African Journal of Biotechnology Vol. 11(25), pp. 6710-6717, 27 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3394
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Identification and molecular analysis of mercury resistant bacteria in Kor River, Iran

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Accepted 17 February, 2012

Mercury (Hg) is one of the most important toxic pollutants widespread in the environment. It is being extensively used in industrial applications (chlor-alkali electrolysis, fungicides, disinfectants, dental products, etc), resulting in local hot spots of pollution and serious effects on biota and humans. The aim of this study was to identify mercury resistant bacteria and extract their plasmids and DNA. In this study, mercury-resistant bacteria were isolated and characterized from mercury-polluted sediments in Kor River in Iran. The samples were cultured in different media cultures, identified using biochemical tests, and due to the relationship between antibiotic and mercury resistance, they were isolated based on these two factors. The plasmids and DNA were extracted from the most resistant bacteria to both antibiotic and mercury and the sizes were determined using agarose gel electrophoresis. A 12.3 Kb plasmid from Serattia sp. and *Escherichia coli* and using Sau3A1 enzyme, some DNA fragments (4, 6, 8 and 10 Kb) from *Pseudomonas* sp., *Serattia* sp. and *Escherichia coli* were also extracted.

Key words: Mercury, resistant, bacteria, DNA, plasmid extraction, restriction endonuclease.

INTRODUCTION

To survive in the presence of mercury in their natural habitats, many microorganisms have evolved an effective resistance mechanism based on the enzymatic transformation of Hg (II) into metallic mercury (Hg (0)). Hg (0) is not toxic for microorganisms and can leave the microbial cell by diffusion. The reductive transformation occurs inside the microbial cell and requires biochemical reduction equivalents ($NADPH_2$). The reaction is catalyzed by the enzyme, mercury reductase, which is the product of the MerA gene. The MerA gene is usually within the mer operon, whose organization and properties have been previously reviewed in detail (Barkay et al., 2003; Hobman and Brown, 1997; Osborn et al., 1997; Silver and Phung, 1996).

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In brief, the MerA gene consists of genes encoding regulatory proteins (MerR and MerD); mercury transport proteins (MerT, MerP, MerF, MerC and MerE) (Liebert et al., 1999; Wilson et al., 2000); and sometimes an organomercurial lyse (Mer B1, MerB3) (Begley et al., 1986; Huang et al., 1999a), which in combination with MerA mediates resistance to organic mercurials. In Gram-positive bacteria, mer operons have been found on plasmids and transposons, including the small *Staphylococcus aureus* plasmid pI285 (Laddaga et al., 1987; Novick and Roth, 1968), on class II transposons in a range of isolates (Bogdanoova et al., 2004; Wang et al., 1999b; Stapleton et al., 2004; Wang et al., 1989) and on a complex composite conjugative chromosomal element in *Enterococcus faecalis*, Tn5385 (Rice and Carias, 1998).

The objective of this study was to identify mercury resistant bacteria in order to apply them in deleting or decreasing mercury in high polluted areas. We also aim

to identify plasmids and transfer them to other mercury sensitive bacteria, to be able to delete mercury too.

MATERIALS AND METHODS

Study area and sampling

The study area stretched from Droodzan Dam to Lake Bakhtegan with four sampling stations including Droodzan (D), Pole Petroshimi (PP), Pole khan (PKh) and Ghavmishi (G). The entire stretch from Droodzan to Lake Bakhtegan is about 120 km and lies between longitude 52°25′ 32″ to 53°25′ 00″E and latitude 29°51′ 00″ to 30°12′22″N. Sampling of surface water and sediments were performed monthly (three times per season) from Summer 2010 to Spring 2011 from the sampling stations. We did sampling in the middle of each month on Tuesdays to avoid bias.

Isolation and identification of mercury resistant bacteria

Isolation of Hg resistant bacteria was performed by primary enrichment method and directly plating on Luria Bertani (LB) broth containing mercury. In this method, 10 g or 10 ml of each sample was added to 90 ml of LB broth (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl) containing 5 mg/L $HgCl_2$ and incubated at 30°C for 48 h. Then 0.1 ml of appropriate dilution was spread on LB agar (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl and 12 g/L agar) supplemented with $HgCl_2$ (5 mg/L). The plates were incubated at 30°C for 48 h (Wangner et al., 2000). After incubation, the appeared colonies were purified and identified with Gram staining, catalase test, oxidase test, and conventional biochemical tests according to the method of Bergey (Prescott and Harley, 2002).

