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Isolation and Molecular Identification of Heavy Metal Resistant Bacteria from Khoshk River in Shiraz, Iran

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Abstract

Heavy metal release is a serious threat to public health because of its persistence in the environment. One of the best ways to remove heavy metals is to use resistant bacteria to metals. The current study was aimed to isolate and identify heavy metal resistant bacteria from the wastes of the Khoshk River in Shiraz, Iran. First, water and sediment samples were collected from stations which had the highest prospect of entering hospital and industrial wastewater in the Khoshk River. The six isolates were selected based on heavy metal resistance. Isolates were identified by morphological and biochemical characteristics and 16S rRNA gene sequencing. The minimal inhibitory concentration for isolates against cadmium, nickel, cobalt, mercury, chromium, zinc, iron and lead was determined. These isolates included *Staphylococcus epidermidis* (R1), *Bacillus subtilis* (R2), *Escherichia coli (R3), Pseudomonas aeruginosa* (R4), *Proteus mirabilis* (R5) and *Proteus vulgaris* (R6). *Pseudomonas aeruginosa, Proteus mirabilis, Proteus vulgaris, Escherichia coli* and *Bacillus subtilis* were shown the highest resistance to mercury and lead. Also, all isolates were resistance to antibiotics Tetracycline and Streptomycin. Therefore, co-resistance of bacteria to both antibiotic and heavy metals was detected in the strains isolated from Khoshk River in Shiraz. The resistance of bacteria against heavy metals may offer a beneficial tool for monitoring of many pollutants in the environment. Thus, these bacterial isolates can be used for the remediation of metals from the natural ecosystems in Iran.

Keywords: *Heavy metals, Khoshk river, Resistant bacteria, 16S rRNA*

Introduction

Heavy metals are defined as compounds with an atomic density higher than 4 g/cm3, or 5 times higher than water (Paul and Sinha, 2015). They are toxic, non biodegradable in the environment, and stored in living organisms (Raghav and Shrivastava, 2016). Accumulations of heavy metals in human tissues and organs have been reported to lead in cardiovascular, nervous system problems, kidney, and bone diseases (Kumar *et al.,* 2011; Sardar *et al.,* 2013). Therefore, it is of absolute importance to prevent contamination or to decontaminate the environment. The process of using microorganisms to decontaminate polluted soil, water or air is called Bioremediation (Murthy *et al.,* 2012), which denotes the procedure of

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breaking down or transforming toxic elements materials into simple nontoxic materials by biological treatments (Murthy *et al.,* 2012). Many organisms have a natural capability to biosorb toxic metals (Abougabal *et al.*, 2018).

Basic mechanisms applied in heavy metal resistant bacteria are metal absorption, metal accumulation outside the cell membrane, mineralization, enzymatic oxidation or reduction and transport of heavy metals from the cell (François *et al.,* 2012; Monteiro *et al.,* 2012). Some of these mechanisms have been recognized as responsible for changing normal cell physiology leading to the development of drug resistance in bacteria (Garhwal *et al.*, 2014).

Rivers act as reservoirs for the resistant bacteria and proliferate their resistant genes to other bacteria and organize communities of heavy metal and antibiotic resistant bacteria (Harris *et al.,* 2012). The Khoshk River passes through Shiraz city, southwest Iran (Moore and Salati, 2006). It flows through populated urban areas of Shiraz and transports different industrial and urban solid and liquid wastes produced by industries and domestic sewage (Moore and Salati, 2006). Another major source of pollution for the Khoshk River is the Namazi hospital, one of the largest health-care facilities in the Fars Province, Iran (Jabbra and Jabbra, 1997). The increasing accumulation of industrial wastes and hospital pollutants generate different contaminations in the Khoshk River, which are dangerous for the ecosystem. The purpose of the present study is to isolate and characterize heavy metal resistant bacteria from water and sediment samples in Khoshk River which can be used for bioremediation of toxic metals from the polluted areas in Iran.

Materials and methods

This study was conducted in 2018 to identify the heavy metal resistant bacteria from Khoshk River in Shiraz. Water and sediment samples were collected from different parts of the Khoshk river, especially from the stations where hospital pollutants and industrial wastewater were introduced. The samples were collected and placed in a sterile plastic container and transported to the laboratory where they were conserved at -4°C for later use. The concentration of heavy metals including cadmium, nickel, cobalt, mercury, chromium, zinc, iron and lead was quantified using a flame atomic absorption spectrophotometer (Perkin Elmer 800, USA).

