RESEARCH ARTICLE

STUDIES ON HEAVY METAL TOLERANCE AND ANTIBIOTIC RESISTANCE PATTERNS OF BACTERIAL POPULATION ISOLATED FROM EFFLUENT TREATED WATER OF DELHI

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ABSTRACT

Along with the growing industrialization, problem of toxic heavy metal contamination is increasing worldwide. Microbes related technologies may provide an alternative or addition to conventional method of metal removal or metal recovery. The present study dealt with isolation, identification and characterization of microorganisms showing tolerance towards several heavy metals and resistance against antibiotics. These bacterial strains were isolated from different effluent treatment plants located in suburb of Delhi. Initially samples were analyzed for the presence of *B.cereus, P.aeruginosa, E. coli* & *S.aureus* through biochemical and morphological methods. Primary screening methods were applied to isolate heavy metals (viz. lead, zinc and cadmium) resistant organisms. Further susceptibility patterns of these isolates were studied towards clinically significant antibiotics such as meropenem, Doxycycline, streptomycin, tetracycline, gentamycin, cloxacillin, amoxicillin, ampicillin, vancomycin, and cephalosporin. All isolates were found to have 100% susceptibility against cadmium & 100% resistance against lead except *P.aeruginosa* (67%). Doxycycline was found to be most inert antibiotic as it was ineffective against all isolates, whereas meropenem was found to be most promising antibiotic followed by cephalosporin, streptomycin, tetracycline, and gentamycin. These heavy metal resistant organisms could be a potential agent for bioremediation of heavy metals polluted environment.

Keywords: Heavy metals, Effluent treatment water, Antibiotics, Susceptibility, Resistance, Bioremediation.

INTRODUCTION:

Pollution in source water is a problem in developing as well as in developed countries (American Society for Microbiology (ASM) Colloquium Report, 1999). In the past few decades, uncontrolled urbanization has caused a serious pollution problem due to the disposal of sewage and industrial effluents to water bodies. Effluent wastewater treatment is the process of removing contaminants from wastewater and household sewage, both runoff (effluents) and domestic. It includes physical, chemical, and biological processes to remove physical, chemical and biological contaminants. Its objective is to produce a waste stream (or treated effluent) and a solid waste or sludge suitable for discharge or reuse back into the environment. This material is often inadvertently contaminated with many toxic organic and inorganic compounds. Unlike many other pollutants such as heavy metals are difficult to remove from the environment [30]. Heavy metals are recognized to be powerful inhibitors of biodegradation activities [11]. These metals cannot be degraded, and are ultimately indestructible. Heavy metal contamination is widespread. In nature, there are about

50 heavy metals of special concern because of their toxicological effect to human beings and other living organisms. Heavy metals such as Lead (*Pb*), Mercury (*Hg*), Cadmium (*Cd*), Nickel (*Ni*), Zinc (*Zn*), Copper (*Cu*), Chromium (*Cr*), when accumulated in soils, water bodies they can also be present in concentrations toxic to plants, animals, humans and aquatic life [12]. At high concentrations these metals form unspecific complex compounds in the microorganism, which leads to toxic effects. The toxic effects of heavy metals result mainly from the interaction of metals with proteins (enzymes) and inhibition of metabolic processes. Each heavy metal has unique bio-functions or bio-toxicities. For example, copper can enhance microbial growth at low concentrations but repress growth at high concentrations [41] and cadmium has high toxicity at low concentrations [22]. Most heavy metals are metabolically poisonous in low concentration. They inhibit the activities of certain enzymes involved in the metabolic processes [24]. By affecting the growth, morphology and biochemical activities, heavy metals influence the microbial population and resulting in decreased biomass as well as diversity. Microbes play massive role in the bio-

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geochemical cycling of toxic heavy metals and also in cleaning up or remediating metal-contaminated environments. Microorganisms have acquired a variety of mechanisms for adaptation to the presence of toxic heavy metals [31]. The general believes are these resistances arisen as a result of human pollution in recent centuries. However, it seems more likely that these resistances arose soon after life began, in a world already polluted by volcanic activities and other geological sources. Similar to antibiotic resistances are preexisted in the pre-antibiotic era.