Antimicrobial susceptibility test

The isolates on LB broth were incubated at 30°C for 24 h. The number of bacteria per ml was prepared according to 0.5 MacFarland standard (1.5×10^8 cfu/ml). Using Lown's method, the bacteria were grown on Mueller-Hinton agar (Merck, Germany). Antimicrobial susceptibility test was carried out using double-disc (DD) synergy test for tetracycline (TE), ampicillin (AM), amikacin (AN), penicillin (P), sulfamethoxazole (SXT), chloramphenicol (C), gentamycin (GM), clindamycin (CC) and erythromycin (E) antibiotics. The antibiotic discs were placed on bacterial culture and then incubated at 30°C for 24 h.

Minimal inhibitory concentration (MIC) of Hg^{2+}

Minimal inhibitory concentration (MIC) of Hg^{2+} was tested by growing the resistant strains on LB broth and incubating it at 30°C for 24 h. To have the desired turbidity, the number of bacteria per ml was prepared according to 0.5 MacFarland standard (1.5×10^8 cfu/ml) with cationic concentrations of 800, 400, 200, 100, 50, 25 and 12.5 µmg/ml for Hg^{2+}. Then 100 µL of bacteria suspension (1.5×10^8 cfu/ml) was added to each tube and the tubes were incubated at 30°C for 24 h.

Plasmid extraction

The resistant isolates were selected for plasmid extraction. The isolates were grown on LB broth containing 5 mg/L $HgCl_2$ and subsequently incubated at 30°C for 24 h. The plasmid extraction was performed according to Kado and Liu method (Kado and Liu, 1981). Finally, the plasmid size was determined using 0.8% agarose gel (Merck, Germany) stained with ethidium bromide.

DNA extraction

The resistant isolates to Hg^{2+} were grown on LB broth containing 5 mg/L $HgCl_2$ and incubated at 30°C for 24 h. DNA extraction was performed using DNP™ Kit (Sinagene).

The effect of restriction endonuclease

The extracted DNA from the resistant isolates was partially digested with the restriction endonuclease *Sau*3AI at 37°C. Aliquots were removed from the digestion at various points. Next, the DNA fragments were visualized by agarose gel electrophoresis on 0.8% gel (Merck, Germany) stained with ethidium bromide.

RESULTS

Since sampling was performed seasonally (three times) in this study, the results are presented according to each season separately. According to spring results, *Pseudomonas* sp1 was more resistant to all antibiotics (Table 1). Spring isolates of *Pseudomonas* sp2 and sp3 showed intermediate resistance just to amikacin and gentamycin antibiotics and were resistant to the other applied antibiotics. Also among all isolates in this season, the most amount of resistance was shown to ampicillin and clindamycin antibiotics with 100% resistance (Figure 1). *Acinetobacter* sp., *Proteus* sp1 and sp2 as well as *Serattia marcescens* were sensitive to just two antibiotics. On the other hand, *Micrococcus* sp1 was the most sensitive bacteria to a wide range of antibiotics including amikacin, erythromycin, sulfamethoxazole, chloramphenicol, penicillin, gentamycin and tetracycline (Table 1).

According to summer results, *S. marcescens* sp3 was not sensitive to any antibiotic. Also *Serattia* sp. 1 showed resistance to most antibiotics except sulfamethoxazole (Table 2). Generally, the bacterial isolates were resistant to ampicillin, penicillin and clindamycin antibiotics with the amount of 100% (Figure 2). Fall results revealed that *Pseudomonas* sp. is the most resistant to all antibiotics, whereas other bacteria have a partial tolerance to applied antibiotics (Table 3). In addition, the bacterial isolates showed the most amounts of resistance to ampicillin and penicillin antibiotics (100%) and the least resistance to amikacin with the amount of 9%. None of the bacterial isolates showed resistance to gentamycin (Figure 3). Moreover, in winter, we witnessed a resistance to some antibiotics among all bacteria (Table 4). Also the results show that all bacterial isolates were 100% resistant to penicillin, ampicillin and clindamycin and sensitive to gentamycin (Figure 4).

