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ASSOCIATION OF ANTIBIOTIC AND HEAVY METAL RESISTANT BACTERIA SCREENED FROM WASTEWATER

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Abstract

Wastewater treatment plant is a potential reservoir contributing to the evolution and spread of heavy metal and antibiotic resistant bacteria. The pollutants such as biocides, antibiotics, heavy metals are to be feared for as they have been known to evoke resistance in microorganisms in such polluted environment. The aim of this study was to the isolate bacteria from the treated wastewater and assess the resistance pattern of the isolates against antibiotics and heavy metals. Grab sampling was performed from April to June 2017, from the treated effluent from the secondary treatment plant. To assess the resistance pattern for antibiotic(s) and heavy metal(s), antibiotic susceptibility test and minimum inhibitory concentration by cup well method were performed respectively. *Staphylococcus aureus, Enterococcus faecalis, Citrobacter freundii, Escherichia coli, Enterobacter aerogenes, Proteus mirabilis, P. vulgaris, Salmonella* Typhi, *Pseudomonas aeruginosa* were isolated. Multi drug and heavy metal resistant isolates were screened. Fisher's exact test revealed that there is a significant association ($p < 0.001$) between antibiotic resistance pattern and resistance patterns at dilution of 2500 g/L (25%). Cramer's V test revealed that the effect size of antibiotic resistance pattern and heavy metal resistance pattern at dilution 2500 g/L is medium. *P. aeruginosa* was able to resist the metal concentration up to 10000 g/L (100%) dilution of Fe^{++}. Heavy metal resistant bacteria can be safely used to lower chemical concentration in the environment once their harmful genes are edited, knocked out etc. so that risks of evoking antibiotic resistance could be minimized.

Keywords: Isolates, antibiotic(s), heavy metal(s), resistant, wastewater

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Introduction

Environment pollution is an increasing problem on a global scale led through urbanization (Henze et al., 2008; Mishra et al., 2017) and sometimes due to natural sources (Allen et al., 2010). Industrial effluents, wastewater, agricultural runoffs, metal processing, mining activities, tanning etc. are some major known pollutants across the globe (van den Berg et al., 2005; Rajbanshi, 2009).

Due to unplanned urbanization in Kathmandu valley (Mishra et al., 2017), wastewater contains discharges from toilets and bathrooms (hormones, anatomical wastes) (Henze et al., 2008), industrial effluents (antibiotics, dyes, detergents, phenols, cyanide) (Leal et al., 2011), agricultural runoffs (pesticides, nitrogen, phosphorous, ammonium) (Eriksson et al., 2002; Henze et al., 2008), ions (such as Cl^-, K^+, Na^+, Fe^{++}, Zn^{++}, Ca^{++}etc) (Eriksson et al., 2002; Oaikhena et al., 2016; Radhi, 2012). Traces of these heavy metal(s) are essential for the survival of microorganisms as they are required for the enzymatic reactions (as cofactors) (Henze et al., 2008). High concentration of these heavy metal(s) have been reported to cause electrolyte imbalance in the microorganisms and even cause extreme toxicity by inhibiting metabolic reactions (Dixit et al., 2015; Eriksson et al., 2002). Bacteria survive in such harsh environment by utilizing genes responsible for mechanisms like efflux pump, biosorption to the cell walls, entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reactions (Chen et al., 2015; Dixit et al., 2015) which might even be cause for antibiotic(s) resistance (Galvin et al., 2010; Munir et al., 2011) and heavy metal(s) (Issazadeh et al., 2014; Klümper et al., 2017).

Antibiotic resistance is common in pathogens, opportunistic pathogens and even non-pathogenic bacteria (Galvin et al., 2010). Among these bacteria, the major etiological agents known for evoking antibiotic resistance are heavy metals and biocides (Rajbanshi, 2009; Yamina et al., 2012) followed by natural causes like genetic jugglery (Davies and Davies, 2010; Gautam and Adhikari, 2018), incorporation of viral genome of a bacteriophage undergoing lysogenic cycle (Gautam et al., 2018; Gautam and Adhikari, 2018; Labrie et al., 2010), plasmid incorporation inside the host cell (Allen et al., 2010; Klümper et al., 2017; Radhi, 2012) and horizontal transfer of resistant genes (Allen et al., 2010; Gautam and Adhikari, 2018; Munir et al., 2011; Shakibaie et al., 2009).