0.1 ml of samples were cultured on Luria Bertani (LB) agar plates (HiMedia, India) supplemented with 5 mg/L of each the following metals: cadmium (Cd), nickel (Ni), cobalt

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(Co), mercury (Hg), chromium (Cr), zinc (Zn), iron (Fe) and lead (Pb), respectively, by the standard pour plate method (Gandhi *et al.,* 2015). Plates were incubated at 35°C for 48 h and colonies were selected by morphology. Then, the colonies were purified by the streak method on LB agar plates containing heavy metals (Chihomvu *et al.,* 2014).

The six isolates were selected based on heavy metal resistance. The shape and colors of the colonies were evaluated by light microscope after gram staining. Also, isolates were considered by biochemical tests such as oxidase, catalase, methyl red, voges-proskauer, indole production, H2S production, motility, triple sugar iron reaction, urea hydrolysis, citrate utilization and carbohydrate utilization according to Bergey's Manual of systemic Bacteriology, the isolates were recognized up to genus level ((Barrow and Feltham, 1993).

Then, genomic DNA was extracted from all the 6 isolates by using the bacterial DNA Extraction Kit (YektaTajhiz, Iran) according to the manufacturer's protocol. The 16SrRNA fragments were amplified using the universal primer combination 27F 5'AGA GTT TAG TCCTGG CTC AG 3' and 1492R 5'GGTTAC CTTGTTACGACT T 3' (Merck Millipore, India) (Chihomvu *et al.,* 2014). Amplification was performed in a 25μL reaction mixture containing 2x PCR master mix (Sina gene, Iran), 17 μL of PCR quality water, 1 μL of each forward and reverse primer (10 pmol), 2 μl DNA template, 0.75 μL MgCl2 (1.5 mM), 0.5 μL dNTPs, 0.25 μL Taq polymerase and 2.5 μL buffer. PCR was performed in a thermocycler (Bio-Rad, Hercules, CA). Thermal cycling conditions were as follows: initial denaturation at 95°C for 4 min followed by 30 cycles consisting of denaturation 95°C for 20 sec, annealing at 58°C for 15 sec, extension at 72°C for 15 sec and a final extension at 72°C for 2 min. PCR products were analyzed in an electrophoresis system and sent for sequencing to Macrogen Company in Korea. The 16SrRNA sequences were aligned and compared with known nucleotide database in the GenBank by using the National Center for Biotechnology Information (NCBI) and Basic Local Alignment Search Tools, BLAST program (Jyothi *et al.,* 2012).

Stock solutions (1M) of cadmium chloride, potassium dichromate, lead chloride, iron sulfate, zinc chloride, copper sulfate, nickel chloride and mercury chloride were prepared with deionized water and sterilized by autoclaving at 121°C for 15 min. The minimum inhibitory concentration (MIC) of the selected isolates was determined against increasing concentrations of Cd, Ni, Co, Hg, Cr, Zn, Fe and Pb on LB agar plates until no growth was observed (Gandhi *et al.,* 2015). Starting with an initial concentration of 0.05mM, further MIC tests were carried out with concentrations of 0.1mM, 0.2mM, 0.4mM, 0.6mM and 0.8mM.

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Cultures that showed growth at a particular concentration were transferred to the next higher concentration.

The antibiotic susceptibility test was performed using disk diffusion method on Mueller-Hinton agar using commercial discs. The antibiotics tested were: Ampicillin (10µg/disk), Tetracycline (30µg/disk), Kanamycin (5µg/disk), Erythromycin (10µg/disk), Streptomycin (10µg/disk), Nalidixic acid (30µg/disk), Vancomycin (30µg/disk), Cephalotin (30µg/disk), Co-Trimoxazole (25µg/disk) and Chloramphenicol (30µg/disk) (Hi-media, India). One hundred microliters of fresh bacterial cultures were spread on Mueller-Hinton agar. The antibiotic's discs were placed on the plate. The plates were incubated at 35ºC for 24 h and observed for inhibition zones. Strains were considered susceptible when the inhibition zone diameter was higher than 12mm (Baquero *et al.,* 1998).