According to MIC results (Table 5) the most resistant bacteria to mercury were as follows: In spring it was the isolates of *Pseudomonas* sp. 1 and sp. 2 (100 mg/L); in

Table 1. Antibiogram test for bacterial isolates in spring.

Genus and species	Resistant	Intermediate	Sensitive
Klebsiella sp.	P, AM, E, CC	-	TE, GM, C, SXT, AN
E. coli 1	P, AM, CC	E	TE, GM, C, SXT, AN
E. coli 2	P, AM, SXT, E, CC	TE	GM, C, AN
Serattia marcescens	TE, P, AM, C, SXT, E, CC	-	GM, AN
Bacillus sp.	AM, CC	-	TE, GM, P, C, SXT, E, AN
Proteus sp. 1	TE, P, AM, C, SXT, E, CC	-	GM, AN
Proteus sp. 2	TE, P, AM, C, SXT, E, CC	-	GM, AN
Acinetobacter sp.	TE, P, AM, C, SXT, E, CC	-	GM, AN
Pseudomonas sp. 1	TE, GM, P, AM, C, SXT, E, AN, CC	-	-
Pseudomonas sp. 2	TE, GM, P, AM, C, SXT, E, CC	AN	-
Pseudomonas sp 3	TE, P, AM, C, SXT, E, CC	GM, AN	-
Staphylococcus sp.	P, AM, CC	-	TE, GM, C, SXT, E, AN
Micrococcus sp.	AM, CC	-	TE, GM, P, C, SXT, E, AN

TE, Tetracycline; AM, ampicillin; AN, amikacin; P, penicillin; SXT, sulfamethoxazole; C, chloramphenicol; GM, gentamycin; CC, clindamycin; E, erythromycin.

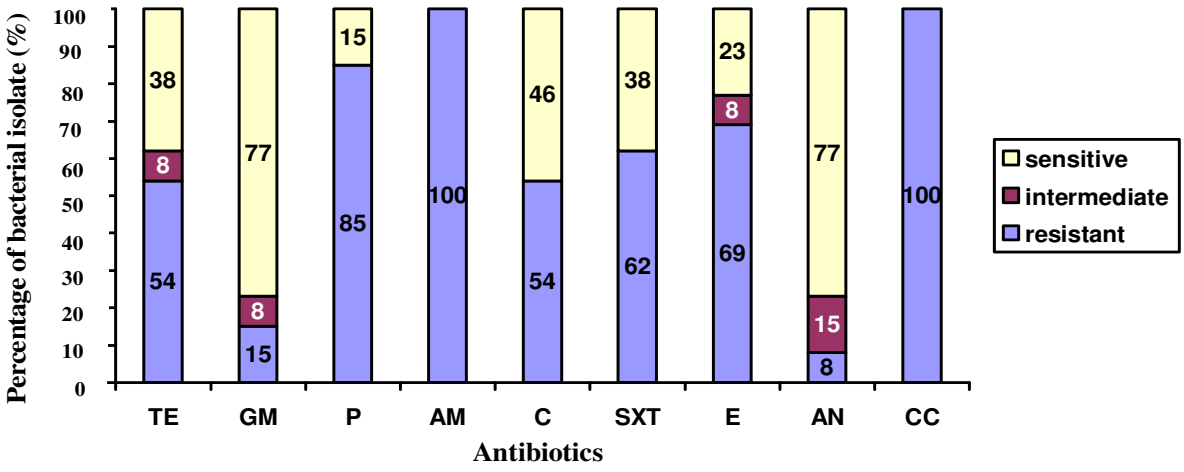

Figure 1. The percentage of all the bacterial isolates resistant to each antibiotic in spring. TE, Tetracycline; AM, ampicillin; AN, amikacin; P, penicillin; SXT, sulfamethoxazole; C, chloramphenicol; GM, gentamycin; CC, clindamycin; E, erythromycin.

summer, the most resistant isolate was *S. marcescens* 1 (50 mg/ml), in fall it was *Pseudomonas* sp. (50 mg/ml) and in winter they were *S. marcescens*, *Pseudomonas* sp. and *Salmonella* sp. (25 mg/ml). Plasmid extraction results showed that a 12.3 Kb plasmid of *Serattia* sp. was extracted (Figure 5). Following extraction, plasmid and DNA of resistant isolates were digested by Sau3A1 enzyme. DNA fragments of about 4, 6, 8 and 10 Kb were separated using gel electrophoresis and were visualized using gel documentation unit (Figure 6).