In the present study, bacteria were isolated from the treated wastewater and resistance pattern of isolates against antibiotics and heavy metals were assessed. The study aims to isolate the pathogenic bacteria from wastewater which are able to resist highest concentration of heavy metal illuminating the fact that bacteria have adaptive mechanisms (metal sorption, mineralization, uptake and accumulation, extracellular precipitation, enzymatic oxidation or reduction to a less toxic form and efflux pump) and thus can be used for bioremediation, biosorption, bio-precipitation etc. As bioaccumulation of heavy metals in plants and animals is one of the global issues which have known to cause morbidity and mortality. In industries, heavy metal resistant bacteria could be used to lower heavy metal concentration of the discharge/effluent so that bioaccumulation of toxic chemical will be low or zilch in the near future. If used then this process

could turn out to cheap, environment friendly and efficient way to clean up chemical rich environment.

Materials and methods

Sample collection and investigation

The study was conducted at Microbiology laboratory, Department of Microbiology, St. Xavier's College, Maitighar, Kathmandu, Nepal during the period of April 16 to June 16, 2017.

For the study, grab sampling was performed from the outlet of the secondary treatment plant (50 mL of secondary treated effluent) in sterile plastic bottles at 9:30 am. The sample was kept in a mini cooler filled with ice-pack and was transported to the laboratory.

Isolation, characterization and biochemical test

With the help of sterile micropipette and sterile glass spreader, 100 µL sample was spread on cetrimide agar, mannitol salt agar and mac conkey agar respectively. 10 mL sample was first enriched in selenite F broth and then was spread on salmonella-shigella agar. 5 mL sample was added in 10 mL azide dextrose broth containing tube. The plates and tubes were incubated at 37°C for 24 hours. A duplicate set of mac conkey and cetrimide agar plates were incubated at 44.5℃and 42℃ for 24 hours respectively. On the following day, the isolated colonies were inoculated in nutrient agar, was incubated at 37°C for 24 hours and then subjected to biochemical tests the next day (Gautam et al., 2017b; Gautam et al., 2018; Gautam and Adhikari, 2018).

Antibiotic susceptibility test

The isolated organisms (turbidity tallied with McFarland standard 0.5) were subjected to antibiotic susceptibility test according to Clinical and Laboratory Standards Institute (CLSI) guidelines 2017. As a control, antibiotic susceptibility test of ATCC 27853 was performed (CLSI, 2017).

Selection and sterilization of heavy metal

The heavy metals tested were sulfate salts: $CdSO_4.2.5H_2O$, $ZnSO_4.5H_2O$, $MnSO_4.H_2O$, and $FeSO_4.H_2O$. Each sulfate salt of 20 g was mixed with 20 mL of distilled water, to prepare a solution of ratio 10000 g/L (100%). The process was repeated for other sulfate salts too and were sterilized at 15lbs at l2l°C for 15 minutes. Double fold dilution was done in sterile distilled water so that the solution was in the ratio of 5000 g/L (50%), 2500 g/L (25%), 1250 g/L (12.5%).

Heavy metal resistance and minimum inhibitory concentration test (MIC)

Isolates were tested for its resistance to heavy metals by well diffusion method (Gautam et al., 2017a). Lawn culture of McFarland standard 0.5 tallied isolated organisms and ATCC 27853 were made on sterile Mueller-Hinton agar plates using a sterile cotton swab. The plates were dried for 15 minutes. Using sterile micropipette 10 µL of different concentration of metal (100%, 50%, 25%, 12.5%) were introduced into each of the wells along with control (sterile distilled water). The process was repeated for all the metal solutions. The plates were incubated at 37ºC for 24 hours. Presence of halo zone and resistance of isolates inside the halo zone were observed.