Results

The analysis of samples collected from Khoshk River showed the presence of cadmium, nickel, cobalt, mercury, chromium, zinc, iron and lead. Then, at the later stage, isolation of heavy metal resistant bacteria was carried out in the culture media containing heavy metals. The Six bacteria that had shown the significant resistance to heavy metals were purified. These microorganisms were selected based on their differential colony characteristics and were coded R1 to R6 respectively. The R1 isolate was a gram positive bacterium and coccus in shape (Figure 1a). The R2 isolate was gram positive and rod shaped (Figure 1b). R3-R6 isolates were gram negative and rod shaped (Figure 1c-f). Therefore, as it could be observed resistance to heavy metals was detected in most of the isolates, either from gram-positive and/or gram-negative genera. Table 1 shows biochemical characteristics in bacterial isolates.

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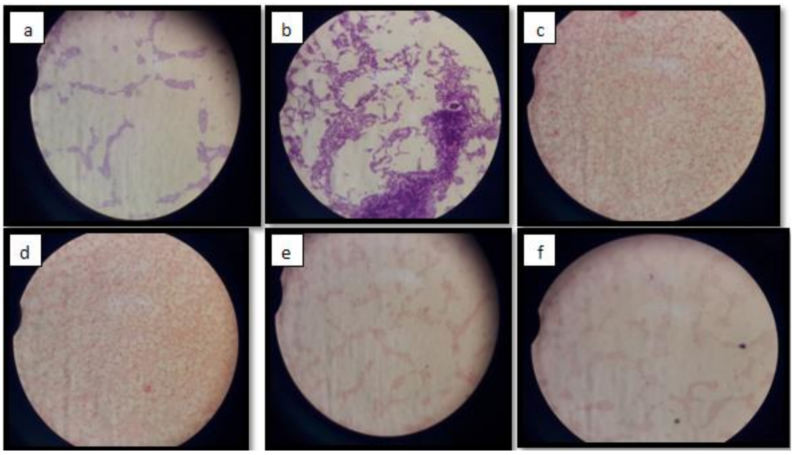

Figure 1. Light microscope images (×100) of bacterial strains : a)R1, b)R2, c)R3, d)R4, e)R5 and f)R6

Table 1. Biochemical characteristics of bacterial isolates

Characters	R1	R2	R3	R4	R5	R6
Catalase	+	+	+	+	+	+
Oxidase	-	+	-	+	-	-
Motility	-	+	+	+	+	+
Methyl red	-	-	+	-	+	+
Voges proskauer	+	+	-	-	-	-
Indole production	-	-	+	-	-	+
H2S production	+	-	-	-	+	+
Triple sugar iron reaction	A/A	A/A	A/A, gas	Alk/Alk	Alk/A	Alk/A
urea hydrolysis	+	-	-	-	+	+
citrate utilization	-	+	-	+	+	-
carbohydrate utilization						
Glucose	+	+	+	+	+	+
Maltose	+	+	-	-	-	-
Lactose	+	-	+	-	-	-

+ positive result; - negative result

PCR amplification of 16S rRNA gene produced fragments of approximately 1506 base pairs in size for the six bacterial isolates (Fig 2). Molecular characterization using 16S rRNA gene sequencing showed that the isolates R1-R6 had the maximum similarity with: *Staphylococcus epidermidis* strain 4S02, *Bacillus subtilis* strain TSA38, *Escherichia coli* strain FHI, *Pseudomonas aeruginosa* strain RSP8, *Proteus mirabilis* strain FA-9 and *Proteus vulgaris* strain CUMBPV 01-A1, respectively (Figure 3).

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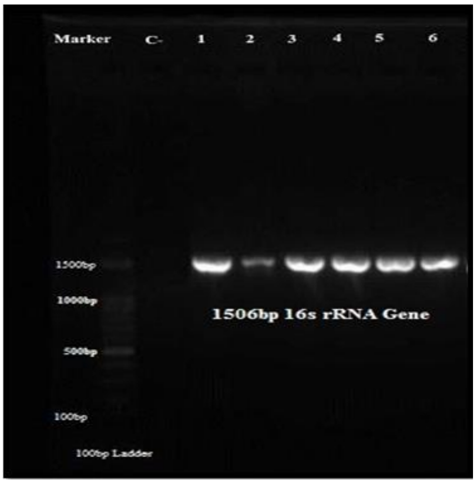

Figure 2. PCR amplicons of 16S rRNA genes in six bacteria isolated from Khoshk river. Lane A: Ladder; lane B: negative control, Lane 1: R1, Lane 2: R2, Lane 3: R3, Lane 4: R4, Lane 5: R5, Lane 6: R6

Figure 3. 16S rRNA Sequences in isolates R1 (*Staphylococcus epidermidis*) (a), R2 (*Bacillus subtilis*) (b), R3 (*Escherichia coli*) (c), R4 (*Pseudomonas aeruginosa*) (d), R5 (*Proteus mirabilis*) (e) and R6 (*Proteus vulgaris*) (f).