DISCUSSION

In this study, we considered two factors for isolation of bacteria. At first we isolated antibiotic resistant bacteria, and then we evaluated the isolated bacteria in respect to mercury resistance factor. According to the previous researches that were confirmed in our study, there is a relationship between antibiotic and mercury resistance in bacteria. Our data shows that for instance in spring, *Pseudomonas* sp. had a high resistance to both antibiotic and mercury, whereas in winter there was less tolerance to antibiotic, and no mercury high resistant bacteria was found. According to our results, we focused on certain bacteria to extract their DNA and plasmids including *Pseudomonas* sp., *Serattia* sp., as the most resistant isolates to antibiotic and mercury, and *E. coli* as a highly resistant isolate to mercury.

Kafilzadeh et al. (2005) on their studies on mercury pollution of the Kor River reported that Pole khan and Pole Petroshimi were the most mercury contaminated

Table 2. Antibiogram test for bacterial isolates in summer.

Genus and species	Resistant	Intermediate	Sensitive
Klebsiella sp.	TE, P, AM, E, CC	-	GM, C, SXT, AN
Citrobacter sp.	TE, P, AM, CC	-	GM, C, SXT, E, AN
E. coli 1	P, AM, CC	-	TE, GM, C, SXT, E, AN
E. coli 2	P, AM, E, CC	-	TE, GM, C, SXT, AN
Enterobacter sp.	P, AM, CC	-	TE, GM, C, SXT, E, AN
Serattia marcescens 1	TE, P, AM, C, E, CC	GM, AN	SXT
Serattia marcescens 2	TE, GM, P, AM, CC	-	C, SXT, E, AN
Serattia marcescens 3	TE, GM, P, AM, C, E, CC	SXT, AN	-
Bacillus sp.	TE, GM, P, AM, CC	E	C, SXT, AN
Proteus sp.	GM, P, AM, E, CC	TE, C	SXT, AN

TE, Tetracycline; AM, ampicillin; AN, amikacin; P, penicillin; SXT, sulfamethoxazole; C, chloramphenicol; GM, gentamycin; CC, clindamycin; E, erythromycin.

