1 and <0.01 mg/lit (table 4). Comparison of these

results with environmental standards of Iran and BPT standards related to US-EPA (table 3) shows that in all of the samples, heavy metals concentration are lower than EPT standard, while there are many workshops and industries in the Arak city that can be cause of

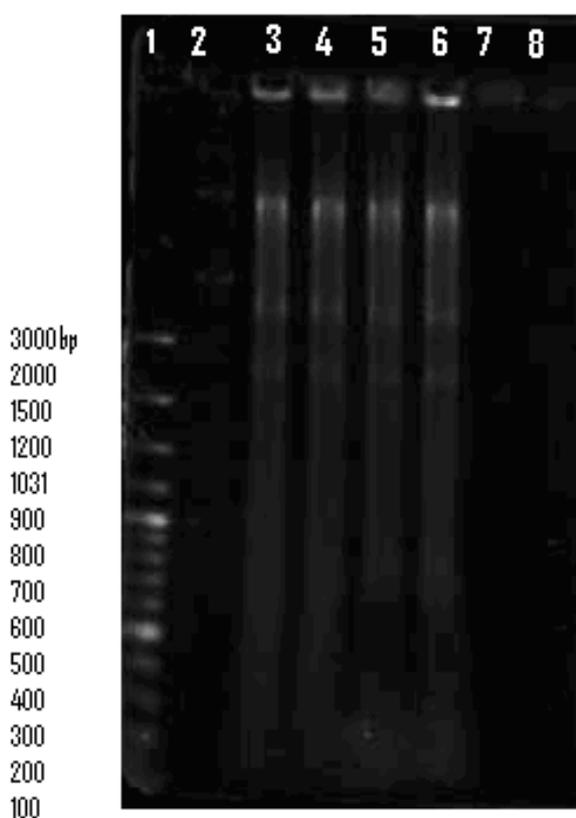


Fig. 8. Agarose gel electrophoresis picture showing line 1= ladder SMO321, line 2= Pet 32 plasmid (4900bp), Lane3= Plasmid profiles of *Klebsiella pneumoniae* isolated from industrial waste water, Lane4=entry of Wastewater treatment plant, Lane5= output of Wastewater treatment plant, line 6= Transformed *E. coli* DH5 α , line7= *E. coli* DH5 α before Transformation, line8= cured *Klebsiella pneumoniae* by SDS

entry heavy metals in urban wastewater, and we expected that heavy metals concentration to be at high level in entry of treatment plant but they were not. This might due to the unauthorized disposal of factories sewage or seasonal closure of workshops in the city.

In the present study, the plasmid DNA was isolated from *K. pneumoniae* size of this plasmid was approximately 4.9 kb and *E. coli* DH5 α (without any plasmids) was transformed with this plasmid *K. pneumoniae*. The similar size of plasmid DNA in *E. coli* DH5 α after transform was identified same size as, which is shown in Fig. 8. The similar size, Curing of plasmid was carried out with SDS. Plasmid curing was achieved only by growing the strain treatment with SDS. A plasmid isolated from *Klebsiella* sp. was treated with 10 % SDS leads to loss of a plasmid.

CONCLUSIONS

The strains isolated from industrial waste water were more resistant to heavy metals and antibiotics than strains from domestic wastewater and various parts of the wastewater treatment system.

Furthermore, we have found that some *K. pneumoniae* strains isolated from waste water have a conjugative plasmid (< 4.9 kb) encoding resistance to antibiotics and heavy metals. (Ghosh *et al.*, 2000). Wild type *K. pneumoniae* isolated from waste water and transformed *E. coli* DH5 α , were resistant to cadmium and ampicillin, but cured *K. pneumoniae* (remove plasmid by curing process) and wild type *E. coli* DH5 α (without any plasmid) were sensitive to cadmium and Ampicillin. From this data, the genes encoding resistance to cadmium and ampicillin are located on the plasmid and resistance genes can be transferred from these organisms to other enteric organisms.

According to the isolated of coliform bacteria strains with very high resistance to heavy metals, we hope this strain be suitable selection for bioremediation industrial wastewater from heavy metals such as cadmium, nickel, mercury and toxic oxyanions of tellurite and chromate. Bioremediation process is a suitable method for remove of heavy metals from wastewater and its cost is much less than the chemical method.

Technology of bioremediation is still in early stages of development; it is expected reduced cost and improved performance but for extensive use is required to more researches. We hope, with promote of this cheap technology, taken an important step to improving the quality of the environment.

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