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Heavy Metals and Antibiotic Co-Resistance in Bacterial Isolates of Industrial Effluents

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Abstract

Heavy metal and antibiotic co-resistance is a global issue. The goal of this research was to explore the heavy metal, also antibiotic resistance patterns of effluent bacterial isolates. Heavy metal resistant bacteria were isolated from effluents and their Minimum Inhibitory Concentration (MIC) was determined. The Multi-Metal resistance (MMR) pattern and antibiotic resistance trait of isolates were defined. The MIC of Cu^{2+}, Pb^{2+}, Cd^{2+} and Zn^{2+} was 4, 8, 12 and 24 mM/L, respectively. Most of the isolates indicated the Cd^{2+}, Pb^{2+} and Zn^{2+} resistance and high resistance to the most tested antibiotics. The 16S rDNA gene sequences of resistant isolates were handed over to NCBI-GenBank as *Staphylococcus sp*. ATHA2(JX120151) and *Klebsiella oxytoca* ATHA1(JQ928574). Correlation was found between metal tolerances, heavy metal concentration, also antibiotic resistance in bacteria. Thus, it is important to not only be aware of antibiotics misapplication, but also respond to excessive discharge of effluent containing heavy metals to the environment.

Keywords: Antibiotic, Bacteria, Co-Resistance, Heavy Metals, Industrial Effluent.

1. Introduction

Chemical contamination compromising pharmaceuticals (Mojiri et al., 2019) and toxic metals, is an issue that affects the hydrosphere and humans negatively (Filali et al., 2000). The existence of pharmaceuticals in environments, such as water and wastewater, has considerable attraction for researchers, because these compounds are biologically active. Amongst them, antibiotics have drawn lots of attention (Tahrani et al., 2015). The environmentally increasing antibiotics occurrence has led to growing bacterial resistance to several kinds of antibiotics which can threaten human health. Lately, World Health Organization[1] has announced that antibiotic resistance is rising, and we are quicklyfacing challenges in dealing with it (Lawe-Davies and Bennett, 2017). Besides pharmaceuticals, the most abundant contaminant in sewage and wastewater, is heavy metals (Filali et al., 2000; Xiong et al., 2019).

Some heavy metals (e.g. copper, cobalt, manganese,

[1] World Health Organization (WHO)

nickel, and zinc), are important for the microbial growth, in trace amounts. They provide the enzyme components (Chen et al., 2006), pigments, structural proteins and retain the cells ionic balance. But they have harmful effects on many organisms and human (Hasan et al., 2019; Verma and Kuila, 2019); also, they can change the ecological balance of the environment, at high concentrations (Nwuche and Ugoji, 2008). The rest of them (like cadmium, lead and other metalloids) are extremely toxic (Yana and Niua, 2019) because they have relative access to biological systems. The environmental existence of the toxic amount of such metal has a limiting effect on most microorganisms. The inhibitory effect is determined as the MIC which is a gold standard, that could represent the resistance of microorganisms (Tamás and Martinoia, 2006). The growing of antibiotic resistant bacteria is increasing together with microbial resistance to metal ions, due to the fact that both traits, microbial resistance to metal ions and antibiotics, are usually connected with each other. Occasionally, these genes are placed on mobile genetic fragment as plasmids, transposable elements and genomic islands. Furthermore, some mechanisms of resistance protect bacteriafrom the lethal impact of antibiotics as well as heavy metal compounds (Pal et al., 2017). As resistant genes can move from bacterium to bacterium, either through a transformation or conjugation, the water resources are considered as reservoirs for the resistant bacteria (Harris et al., 2012). Consequently, organisms should maintain homeostasis of metal within physiological or sub-toxic levels, and develop the resistance mechanisms that end in the resistant variant selection (Tamás and Martinoia, 2006). Bacteria might become resistant to metals and antibiotics through mutations or the gaining resistance genes from their counterparts. In other words, environmental bacteria are the main sources of resistance genes, and can play a role as resistance genes in recipient pathogens (Sinegani and Younessi, 2017). Additionally, heavy metal resistant bacteria may also assist the strength of antibiotic resistance genes. Metal and antibiotic resistance are correlated owing to the possibility that genes responsible for both antibiotics and heavy metals may exist on the similar plasmid (Knapp et al., 2011; Li et al., 2017). Resistant bacteria to antimicrobial agents are ubiquitous microorganisms which are found in water, soil, air, human, food chain, animals, plants, and can shift between ecological niches. When bacteria (and other microorganisms) are subjected to antimicrobial agents, resistance naturally appears and it leads to unsuccessful infection treatments (World Health Organization, 2015). Nowadays it is obvious that the misapplication and overuse of antibiotics in human, animals and agriculture are the leading causes of appearance of resistant bacteria (Khan et al., 2013). However, the microbial biomass (e.g. fungal, algal and bacterial) application, especially the resistant ones, for the toxic metal reduction from aqueous systems in terms of bioremediation, attracts attention because it is both safe and inexpensive (Ansari and Malik, 2007; Verma and Kuila, 2019). So, this research aims to survey the existence of antibiotic/heavy metal co-resistance in the bacterial isolates of a polluted industrial effluent.