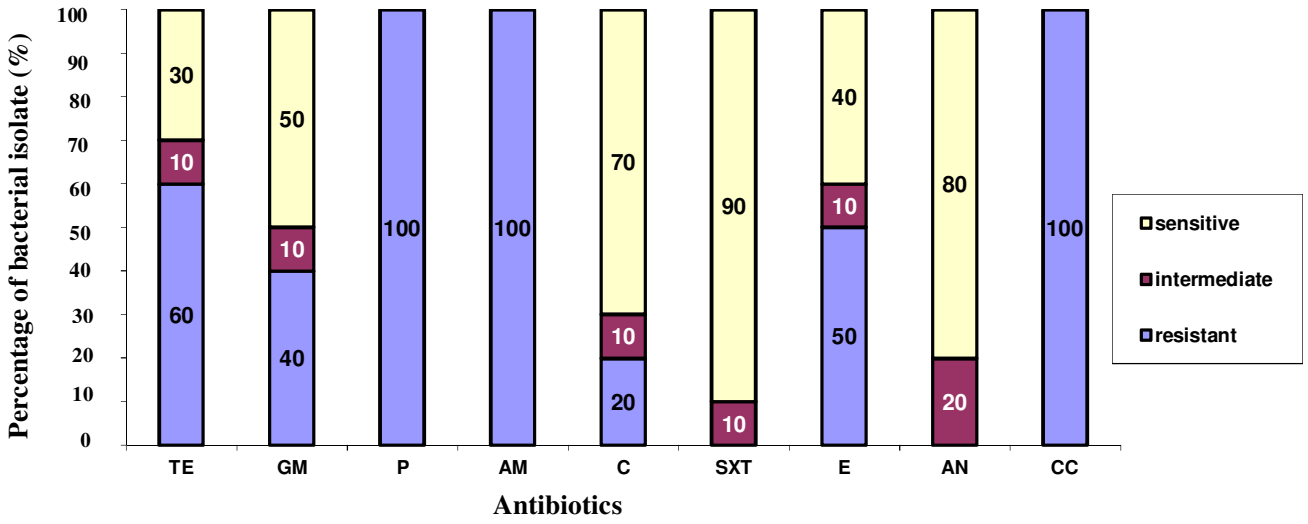

Figure 2. The percentage of all the bacterial isolates resistances to each antibiotic in summer. TE, Tetracycline; AM, ampicillin; AN, amikacin; P, penicillin; SXT, sulfamethoxazole; C, chloramphenicol; GM, gentamycin; CC, clindamycin; E, erythromycin.

areas of the Kor River. So we decided to select this area as a target for our study. MIC values for Hg in bacterial isolates and high level of resistance to mercury in the bacterial samples of the river obtained in this study confirmed a high pollution of mercury in this river. The placement of the Petroshimi industrial factory in the vicinity of the river and discharging of urban waste water and industrial town waste water into this river is the reason for this high pollution. Earlier studies have reported the genera of *Pseudomonas, Staphylococcus, Bacillus, Proteus, Citrobacter* and *Corynebacterium* as the most resistant bacteria to mercury (Keramati et al., 2011). The most amount of mercury resistance was shown by *Pseudomonas* sp2 with the amount of 100 mg/L $HgCl_2$. Horn et al. (1994) reported mercury resistance level among different *Pseudomonas putida* strains in range of 35 to 65 mg/L $HgCl_2$. Also, Ogunseitan (2002) showed a 100 mg/L mercury resistance to *Pseudomonas aeruginosa* in environmental isolates by a 142.5 kb plasmid called Rip64. In another research on *E. coli* strains, the range of MIC for $HgCl_2$ was between 25 to 55 mg/L (Zeyullah et al., 2010). Employing the primary enrichment method in this study, the high level of mercury pollution in Pole khan and Pole Petroshimi stations were the reasons for the difference in MIC values of the mercury resistant bacterial isolates.

Moreover, Horn et al. (1994) showed that toxic concentrations of mercury do not affect growth of mercury resistant bacteria. Addition of mercury to the medium of the resistant bacteria cannot prevent their growth. Mercury resistant bacteria are able to remove mercury and grow in presence of this toxicant. Tothova et al. (2006) reported that some of the sensitive bacteria can tolerate low concentrations of Hg. Interestingly, expression of mercury resistant genes can be induced by Hg (2) (Barkay et al., 2003). The genes coding for mercury

Table 3. Antibiogram test for bacterial isolates in fall.

Genus and species	Resistant	Intermediate	Sensitive
E. coli	P, AM, E, CC	-	TE, GM, C, SXT, AN
Enterobacter sp.	P, AM, E, CC	TE	GM, C, SXT, AN
Serattia marcescens 1	P, AM, AN CC	GM, E	TE, C, SXT
Serattia marcescens 2	TE, P, AM, E, CC	AN	GM, C, SXT
Bacillus sp.	P, AM	CC	TE, GM, C, SXT, E, AN
Proteus sp.	TE, P, AM, C, SXT, E, CC	-	GM, AN
Alcaligenes sp.	P, AM, C, E, CC	-	TE, GM, SXT, AN
Acinetobacter sp 1	P, AM, E, CC	-	TE, GM, C, SXT, AN
Acinetobacter sp 2	P, AM, C, CC	E	TE, GM, SXT, AN
Salmonella sp.	P, AM, CC	-	TE, GM, C, SXT, E, AN
Pseudomonas sp.	TE, P, AM, C, SXT, E, CC	GM, AN	-

TE, Tetracycline; AM, ampicillin; AN, amikacin; P, penicillin; SXT, sulfamethoxazole; C, chloramphenicol; GM, gentamycin; CC, clindamycin; E, erythromycin.