Bacteria develop heavy-metal resistance mostly for their survivals, especially a significant portion of the resistant phenomena was found in the environmental strains (with or without the presence of heavy metals). One theory for bacterial heavy-metal resistance evolved is due to the use of antibiotics. For example, bacterial antibiotic-plasmids (sometime these plasmids are very big and called mega-plasmid) existed in bacteria before the antibiotic era but their presence was brought into prominence by the use of antibiotics, which selected for antibiotic resistant strains. Aquatic microbes become resistant to antibiotics and metals as a result of contamination with effluents [38]. The mechanism of heavy metal resistance is found to enhance the antibiotic resistance ability of microorganisms [13]. and they are strongly correlated between each other [6]. The number of antimicrobial-resistant (AMR) bacteria in the environment increases exponentially with the use of antimicrobials, as a result of increasing selective pressure on bacterial populations [32, 25 and 39] and its spread between different bacterial strains in different habitats has also been demonstrated [40, 34 and 33]. The resistant to antibiotics and heavy metal are found to be plasmid mediated. The genetic determinant responsible for the heavy metal resistance often resides on plasmids which mediate antibiotic resistance. The resistance development may be due to nonspecific mechanism with gene regulation of plasmids

and chromosomes, which may be heritable or transferable due to the presence of a resistance (R-factor) factor [35]. Bacterial species had been isolated from drinking water that was tolerant to metals and antibiotics [7]. The significant increase of Multiple Antibiotic Resistant (MAR) bacteria are observed in various aquatic systems. Human infections caused by such bacteria could be difficult to treat with drugs [8, 10, 23, and 14]. To survive under metal-stressed conditions, bacteria have evolved several types of mechanisms to tolerate and uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, and reduction of the heavy metal ions to a less toxic state [27 and 36].

The aim of our study was to evaluate the antibiotic and heavy metal resistance patterns of bacteria which were isolated from ten different prominently critical Effluent treated water from suburb of Delhi. The study involves; (a) Collection of water samples from different effluent treatment plants (b) Isolation of *E.coli, S.aureus, B.cereus, P.aeruginosa,* from water samples. (c) Evaluation the % of resistance against Lead (*Pb*), Zinc (*Zn*) and Cadmium (*Cd*) by primary screening method. (d) Determination of susceptibility and resistance pattern against ten different antibiotics by agar well diffusion assay (e) Interpretation of the data generated to determine the resistance patterns of the isolated bacteria towards three heavy metals (*Zn, Pb , Cd*) and to correlate metal and antibiotic resistance for the benefit of human welfare by increasing general awareness among the people.

MATERIALS AND METHODS:

Collection of water samples: Gamma irradiated, clean and sterilized bottles (1lt capacity) were used for sampling of water from ten different effluent treatment plant located in suburb of Delhi are shown in Table 1. Samples after collection were encoded with ETPW01 to ETPW10.

Table 1: Sites for Sample Collection with Sample code

S. No.	Sample Location	Sample Code
1	Noida Industrial Effluents	ETPW01
2	Badarpur Power out	ETPW02
3	Badarpur Power in	ETPW03
4	Okhla Head	ETPW04
5	DND Highway	ETPW05
6	Nizamuddin	ETPW06
7	IP Powerhouse	ETPW07
8	Rajghat Power House	ETPW08
9	Wazirabad Highway	ETPW09
10	Nijafarbad Industrial Effluents	ETPW10

For dechlorination sodium thiosulphate was added to the clean, dry sampling bottles before gamma sterilization in an amount to provide an approximate concentration of 100mg/lit in the sample. Aseptic conditions were maintained during the collection of samples. The samples were kept in an ice pack to prevent any changes in the microbial flora of the samples during the transportation. The water samples were transported to the lab in vertical position maintaining the temperature 1-4°C with ice pack enveloped conditions. Samples were analyzed within 6 h of collection.

Isolation and Identification of *E.coli*: 250 ml of each water samples were filtered through 0.45 micron filter paper, after filtration filter paper was aseptically transfer to MacConkey Broth and incubated at 37ºC for 48 hrs. Loopful culture from the flasks showing acid and gas was subcultured on Eosine Methylene Blue agar (EMBA) as well as on Mac Conkey agar (MCA) plates and were incubated at 37ºC for 24 hrs. Characteristic colonies were further confirmed by biochemical test using HiIMViC test kit (Hi media) as per Indian Standard [18].