2. Materials and Methods

2.1. Chemicals, sampling, and bacterial analysis

The used heavy metals were (Merck Co. Germany): $ZnSO_4$. $7H_2O$, $CuSO_4$. $5H_2O$, $Cd(NO_3)2$. $4H_2O$, $Pb(NO_3)2$ with the concentrations of 0.5, 1, 2, 4, 8, 12, 16, 24 and 32(mm/L). Stocks were made in dH_2O, sterilized by using Millipore filters (0.22 µm pore size). All glasses were leached in HNO_3 (2N) and rinsed with dH_2O several times to prevent metal contamination.

Two effluents samples (acrylic/human and acrylic effluents), from weaving factory located in Isfahan, Iran were gathered in sterile glass bottles (1 Liter). The bottles were sent to the laboratory in ice box, the pollution amounts were measured as BOD_5, COD and heavy metal concentrations (Cd^{2+}, Pb^{2+}, Zn^{2+} and Cu^{2+}). The heavy metal concentration measurements were performed by Atomic absorption spectrophotometer, Buck Scientific, based on (Rice et al., 2012). The amount of EC (electrical conductivity) and pH were also assessed by EC and pH meter (Metrohm).

Then, the total bacterial number of samples was counted as CFU/ml within 4-6 hours of sampling. This was done by Pour plate method (APHA, 9215B) and Spread plate method (APHA, 9215C) for heterotrophic bacterial count (HBC), on nutrient agar medium, and Replica plating method for resistant bacteria count (RBC), on PHG II agar medium. The PHG II agar medium contained peptone, yeast extract, glucose, agar, and different concentration of metals.

The plates were kept in an incubator (35 ^{o}C) for 3-5 days (Chen et al., 2006; Ansari and Malik, 2007). The method of streak plate was employed for further isolation and purification of bacterial colonies. Finally, the isolates were identified based on gram staining, morphological, cultural and biochemical characteristics in accordance with Bergey's Manual of Systematic Bacteriology (Vos et al., 2011).

2.2. MIC and MMR determination

The minimum heavy metal concentration which inhibits growth of isolates (MIC) was obtained using the agar dilution mehod. Plates containing PHG II agar, plus the different heavy metal concentrations, were attentively inoculated with each isolate (log phase) and incubated (35 ^{o}C, 36-48 h).

For evaluation of MMR (multi-metal resistance) ability, PHG II agar plates containing the heavy metal ions were inoculated by four selected resistant isolates in

radial streaks (Alboghobeish et al., 2014) and incubated at the same condition.

2.3. Antibiotic resistance

The most resistant isolates were investigated for their antibiotic resistance ability using the disk diffusion method and lawn culturing. The saturated discs of antibiotic (Padtan Teb Co., Iran) and Muller Hinton Agar (Merk Co. Germany) plates were used for this experiment. The results of formed inhibition zones around each disk were recorded after 24h incubation at 35 °C. Discs contained the following antibiotics (µg/disc): Gentamycin (10), Ampicillin(10), Erithromycin (15), Vancomycin (30), Carbencilin (100), Penicillin G (10 U) and Cefalothin (30).

2.4. Molecular identification of selected isolates

Finally, further molecular identification of the most resistant isolates (to both antibiotic and metals) with clinical importance (A6 and A7) was performed. So, the DNA of each pure isolate was extracted by the help of a bacterial DNA extraction kit (Qiagen) and the 16S rDNA gene fragment amplification was done by a thermocycler, (PCR, Eppendorph 632500) with each of the Forward and Reverse primers: 27F (5′AGAGTTTGATCCTGGCTCAG-3′) and 1495R (5-GGTTACCTTGTTACGACTT-3). The amplification program was as follows: Initial denaturation step of 95 °C (5 min), then, 30 cycles including, 95 °C (1 min), 60 °C (30 s), 72 °C (35 s). The final step of 72 °C (5 min) was used for extension. The amplified targets were sequenced after purification and Nucleotide sequence similarities were determined by the BLAST software of the NCBI database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST). Then, the 16S rDNA gene sequences were handed over to the GenBank via the BankIt service. The neighbor–joining method was also used to create phylogenetic trees from the distance matrices.