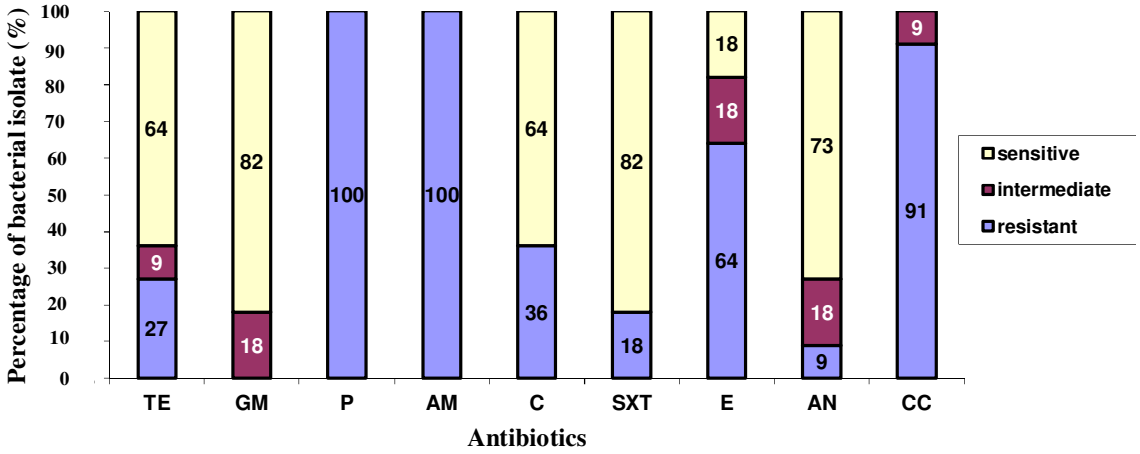

Figure 3. The percentage of all the bacterial isolates resistances to each antibiotic in fall. TE, Tetracycline; AM, ampicillin; AN, amikacin; P, penicillin; SXT, sulfamethoxazole; C, chloramphenicol; GM, gentamycin; CC, clindamycin; E, erythromycin.

Table 4. Antibiogram test for bacterial isolates in winter.

Genus and species	Resistant	Intermediate	Sensitive
Klebsiella sp.	P, AM, E, CC	-	TE, GM, C, SXT, AN
E. coli 1	P, AM, CC	C	TE, GM, SXT, E, AN
E. coli 2	P, AM, CC	TE, E	GM, C, SXT, AN
Serattia marcescens	P, AM, CC	TE, E, AN	GM, C, SXT
Bacillus sp 1	TE, P, AM, C, E, CC	SXT	GM, AN
Bacillus sp 2	TE, P, AM, CC	-	GM, C, SXT, E, AN
Alcaligenes sp.	P, AM, E, CC	-	TE, GM, C, SXT, AN
Acinetobacter sp. 1	P, AM, CC	E	TE, GM, C, SXT, AN
Acinetobacter sp. 2	P, AM, C, E, CC	AN	TE, GM, SXT
Salmonella sp.	TE, P, AM, CC	SXT, E, AN	GM, C
Pseudomonas sp.	TE, P, AM, SXT, E, CC	-	GM, C, AN
Staphylococcus sp.	TE, P, AM, SXT, E, CC	-	GM, C, AN

TE, Tetracycline; AM, ampicillin; AN, amikacin; P, penicillin; SXT, sulfamethoxazole; C, chloramphenicol; GM, gentamycin; CC, clindamycin; E, erythromycin.

