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). Between metal tolerances, heavy metal concentration also antibiotic resistance in bacteria existed a correlation. Thus, we'd better not only be alert of antibiotics misapplication, but also responsive of over 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 great 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 threat human health. Lately, World Health Organization[1] publicized that antibiotic resistance is rising, and we are fast facing up regarding dealing ways (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, 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 affect on many organisms and human harmfully (Hasan et al., 2019, Verma and Kuila, 2019) also, 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 really toxic (Yana and Niua, 2019) because they have relatively accessed to biological systems. The environmentally existence of the toxic amount of such metal has a limiting effect on the most microorganisms. The inhibitory effect is determined as the MIC which is a

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[1] World Health Organization (WHO)

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 nearly placed on mobile genetic fragment as plasmids, transposable elements and genomic islands. Furthermore, some mechanisms of resistance keep bacteria protected from 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 end in the resistant variant selection (Tamás and Martinoia, 2006). Bacteria might become resistant to metals and antibiotics through mutations or the gaining of resistance genes from their counterparts. In another word, environmental bacteria are the main sources of genes for resistance, 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 be existing closely in concert 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 transfer between ecological niches. When bacteria (also other microorganisms) are subjected to antimicrobial agents, resistance naturally appears and it leads to unsuccessful treatments of infection (World Health Organization, 2015). Nowadays it is obvious that the misapplication and overuse of antibiotics in human, animals and agriculture are the leading cause of resistant bacteria appearance (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 the 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): $ZnSO_4 . 7H_2O$, $CuSO_4 . 5H_2O$, $Cd(NO_3)2 . 4H_2O$, $Pb(NO_3)2$ by 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 the (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 systematic bacteriology Bergey's manual (Vos et al., 2011).

2.2. MIC and MMR determination

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

For evaluation of MMR (multi-metal resistance) ability, PHG II agar plates containing each 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 ^{o}C. Discs contained the following antibiotics (µg/disc): Gentamycin (10), Ampicilin(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 amounts of pH, EC, BOD_5, and COD of the effluents are presented in Table 1. The pH of the both effluents was around 6.5-6.8. Mustapha and Halimoon (2015) stated that the optimal pH of bacterial growth is around 6.5 to 7.5.

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 figure 1. It is apparent that, the effluents were much polluted which is maybe due to the existence of several bio-refractory organic contaminants (like: alkanes, organic nitriles, aromatic compounds, phenols, esters and amides) in such wastewater like acrylic fiber manufacturing industries (Zheng et al., 2015). The figure 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.

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 associated with Zinc. The maximum MIC of 24 mM/L was recorded and it was related to some bacteria, including; Staphylococcus (Fig3), Corynebacterium and Enterococus. Citrobacter showed the minimum MIC of 8 mM/L.

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.

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

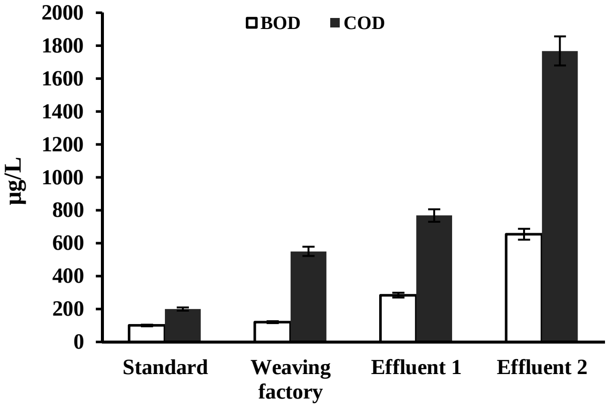

Fig. 1. BOD_5 and COD of the effluents in comparison with the standard amounts and the average of BOD_5 & COD of Tehran`s weaving factories

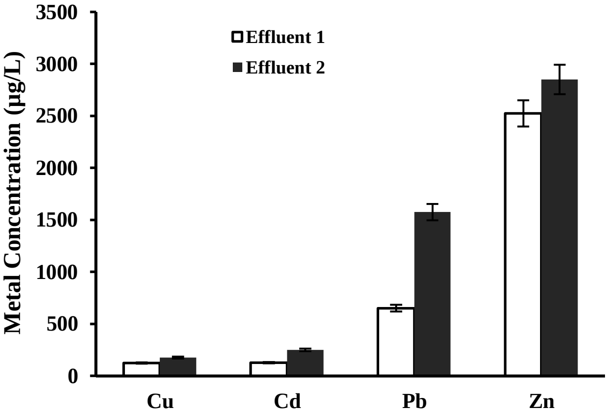

Fig. 2. The amount of heavy metals in the effluents

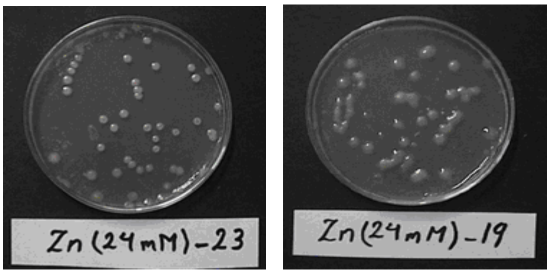

Fig. 3. Zinc resistant colonies of *Staphylococcus* on PHG II agar plates supplemented with 24 mM/L of zinc after 24 h incubation in 35°C

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

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 no. 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 conce.	*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

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	+	+	_	+

copper resistant bacteria mostly were gram negative. According to the 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, 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.

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: Ampicilin(10 µg). R: Resistant, S: Sensitive and D: diameter

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

Bacteria morphological	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	-	+	-	-	+	-
Citrat	+	+	+	-	_	+	+
Nitrat	+	+	+	+	_	+	+
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

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 much polluted. The standard value of pH for fresh water is between 5.5 to 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., 2017) 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). Kacar and Kocyigit (2013) 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 Bacillus different strains. The maximum MIC of 200 µg/ml for Cd^{2+}, 400 µg/ml for Zn^{2+} and Cu and 1600 µg/ml for Pb 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 is maybe 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 (2008) 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, the 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 also showed the different MMR patterns among isolated bacteria of dental clinic effluents.

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, 2017) 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, also 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. 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 resistant bacteria 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 also can be increased as the bacteria multiply and subjected to evolution. Such sharing of genetic elements is an important factor in the spreading 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 the spread of antibiotic resistant pathogenic bacteria, infectious diseases are becoming more complicated and costly to be treated.

Finally, these resistant isolates can be used as the indicator of heavy metal or antibiotic contamination in the ecosystem which can lead to spreading of antibiotic resistant genes, that is really harmful to humankind.

Thus, we should be more cautious about the antibiotic drastic overuse or abuse 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 of view, these resistant strains might be safely applied to 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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