Quality control and statistical analysis

A sample was triplicated and was repeated 2 times in an interval of a week. Purity plating was performed for the media plates and equipment were calibrated. Before the use of metal, all the glassware was leached with 2N HNO_3. Data analysis was done using SPSS version 19.

Results

Biochemical tests revealed that the isolated bacteria were *S. aureus*, *E. faecalis*, *C. freundii*, *E. coli*, *E. aerogenes*, *P. mirabilis*, *P. vulgaris*, *Salmonella* Typhi, *P. aeruginosa*. Antibiotic susceptibility test revealed that *E. coli* were found to be 100%, 15%, 15% and 15% resistant to ampicillin, imipenem, tetracycline and gentamicin respectively. *E. aerogenes* were found to be 100%, 35% and 25% resistant to ampicillin, imipenem and gentamicin respectively. *C. frundii* were found to be 15%, 5% and 15% resistant to ampicillin, imipenem and gentamicin. *P. mirabulis* and *P. mirabulis* were found to be 25% and 37.5%; 25% and 125.5%; 16.67% and 37.5% resistant to ampicillin, imipenem and gentamicin respectively. *P. aeruginosa* were found to be 25%, 10%, 10%, 5% resistant to imipenem, amikacin, piperallicin and ceftazidime respectively. *Salmonella* Typhi were found to be 5.88%, 11.76%, 23.53% resistant to tetracycline, gentamicin and resistant to nalidixic acid respectively. *E. fecalis* were found to be 64.28%, 7.14% resistant to ampicillin and tetracycline respectively. *S. aureus* was found to be 45% resistant to penicillin G. This data is presented in Table 1 and represented in Figure 1.

Table 1: Bacteria isolated from the treated wastewater along with their antibiotic resistance pattern

Sn	Isolates	Z(n=20)	Antibiotics	P	G	A	T	V	C	I	Ak	Pi	Cz	Na	N
1	*S. aureus*	20	P, G, V, C	9	0	-	-	0	0	-	-	-	-	-	-
2	*E. faecalis*	14	T, A, V, N	-	-	9	1	0	-	-	-	-	-	-	0
3	*E. coli*	20	A, I, T, G	-	3	20	3	-	-	3	-	-	-	-	-
4	*E. aerogenes*	20	A, I, T, G	-	5	20	0	-	-	7	-	-	-	-	-
5	*C. freundii*	20	A, I, T, G	-	3	3	0	-	-	1	-	-	-	-	-
6	*Proteus*	12	A, I, T, G	-	2	3	0	-	-	3	-	-	-	-	-
	P. mirabilis														
	P. vulgaris	8	A, I, T, G	-	1	2	0	-	-	2	-	-	-	-	-
7	*Salmonella* Typhi	17	G, I, T, Na	-	2	-	1	-	-	0	-	-	-	4	-
8	*P. aeruginosa*	20	Ak, I, Pi, Cz	-	-	-	-	-	-	5	2	2	1	-	-

P= Penicillin (1 unit), G= Gentamicin (10 µg), A= Ampicillin (10 µg), T= Tetracycline (30 µg), V= Vancomycin (30 µg), C=Cefoxitin (30 µg), I= Imipenem (10 µg), Ak= Amikacin (30 µg), Pi= Piperacillin (100 µg), Cz= Ceftazidime (10 µg), Na= Nalidixic acid (30 µg), N= Nitrofurantoin (300 µg), n= sample size, Z= isolated frequency