The Fe, Hg, Pb, Zn, Cd, Ni, Cr and Co concentrations used during screening ranged from 0.05 – 1 mM. Each isolate differed in their MIC values for different metals but the general order of resistance to the metals was found to be as Pb > Hg > Ni > Co > Cr > Cd >

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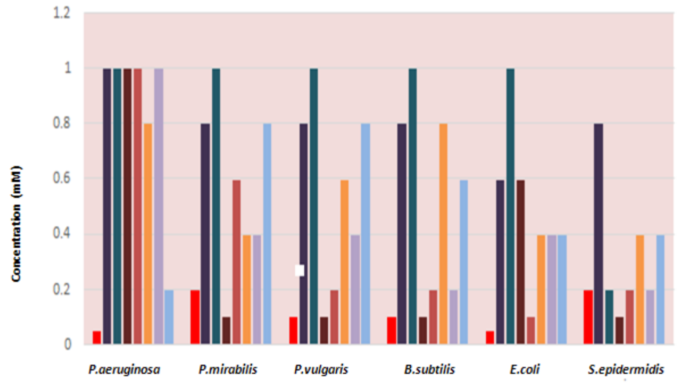
Zn > Fe. Bacterial isolates showed high resistance to heavy metals except for Fe and Zn. The results indicate that these strains are multi-resistant. *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Proteus vulgaris* were shown the highest resistance to heavy metals. *Pseudomonas aeruginosa* had MIC of 1 mM for mercury, lead, zinc, cadmium and chromium. Also, *Proteus mirabilis*, *Proteus vulgaris*, *Escherichia coli* and *Bacillus subtilis* had MIC of 1 mM for lead. *Proteus mirabilis* and *Proteus vulgaris* had MIC of 0.8 mM for mercury and cobalt (Figure 4).



	P.aeruginosa	P.mirabilis	P.vulgaris	B.subtilis	E.coli	S.epidermidis
Fe	0.05	0.2	0.1	0.1	0.05	0.2
Hg	1	0.8	0.8	0.8	0.6	0.8
Pb	1	1	1	1	1	0.2
Zn	1	0.1	0.1	0.1	0.6	0.1
Cd	1	0.6	0.2	0.2	0.1	0.2
Ni	0.8	0.4	0.6	0.8	0.4	0.4
Cr	1	0.4	0.4	0.2	0.4	0.2
Co	0.2	0.8	0.8	0.6	0.4	0.4

Figure 4. MICs for heavy metal resistant bacteria isolated from Khoshk River

Furthermore, the isolates were shown different degrees of resistance to antibiotics (Table 2). All the isolates were resistant to Tetracycline and Streptomycin. Also, gram-negative bacteria were resistant to Erythromycin, Vancomycin and Co-Trimoxazole. *Pseudomonas aeruginosa* showed resistance to all the existing antibiotics except Ampicillin.

Table 2. Antibiotic sensitivity profiles of heavy metal resistant bacteria isolated from the Khoshk River

Strains / Antibiotics	*Staphylococcus. epidermidis*	*Bacillus . subtilis*	*Escherichia. coli*	*Pseudomonas. aeruginosa*	*Proteus. mirabilis*	*Proteus. vulgaris*
Ampicillin	S	R	R	S	S	R
Tetracycline	R	R	R	R	R	R
Kanamycin	S	S	S	R	S	R
Erythromycin	S	S	R	R	R	R
Streptomycin	R	R	R	R	R	R

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Nalidixic acid	R	S	S	R	S	R
Vancomycin	S	S	R	R	R	R
Cephalotin	S	R	R	R	S	S
Co-Trimoxazole	S	R	R	R	R	R
Chloramphenicol	S	S	S	R	S	R

R=Resistance, S = Sensitive.