3. Results

3.1. Pollution evaluation and bacterial analysis

The pH, EC, BOD_5, and COD effluents amounts are presented in Table 1. The pH of both effluents was around 6.5-6.8. Mustapha and Halimoon stated that the optimal pH of bacterial growth is around 6.5 to 7.5. (Mustapha and Halimon, 2015).

COD was 767.7 and 1767.6 for acrylic and human effluents and acrylic effluent, respectively. (Ray et al., 2006) reported 663 mg/L to 1002 mg/L of COD for an acrylic industry effluent which agrees with our finding. The effluents BOD_5 and COD with regard to the standard amounts and the average of BOD_5 and COD of other weaving factories are shown in Fig. 1. It is apparent that, the effluents were very polluted, which may be due to the existence of several bio-refractory organic contaminants (alkanes, organic nitriles, aromatic compounds, phenols, esters and amides) in such wastewater like acrylic fiber manufacturing industries (Zheng et al., 2015). Fig. 2 shows the heavy metal concentrations of samples.

The averages of HBC and RBC of two effluents (1 and 2) are given in Table 2. No significant difference was noticed between the effluents. For Pb, Cu and HBC, the bacterial count obtained from the acrylic/human effluents was more than the acrylic effluent, but for Zn and Cd it was a little lower in acrylic and human effluents.

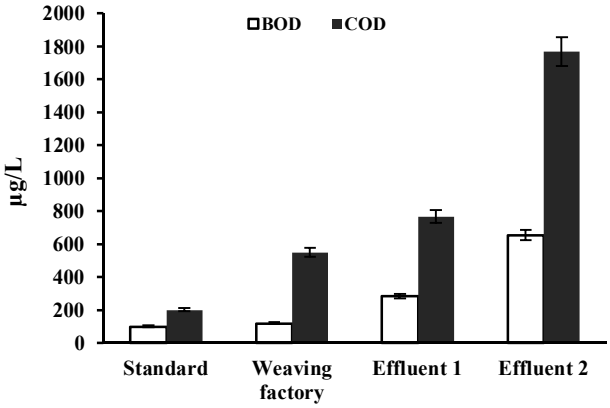

Fig. 1. BOD_5 and COD of the effluents in comparison with the standard amounts and the average of BOD_5 & COD of Tehran`s weaving factories

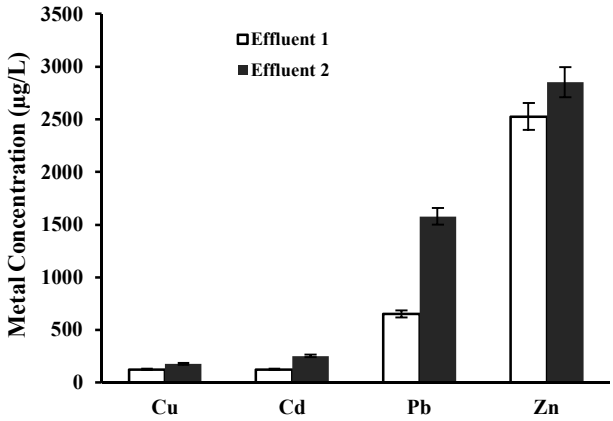

Fig. 2. The amount of heavy metals in the effluents

3.2. MIC, MMR and antibiotic resistance analysis

The MIC and resistant strains percentages to different heavy metal concentrations are presented in Table 3. The maximum resistance level is associated with Zinc. The maximum MIC of 24 mM/L was recorded and it was related to some bacteria, including: Staphylococcus (Fig. 3), Corynebacterium and Enterococus. Citrobacter showed the minimum MIC of 8 mM/L.