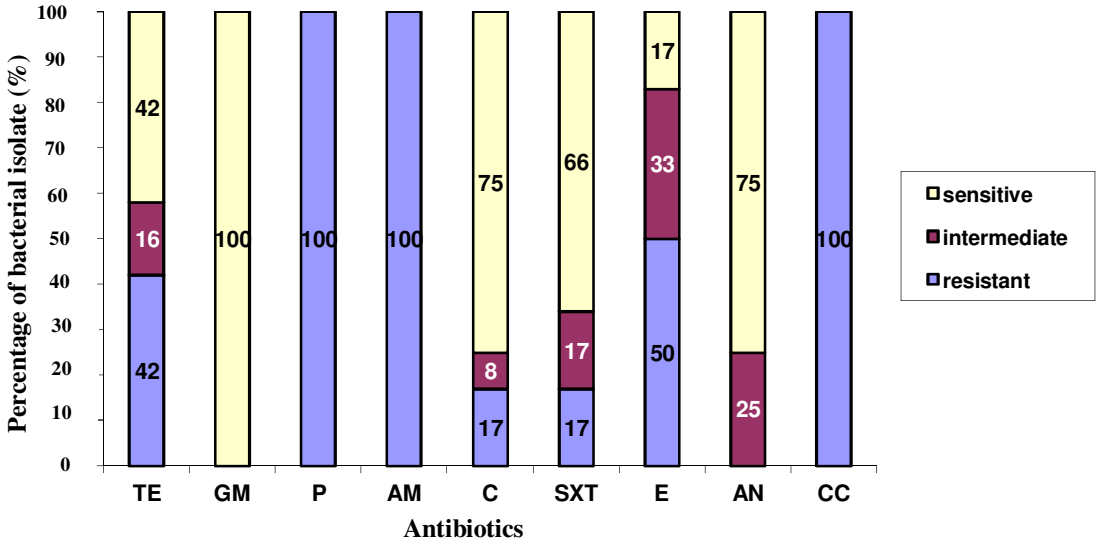

Figure 4. The percentage of all the bacterial isolates resistances to each antibiotic in winter. TE, Tetracycline; AM, ampicillin; AN, amikacin; P, penicillin; SXT, sulfamethoxazole; C, chloramphenicol; GM, gentamycin; CC, clindamycin; E, erythromycin.

Table 5. The results of MIC values of Hg^{2+} for different seasons.

$HgCl_2$ (mg/L)	Genus and species (spring)	$HgCl_2$ (mg/L)	Genus and species (summer)	$HgCl_2$ (mg/L)	Genus and species (fall)	$HgCl_2$ (mg/L)	Genus and species (winter)
6.25	*Klebsiella* sp.	12.5	*Klebsiella* sp.	12.5	*E. coli*	6.25	*Klebsiella* sp.
12.5	*E. coli* 1	12.5	*Citrobacter* sp.	6.25	*Enterobacter* sp.	12.5	*E. coli* 1
50	*E. coli* 2	25	*E. coli* 1	12.5	*Serattia marcescens* 1	12.5	*E. coli* 2
50	*Serattia marcescens*	25	*E. coli* 2	25	*Serattia marcescens* 2	25	*Serattia marcescens*
6.25	*Bacillus* sp.	12.5	*Enterobacter* sp.	6.25	*Bacillus* sp.	12.5	*Bacillus* sp. 1
50	*Proteus* sp. 1	50	*Serattia marcescens* 1	25	*Proteus* sp.	6.25	*Bacillus* sp. 2
25	*Proteus* sp. 2	25	*Serattia marcescens* 2	12.5	*Alcaligenes* sp.	6.25	*Alcaligenes* sp.
25	*Acinetobacter* sp.	25	*Serattia marcescens* 3	12.5	*Acinetobacter* sp. 1	6.25	*Acinetobacter* sp. 1
100	*Pseudomonas* sp. 1	6.25	*Bacillus* sp.	25	*Acinetobacter* sp. 2	12.5	*Acinetobacter* sp. 2
100	*Pseudomonas* sp. 2	25	*Proteus* sp.	12.5	*Salmonella* sp	25	*Salmonella* sp.
50	*Pseudomonas* sp. 3			50	*Pseudomonas* sp.	25	*Pseudomonas* sp.
6.25	*Staphylococcus* sp.					12.5	*Staphylococcus* sp.
6.25	*Micrococcus* sp.						

The most resistant isolates were *Pseudomonas* sp. 1 and sp. 2 (100 mg/ml) in spring.