Discussion

Bacteria may be able to tolerate certain levels of heavy metal concentrations in their contaminated environments, such bacteria can be used for heavy metal removal from polluted habitats (Mihdhir *et al.,* 2016). Being an industrial city, Shiraz in southern west of Iran is facing pollution problems. Heavy metals are discharged in the Khoshk River of Shiraz from industrial and domestic sewage systems and hospital waste materials. This study is mainly focued on isolating and identifying the bioremediating bacteria from the Khoshk River that can help the improvement of agricultural and residential environments. Based on data collected for this purpose, three (50%) of the total 6 identified isolates belong to the *Enterobacteriaceae* family. The isolates were closely related to *Staphylococcus epidermidis*، *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis* and *Proteus vulgaris***.**

Similarly results were found in different studies in Iran indicating that *Pseudomonas aeruginosa* and *Bacillus* were the most resistant bacteria to heavy metals (Kasra Kermanshahi *et al.,* 2007; Shakibaie *et al.,* 2008; Kafilzadeh and Saberifard, 2016). Also, heavy metal resistant bacteria isolated from rivers in northern Pakistan were related to *Escherichia coli, Pseudomonas* and *Proteus* (Sair and Khan., 2018). Whereas in other countries, certain genera of microorganisms such as *Citrobacter, Thiobacillus, Bacillus, Pseudomonas, Micrococcus, Acinetobacter*, *Ochrobactrum* and *Arthrobacter* isolated from contaminated soil with heavy metals were demonstrated to reduce environmental pollution (Mohamed, 2016; Qayyum *et al.,* 2016; Andriamafana *et al.,* 2018).

Pseudomonas aeruginosa, Proteus mirabilis, *Proteus vulgaris*, *Bacillus subtilis* and *Escherichia coli* showed the highest resistance to mercury and lead. Similarly, Kasra Kermanshahi *et al*. (2007) showed that the most abundant type of bacteria resistant to lead was *Bacillus* in the soils of Isfahan province, Iran. Also, Chihomvu *et al*. (2014) demonstrated that *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Proteus* can tolerate high concentrations of heavy metals. Kacar and Kocyigit (2013) showed that

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Bacillus strains isolated from Eastern Aegean Sea in Turkey were highly resistant to Pb but were sensitive to Hg. In gram-negative enteric bacteria, mercuric resistance genes are often located on plasmids and are associated with transposons (Yazdankhah *et al.,* 2018). The mechanism of resistance to mercury in gram-positive bacteria is broadly similar to that in gram-negative bacteria (Hobman and Crossman, 2014). The main mechanisms of lead resistance participate adsorption by extracellular polysaccharides, cell exclusion and ion transport to the cell exterior (Naik and Dubey, 2013; Jaroslawiecka and Piotrowska-Seget, 2014). Also, studies by Naik and Dubey (2011) revealed that the production of siderophores by *Pseudomonas aeruginosa* may play a role in response to lead exposure. Furthermore, efflux pumps in *Staphylococcus, E. coli* and *Pseudomonas* transport lead to the periplasm (Jarosławiecka and Piotrowska-Seget, 2014).

The antibiotic resistance of the isolates in this study may be due to the presence of antibiotics in the Khoshk River. Tetracycline and Streptomycin are broad spectrum antibiotics which inhibit both gram- positive and negative bacteria (Chihomvu *et al.,* 2014). However, in the present study all isolates were resistant to these drugs. Similarly, resistance against highly important antibiotics was identified in some isolates (Qian *et al.,* 2016; Yazdankhah *et al.,* 2018). *Pseudomonas aeruginosa* and *Escherichia coli* were the most important bacteria with the occurrence of resistance to heavy metals and antibiotics (Nguyen *et al.,* 2019). It is known that genes of resistance to heavy metal and antibiotic are often genetically related and located on mobile elements (i.e., plasmids, transposons, and integrons), some of which are easily transported among phylogenetically distant bacteria (Davies and Davies, 2010). Therefore, antibiotic and heavy metal resistances in bacteria may be due to the presence of R-plasmid-containing genes (Neethu *et al.,* 2015).

Conclusion

Occurrence of antibiotic and heavy metal resistant bacteria in the Khoshk River of Shiraz indicated the impact of human activities on the environment that may endanger public health. In this study, the isolates from the above-mentioned site were studied to select the best bacterial strains that might be of further use for the bioremediation of heavy metal pollutants in the natural ecosystems of Iran. Antibiotic resistance was witnessed in the bacterial isolates. Therefore, there seems to be a relationship between bacterial resistance to both heavy metals and antibiotics. However, the findings of this study are not conclusive and more future studies are needed to better understand bacterial resistance mechanisms to heavy metals and antibiotics.

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