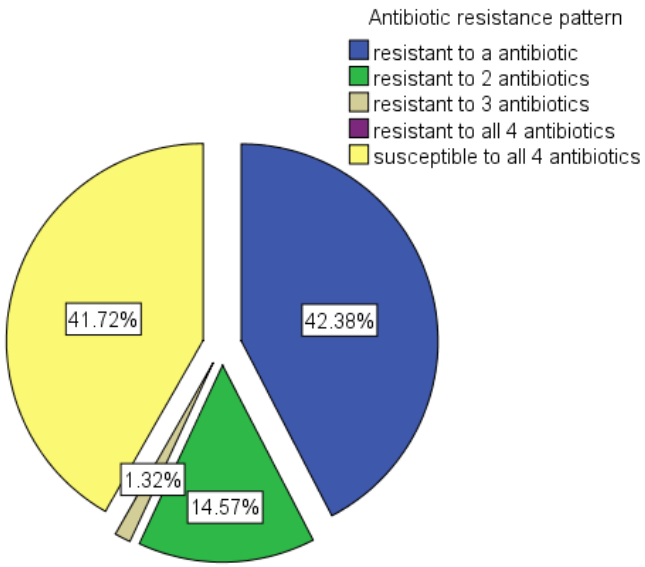

Figure 1: Antibiotic resistance pattern of the isolates against different antibiotics.

Fisher's exact test shows that there is a significant association ($p<0.001$ at 95% confidence interval) between isolates and antibiotic resistance pattern; isolates and heavy metal resistance pattern at dilution 10000 g/L, 5000 g/L, 2500 g/L and 1250 g/L. Cramer's V test reveals that the effect size between isolates and antibiotic resistance pattern is medium ($\varphi_c = 0.44$); the effect size between isolates and heavy metal resistance pattern of all dilutions (10000 g/L, 5000 g/L, 2500 g/L and 1250 g/L) is large ($\varphi_c = 1.00$) respectively.

From MIC test (Table 2 and Figure 2, 3, 4, 5) it is clear that with the exception of few bacterial isolates can resist up to dilution 5000 g/L; while all the isolates were resistant up to dilution 1:8 (Figure 6, 7, 8). *P. aeruginosa* isolates were able to resist up to dilution 1:1 (Figure 9).

Table 2: Isolates and their respective MIC dilution of different heavy metals

Sn	Heavy metal		*E. coli*	*P. aeruginosa*	*C. fruendi*	*E. aerogens*	*S. aureus*	*Proteus*		*E. faecalis*	*Salmonella* Typhi
								P. mirabulis	*P. vulgaris*		
			n= 20					n= 12	n= 8	n= 14	n= 17
1	MIC dilution of	Cd^{++}	A	B	A	A	A	A	A	B	B
2		Mn^{++}	A	A	A	A	A	A	A	A	A
3		Fe^{++}	B	*	A	B	A	A	A	A	A
4		Zn^{++}	A	A	A	A	A	A	A	A	A

*= resistant to all dilution n= sample frequency A= 5000 g/L (50%) B= 2500 g/L (25%)

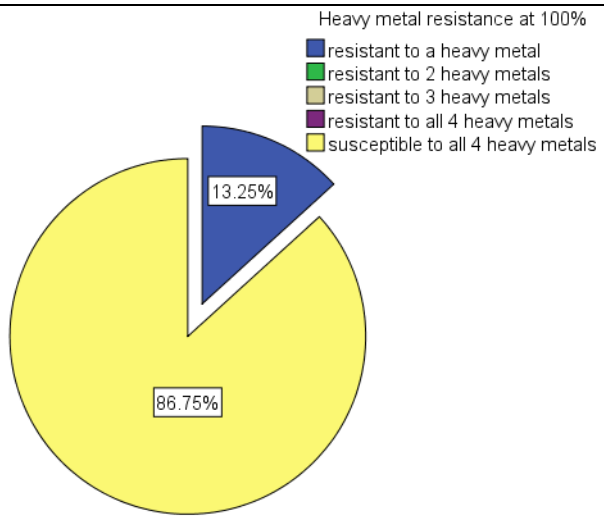

Figure 2: Heavy metal resistance pattern of the isolates against dilution 10000 g/L (100%) of different heavy metals.