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Table 1. Some of the physicochemical characteristics of industrial wastewaters

Effluents	BOD_5 mg/L	COD mg/L	TOC mg/L	pH	EC ds/m
1: Acrylic and human	284.1	767.76	119.86	6.8	2.2
2: Acrylic	654.04	1767.67	460	6.5	2.2

Table 2. The average number of heavy metal resistant bacteria in the effluents (CFU/ml) on PHG II Agar supplemented with 0.5 mM/L of each heavy metal and heterotrophic bacteria (HPC) (CFU/ml) in the Nutrient Agar plates

Effluent	Cd^{2+}	Pb^{2+}	Zn^{2+}	Cu^{2+}	HPC
1	1.33×10^5	6.6×10^5	7.5×10^5	1.2×10^5	1.33×10^6
2	2×10^5	4×10^5	8×10^5	1×10^5	1.2×10^6

HPC: Heterotrophic plate count

Table 3. The number and percentage of heavy metal resistant strains in specified MIC

Metal / Metal conc.		*1	2	4	8	12	16	24	Total No.
Cd^{2+}	No. of strain	-	-	-	7	2	-	-	9
	Percentage of isolated strain	-	-	-	(77.8)	(22.2)	-	-	100
Pb^{2+}	No. of strain	-	-	-	14	-	-	-	14
	Percentage of isolated strain				(100)				100
Zn^{2+}	No. of strain	-	-	-	2	10	5	3	20
	Percentage of isolated strain				(10)	(50)	(25)	(15)	100
Cu^{2+}	No. of strain	1	6	3	-	-	-	-	10
	Percentage of isolated strain	10	60	30					100

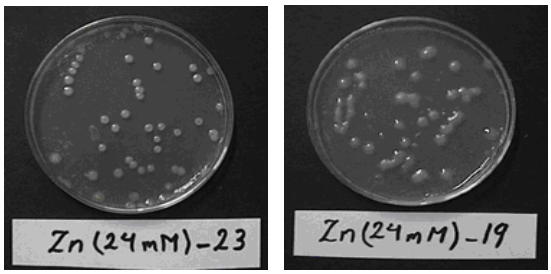

Fig. 3. Zinc resistant colonies of *Staphylococcus* on PHG II agar plates supplemented with 24 mM/L of zinc after 24 h incubation in 35 °C

Cadmium resistant bacteria showed the maximum and minimum MICs of 16 mM/L (Corynebacterium) and 8 mm/L (Bacillus and Corynebacterium). All of the lead resistant bacteria had the MIC of 8 mM/L. The Cu resistant bacteria showed the lowest resistance degree.

Maximum MIC was 4mM/L (Moraxella and Pseudomonas) and minimum is determined as 1mM/L (Klebsiella), and 2 mM/L (Bacillus, Providencia and Staphylococcus).

In MMR evaluation, all isolates (54) except one, showed resistance to Zn^{2+}, Pb^{2+}, Cd^{2+} and the majority of them were gram positive. All of the resistant isolates were not able to resist Cu^{2+} concentrations more than 4 mM/L. All of the Cu^{2+} resistant isolates (with MIC of 2 & 4 mM/L) could grow in the presence of others (Zn^{2+}, Pb^{2+}, Cd^{2+}) at a concentration of 8 mM/L (Table 4). The copper resistant bacteria were mostly gram negative. According to Table 4, most of the isolates (81.49%) were resistant to three metal ions (Zn^{2+}, Pb^{2+}, Cd^{2+}) and considered Tri-R, while 16.6% were tetra-R or resistant to all of the four metal ions tested.

The antibiotic resistance ability of metal resistant isolates was examined and the results are shown in Table 5. The results showed that, the antibiotic resistance was very high among the isolates. All of the isolates showed resistance to antibiotics such as Carbencilin, Vancomycin, Ampicilin, Cefalothin, and Penicillin G and sensitivity against Gentamycin.

3.3. Bacterial identification

Characteristics of the most resistant isolates (A1-A7) are presented in Table 6. According to the results, they belonged to different genera as follows: Moraxella, Pseudomonas, Bacillus, Enterococcus, Micrococcus,

Table 4. Multiple metal resistance pattern of the effluents bacterial isolates

Isolates			Resistance pattern			
MMR Type	No.	%	Zn^{2+}	Pb^{2+}	Cd^{2+}	Cu^{2+}
Tetra – R	9	16.6	+	+	+	+
Tri – R	44	81.49	+	+	+	_
Tri – R	1	1.85	+	+	_	+

Table 5. Susceptibility of some of the heavy metals resistant bacteria against antibiotic discs on Muller Hinton Agar plates