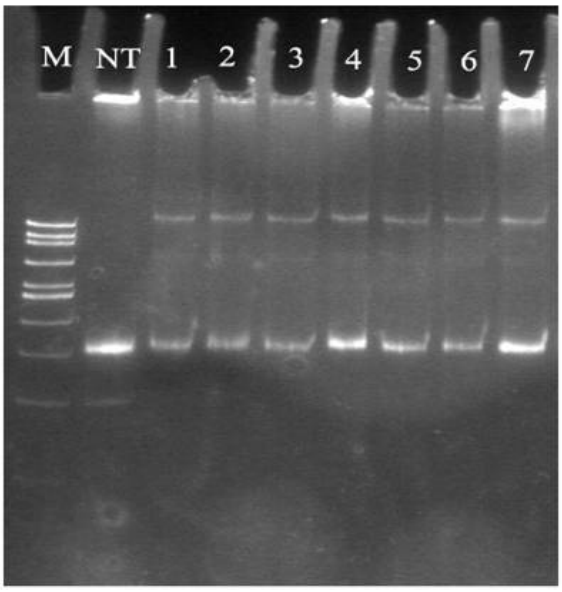

Figure 5. Plasmid extraction from resistant bacteria isolates. M, marker; NT, positive samples isolated from *Pseudomonas* sp. 3. Lanes 1 to 3, Plasmids of 12300 bp isolated from *Serattia* sp.; lanes 4 to 7, plasmids of 12300 bp isolated from *E. coli*.

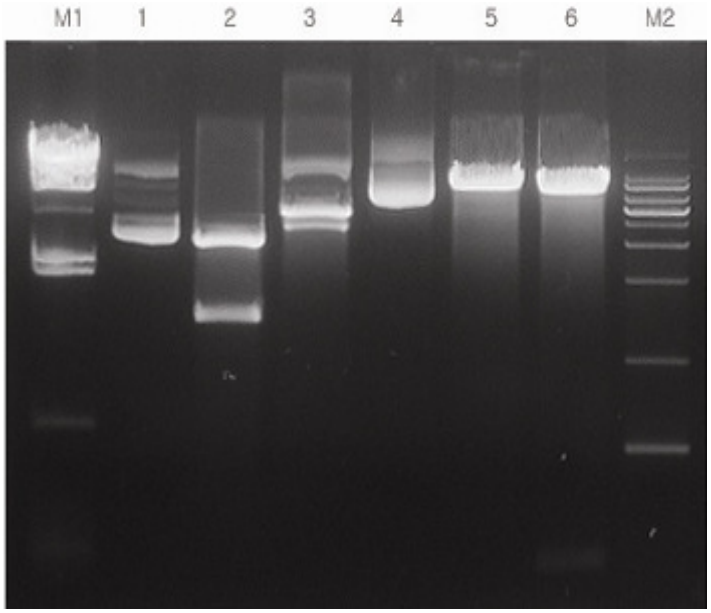

Figure 6. Effect of sau3AI enzyme on the resistant bacteria isolates DNA. M2, Marker; lanes: 1, *Serattia* marcesc*ens* 1 (6 and 8 Kb); 2, *S. marcescens* 2 (4 and 6 Kb); 3, *Pseudomonas* sp. 1 (6 Kb); 4, *Pseudomonas* sp. 2 (8 Kb); 5 and 6, *E. coli* (10 Kb).

resistance (the *mer* operon) have been shown to be carried on plasmids (Summers et al., 1978) and transposons (Stanisich et al., 1997). The results show that plasmids of 12 and 15 Kb were isolated from the resistant isolates.

Furthermore, our research also confirmed that the most amount of mercury resistance and the most number of isolated plasmids were achieved in Gram negative bacteria. The distinction between the mercury resistance systems of Gram negative and positive organisms was

revealed by DNA–DNA hybridization (Barkay et al., 1989). Mercuric reductases originating in Gram negative organisms are heat stable (or even heat stimulated), whereas those produced by Gram positive organisms are inactivated at 60°C (Olson et al., 1982). The results achieved in this study show high pollution of the Kor River to mercury, which can be removed using the resistant bacteria. Hence, pollution eradication can be achieved by designing a bioreactor model using high mercury resistant bacteria such as *Pseudomonas* sp. and *Serattia* sp.

ACKNOWLEDGEMENTS

This study was conducted in the research laboratory of Islamic Azad University of Jahrom. We are thankful to the research assistant secretary for the financial support towards this research.

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