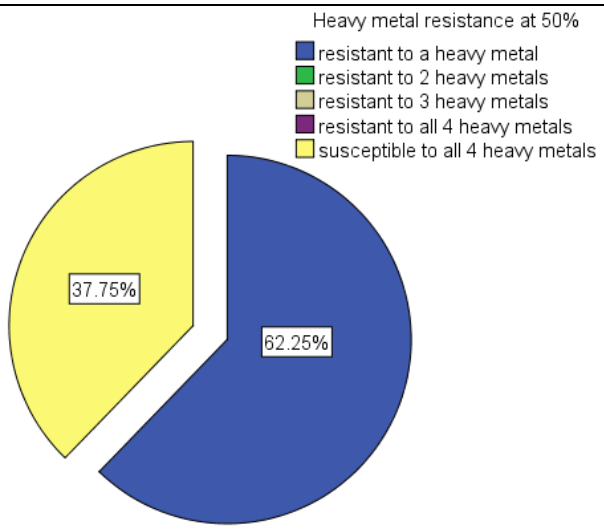

Figure 3: Heavy metal resistance pattern of the isolates against dilution 5000 g/L (50%) of different heavy metals.

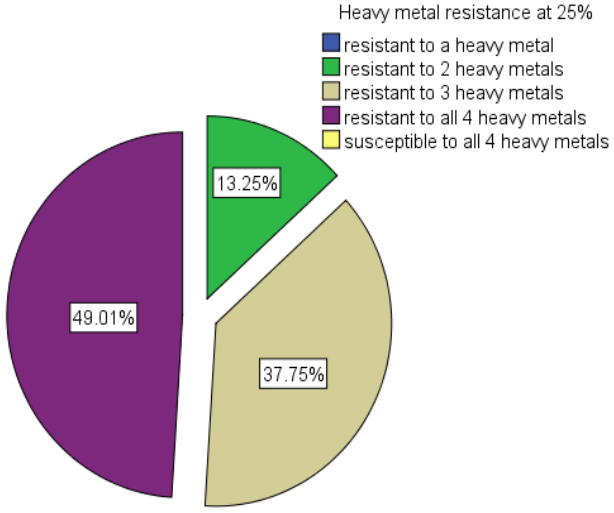

Figure 4: Heavy metal resistance pattern of the isolates against dilution 2500 g/L (25%) of different heavy metals.

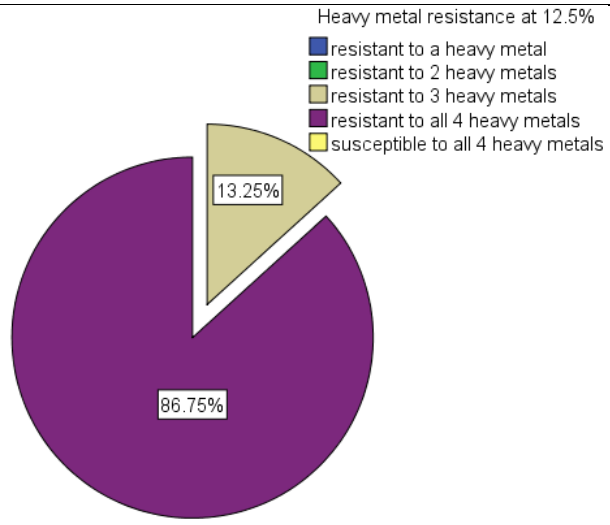

Figure 5: Heavy metal resistance pattern of the isolates against dilution 1250 g/L (12.5%) of different heavy metals.

Fisher's exact test shows that there is a significant association ($p < 0.001$ at 95% confidence interval) between antibiotic resistance pattern and resistance patterns at dilution 2500 g/L (25%) and no significant association between antibiotic resistance and heavy metal resistance pattern at dilution 10000 g/L (100%), 5000 g/L (50%), 1250 g/L (12.5%) ($p = 0.397, 0.126, 0.397$ at 95% confidence interval) respectively. Cramer's V test reveals that the effect size of antibiotic resistance pattern and heavy metal resistance pattern at dilution 1250 g/L is medium ($\varphi_c = 0.34$).