Antibiotic tested isolates	Cf	CB	E	PnG	V	Gm	AMP
Bacillus (cereus)	R	R	R	R	R	S D=20mm	R
Enterococcus(faecalis)	R	R	S D=28mm	R	R	S D=15mm	R
Klebsiella	R	R	R	R	R	ND	R
Moraxella	R	R	S D=15mm	R	R	S D=15mm	R
Pseudomonas	R	R	R	R	R	S D=25mm	R
Micrococcus (luteus)	R	R	R	R	R	S D=20mm	R
Staphylococcus	R	R	S D=12mm	R	R	S D=22mm	R

Cf: Cefalothin(30 µg), CB: Carbencilin(100µg), E: Erithromycin(15µg), PnG: PenicillinG(10 U), V: Vancomycin(30 µg), Gm: Gentamycin(10 µg), and AMP: Ampicillin(10 µg). R: Resistant, S: Sensitive and D: Diameter

Table 6. Morphological and biochemical characteristics of the most resistant isolates

Bacteria morphology	A1	A2	A3	A4	A5	A6	A7
Cell morphology	Cocci	Rod	Rod	Cocci	Cocci	Cocci	Rod
Gram reaction	-	-	+	+	+	+	-
Motility	+	+	+	-	_	-	-
Biochemical							
Catalase	+	+	+	-	+	+	+
Oxidase	+	+	_	-	+	-	-
Indole	-	-	_	-	-	=	+
VP	ND	_	+	+	+	+	+
MR	ND	-	+	-	-	+	-
Citrate	+	+	+	-	_	+	+
Nitrate	+	+	+	+	_	+	+
Utilization of							
Manitol	-	-	_	+	+	+	+
Glucose	-	+	+	+	_	+	+
Fructose	-	+	+	+	ND	+	+
Lactose	-	-	-	+	_	+	+
Result	**Moraxella**	**Pseudomonas**	**Bacillus (cereus)**	**Enterococcus (faecalis)**	**Micrococcus (luteus)**	**Staphylococcus**	**Klebsiella**
Molecular Identification	ND	ND	ND	ND	ND	Staphylococcus sp. ATHA2	Klebsiella oxytoca ATHA1
Accession no.						JX120151	JQ928574

Symbols: (-): Negative (+): Positive (ND): Not determined

Staphylococcus, and Klebsiella.

The further identification of the most resistance isolates (A6 and A7) with clinical importance was performed based on 16S rDNA gene analysis. The comparison of obtained sequences with the sequences in GenBank revealed that the A6 and A7 isolates exhibited the maximum similarity to different strains of Staphylococcus and Klebsiella oxytoca, respectively. So, they were submitted via BankIt service to GenBank as Staphylococcus sp. ATHA2 and Klebsiella oxytoca ATHA1 under the accession numbers of JX120151 and JQ928574, respectively.

4. Discussion

The analysis of heavy metals concentrations in the effluents showed a high level of Zn^{2+}> Pb^{2+}> Cd^{2+} > and Cu^{2+}. Some of the measured heavy metals are supposed to be toxic to biological systems. According to the results (Fig. 1 & Fig. 2), it is apparent that the studied effluents were extremely polluted. The standard value of pH for fresh water is between 5.5 and 8.5 (Esa et al., 2013). In this study, the pH values were neutral.

The significant lower count of RBC in comparison with HBC showed that high concentrations of heavy metals, especially cadmium and lead, had toxic effect and only some bacteria could adapt and grow in such condition. The relatively high RBC count might have been due to the environmental factors of the study area. (Chen et al., 2006) studied the heavy metal (Mn^{2+}, Zn^{2+}, Co^{2+}, and Cd^{2+}) toxicity to P. aeruginosa strain PU21. They recognized that, the PU21 metal tolerance was strongly dependant on its conditions like the existing metal type and the composition of the used medium. It was stated that the presence of some toxic contaminants such as heavy metals in the bacterial niches can provide a selective advantage for developing resistance (Prasanth and Mahesh, 2016). The present results show a positive relationship between resistant bacteria percentage and heavy metal concentrations.

All the isolates of this study could grow at low concentrations of the metal ions (1, 2 and 4 mM).

The growth of isolates was completely inhibited at 8 mM Cu^{2+} concentration while Pb^{2+} was well tolerated, even at this concentration (100%). Growth was fully repressed at 16 and 24 mM of metal ions except Zn^{2+}. The highest to lowest degree of tolerance was detected with Zinc (MIC range from 8-24 mM) followed by cadmium (MIC range from 8-12 mM), Lead (8 mM) and copper (1-4mm).