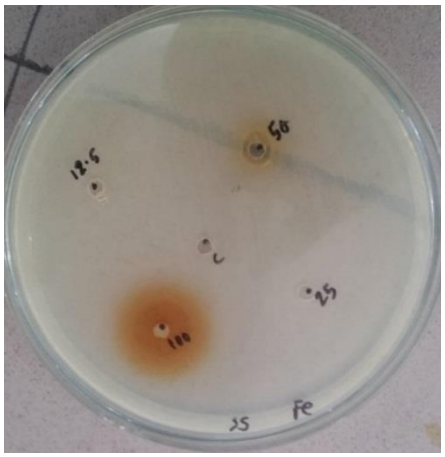

Figure 6: MIC for isolate SS against Fe^{++}
The isolate is resistant against Fe^{++} of dilution 2500 g/L (25%) and 1250 g/L (12.5%).

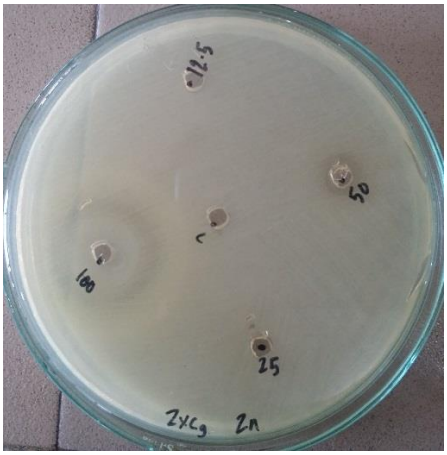

Figure 7: MIC for isolate Zxc_9 against Zn^{++}
The isolate is resistant against Zn^{++} of dilution 2500 g/L (25%) and 1250 g/L (12.5%).

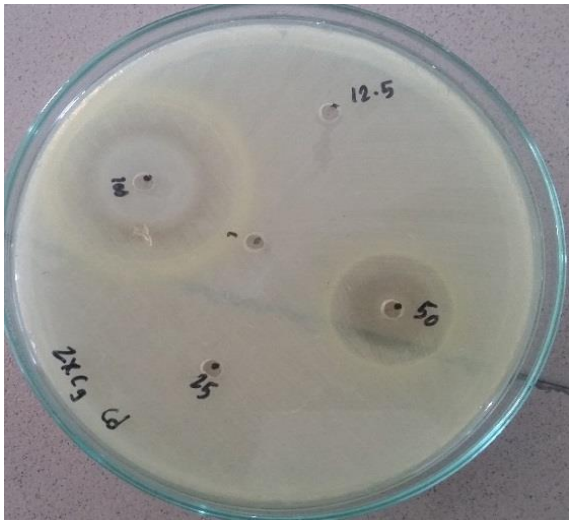

Figure 8: MIC for isolate Zxc_9against Cd^{++}
The isolate is resistant against Cd^{++} of dilution 2500 g/L (25%) and 1250 g/L (12.5%).

Figure 9: MIC for isolate P against Fe^{++}
The isolate is resistant against Fe^{++} even at dilution 10000 g/L and the mutated colony can be seen in the halo zone in dilution 10000 g/L (100%), 5000 g/L (50%), 2500 g/L (25%).

Discussion

The isolated bacteria from the effluent after treatment were *S. aureus, E. faecalis, C. freundii, E. coli, E. aerogenes, P. mirabilis, P. vulgaris, Salmonella* Typhi, *P. aeruginosa* (Table 1). The antibiotic susceptibility test revealed that some isolates were multi drug resistant (Table 1). The heavy metal resistance assessment assayed through MIC revealed that some isolates could resist low dilution (12.5%) of heavy metal(s), while some could resist to the highest dilution (100%) of heavy metal(s) (Table 2, Figure 4). Table 1, 2 and figure 1, 2, 3, 4, 5 also isolates showed multiple tolerances to both heavy metal(s) and antibiotic(s).