Haroun et al., showed the order of heavy metal tolerance by the Pseudomonas strains (the highest to lowest tolerance degree) as follows: Zn (range 1-10 mM) >Pb>Cd>Cu (range 1-5mM) (Haroun et al., 2017). Kacar and Kocyigit also reported MIC 10mM for Lead, 1-4 mM for Zinc, 1-2 mM for copper and from 0.08 to 0.6 mM/L for cadmium that is related to different strains of Bacillus. (Kacar and Kocyigit, 2013). The maximum MIC of 200 µg/ml for Cd^{2+}, 400 µg/ml for Zn^{2+} and Cu and 1600 µg/ml for Pb was also reported by (Ansari and Malik, 2007). Comparing the obtained MICs in this study with others (mentioned above) it is obvious that the isolates of this study showed more resistance that may be due to their genetic structure, natural habitat and other environmental factors. Furthermore, it is obvious that, the heavy metal existence exerts influence on bacterial activity in soil and other environments, significantly. Nwuche and Ugoji showed the metals additive or synergistic impacts. It is also reported that heavy metals can interfere with the biochemical properties of different microbial groups isolated from their niche (Nwuche and Ugoji, 2008; Utgikar et al., 2004).

In our study, the multi-metal resistance of bacterial strains was analyzed against zinc, cadmium, lead and copper at different concentrations. Based on the results, most strains (81.4%) showed Tri-R resistance pattern (Zn, Pb, Cd). A positive relationship was also observed between the bacterial resistance to high concentrations of heavy metals and MMR.

The bacterial MMR patterns were different and this property (MMR) is often conferred by a single plasmid (Prasanth and Mahesh, 2016; Keramati et al., 2011); in examining multi-metal resistances the different MMR patterns among isolated bacteria of dental clinic effluents were also shown.

The heavy metal resistant isolates of this study exhibited high resistance to several antibiotics such as, Carbencilin, Vancomycin, Ampicilin, Cefalothin, Clindamycin and Penicillin G, as well.

Sinegani and Younessi, showed the multiple antibiotic resistance patterns in the isolates of agricultural soils (Ampicillin, Amoxicillin, Vancomycin, Tetracycline, Doxycycline, and Streptomycin). They also presented high rate of co-resistance to mercury and antibiotics amongst the gram-negative strains, as well as to the beta-lactam antibiotics, zinc, mercury and nickel amongst the gram-positive strains. Based on the observations of this study, the adaptive responses of bacteria to stress factors seems to be the outcome of factors such as inappropriate sewage/effluent disposal and misusing antibiotics in human and a number of non-human applications (Sinegani and Younessi, 2017).

Recently, the multidrug resistant bacteria and infectious diseases have increased. Many researchers have explained the coexistence of metal and antibiotic resistant bacteria. It also showed that bacteria resistant to antibiotic might arise in the environment throughout the cross- or co-resistance to metals or resistance mechanisms co-regulation (Abidin and Chowdhury, 2018; Azam et al., 2018; Kacar and Kocyigit, 2013; Knapp et al., 2011; Sair and Khan, 2018).

These studies also confirmed that, the coexistence of

resistance to metal and antibiotic frequently occurs within the same bacteria. This occurrence and also a high frequency of multiple resistant strains existence may have an apparent effect on public health. Unlike antibiotics and organic pollutants with specified half-life, bacteria grow in the nature and resistant genes can also be increased as the bacteria multiply and subjected to evolution. Such sharing of genetic elements is an important factor in the spread of the co-resistance of metal and antibiotic (Sair and Khan, 2018) which occurs more in aquatic environments. Such environments can provide a perfect situation for the resistance dissemination and acquisition (Marti et al., 2014).

5. Conclusion

This study has indicated that the bacteria have developed their resistance mechanisms in the face of toxic stresses to cope with metal toxicity. Thus, the obvious correlation between bacterial metal tolerance and antibiotic resistance exists. So, if heavy metal increases in the environment, bacterial resistance will increase too. Afterwards by increasing As the spread of antibiotic resistant pathogenic bacteria increases, infectious diseases are becoming more complicated and are costly to treat.

Finally, these resistant isolates can be used as the indicator of heavy metal or antibiotic contamination in the ecosystem which can lead to spread of antibiotic resistant genes that are very harmful to humankind. Thus, more caution should be taken about the drastic overuse or abuse of antibiotic in our society and be aware of other compounds like heavy metals that are released into the nature throughout polluted effluents before treating them. As a positive point, these resistant strains might be safely applied at lower chemical concentration and clean the metals contaminated areas when their harmful genes are edited or knocked out so that the induction of antibiotic resistance risks could be diminished.

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