The result of this study agrees with the study of Yamina et al. (2012), Selvi et al. (2012), Rajbhanshi (2008) were multi drug resistant isolates (*P. aeruginosa, Staphylococcus* spp, *E. coli, Enterobacter* spp, *Proteus* spp, *S. aureus*) were found to be resistant towards heavy metals. This could be due to the screening of similar isolates and using similar heavy metals (Adekanmbi and Falodun, 2015; Rajbanshi, 2009; Selvi et al., 2012).

The result of this study agrees with the finding of the study conducted by Oaikhena et al. (2016), Yamina et al. (2012), Radhi et al. (2012), Adekanmbi et al. (2015), Shakibaei et al. (2008) where isolates (*P. aeruginosa, P. vulgaris, E. coli* and *S. aureus*) were resistant to heavy metals and antibiotics. The reason could be the similar selection of heavy metals, it's concentration and screening of similar isolates (Adekanmbi and Falodun, 2015; Oaikhena et al., 2016; Radhi, 2012; Yamina et al., 2012) and/or due to the little known antibiotic resistance of the majority of environmental isolates (Allen et al., 2010). The microbial resistance to heavy metal is attributed to a variety of detoxifying mechanisms like complexation by exopolysaccharides, binding with bacterial cell envelopes, metal reduction, metal efflux etc (Chen et al., 2015; Dixit et al., 2015). Since heavy metals are similar in their toxic mechanism, multiple tolerances are common phenomena among heavy metal resistant bacteria (Al-Gheethi et al., 2015; Dixit et al., 2015; Oaikhena et al., 2016). If the environmental factors are responsible for antibiotic resistance then superbugs and super resistance in the bacteria which can be considered as virulence factors (Davies and Davies, 2010; Gautam et al., 2018; Gautam and Adhikari, 2018).

The result of this study disagrees with the study of Naz et al. (2015), where isolates *Pseudomonas* spp, *Citrobacter* spp and *Enterobacter* spp were found to be resistant only up to 600 ppm, this could be due to the selection of different heavy metal salts.

In an investigation conducted by Tyrrel and Quinton (2003), wastewater containing pathogens of fecal origin were used in irrigation increasing the threat to the public masses consuming such goods, which could be minimized if the wastewater is treated with UV rays of sunlight (Sinton et al., 2002). When such wastewater was used for irrigation purpose in Pakistan, heavy metals accumulated in spinach and were the metal concentration in spinach was unsafe for human consumption as it may lead to metabolic and physiological disorders (Lone et al., 2003). These heavy metal resistant microorganism could be used for bioremediation (Al-Gheethi et al., 2015; Lone et al., 2003; Rajbanshi, 2009; Selvi et al., 2012; Shakibaei et al., 2008) without the fear of antibiotic resistance thanks to the techniques involving gene knockout (Chen et al., 2005; Heap et al., 2007; Liu et al., 2010), genome editing using Clustered Regular Interspaced Palindromic Repeats (CRISPR)/Cas9 (CRISPR- associated protein) (Friedland et al., 2013; Jiang et al., 2013; Rho et al., 2012; Sander and Joung, 2014). Since these heavy-metal resistance bacteria can tolerate or resist high concentration of heavy metals; expensive metals can be recovered, chemicals in effluents can be treated/leached (Rajbanshi, 2009; Shakibaei et al., 2008), toxic metal contaminated areas can be nullified/leached, site of oil and lead paint spillages sites can be treated to lower environmental poisoning (Dixit et al., 2015; Naz et al., 2016; Oaikhena et al., 2016; Rajbanshi, 2009). The heavy metal resistant microorganisms require abundant water (as indicated by the MIC test, Table 2, Figure 6-9) for the bioremediation purpose, this could be due to the fact that water will aid to somehow balance electrolyte of the microorganism.

Conclusion

Among the pathogenic isolates screened from the treated wastewater, few strains were resistant to multiple antibiotic(s) and heavy metal(s). Chemical concentration can be lowered using heavy metal resistant organisms but the general population will be at risk as heavy metals might evoke antibiotic resistance. The Microbes exposed to pollutants have higher heavy metal resisting capacity and can be used for environmental cleanup once their genes are knocked out and edited.

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