Isolation and Identification of *S.aureus*: 250 ml of each water samples were filtered through 0.45 micron filter paper, after filtration filter paper was aseptically transfer to Cooked meat medium with 10% salt and incubated at 37ºC for 24 hrs. Subcultured on Baird Parker Agar (BPA) and incubated at 37ºC for 30 hrs. Characteristic black shiny colonies with grey margin were further confirmed by Gram's staining and biochemical test as per Indian Standard [21].

Isolation and Identification of *Pseudomonas aeruginosa*: 250 ml of each water samples were filtered through 0.45 micron filter paper after filtration filter paper was aseptically transfer to Cetrimide Broth and then incubated at 37 ^{0}C for 48 hrs. Subcultured on the plates of Skim Milk Agar (SMA) and further confirmation was done by Gram's staining and biochemical tests as per Indian Standard [20].

Isolation and Identification of *Bacillus cereus*: 250 ml of each water samples were filtered through 0.45 micron filter paper; after filtration filter paper was aseptically transfer to Buffered peptone water (BPW) and incubated all flasks at 37ºC for 48 hrs. Subcultured on the plates of Mannitol Yolk Polymixin B agar (MYPA) and further confirmation was done by Gram's staining and biochemically by using several analytical methods as per guidelines of Indian Standard [19].

Primary Screening against Heavy metals: Resistance pattern of above isolated strains were studied against Zinc (*Zn*), Cadmium (*Cd*) and Lead (*Pb*). 1000 ppm stock solution was prepared for these metals.50 ppm concentration of working solution was prepared from each stock solution .This solution was used for primary screening technique in this study.150 µl of each working solution was added to 150 ml of nutrient agar media. All bacterial isolates were streaked on nutrient agar plates and plates were incubated at 37°C for 24 hrs. After incubation % of resistance against three heavy metals was evaluated for microbial cultures used for this study.

Antibiotics and their solutions: Ten antibiotics like – STREPTOMYCIN (Streptomycin IP, Mfd by: Nicolas Piramal India LTD), TETRACYCLINE (Tetracycline hydrochloride capsules IP 250mg, Mfd by-Cipla limited), AMPICILLIN (Ampicillin hydrochloride capsules IP 250mg, Mfd by-Cipla limited), AMOXICILLIN (Amoxicillin Trihydrate capsules IP 250mg, Mfd by-Cipla limited), GENTAMYCIN (Gentamycin IP, Mfd by: Nicolas Piramal India LTD), DOXYCYCLINE (Doxycycline hydrochloride IP 100mg, Mfd by-Cipla limited), CLOXACILLIN (Cloxacillin sodium IP 500mg, Mfd by: Nicolas Piramal India LTD), CEPHALOSPORIN (Cephalosporin IP 400mg, Mfd by-Eurolife Health Care), VANCOMYCIN (Vancomycin IP, Mfd by-Cipla limited) and MEROPENEM (Meropenem buffer sterile USP, Mfd by- Shenzhen haibin pharmaceuticals) were used to check susceptibility and resistance pattern of above four bacterial isolates. All these antibiotics were obtained from local pharmacy store and working solution having 10mg/ml concentration of each antibiotic was used for the study.

Inoculum Preparation: Above four bacterial isolates were sub cultured on non selective nutrient agar slants. The bacterial cultures were incubated overnight at 37°C. 0.5 McFarland density of bacterial isolates was adjusted using normal saline (0.85% NaCl) using densitometer to get bacterial population of 1.0×10^8 cfu/ml.

Agar Well Diffusion Assay (Zone of Inhibition Evaluation): Antibiotic susceptibility and resistance were evaluated by agar well diffusion assay [9]. 100µl of each of the adjusted cultures were mixed into separate 100 ml of sterile, molten, cool Muller Hinton Agar (MHA), mixed well and poured into sterile petri plates. These were allowed to solidify and then individual plates were marked for each individual bacterial isolates. Each plate was punched to make wells of 6 mm diameter with the help of sterile cork borer at different sites of the plates. 100 µl of respective antibiotic solutions were pipette into

the well in assay plates. Plates were incubated overnight at 37°C. Following incubation, petri-plates were observed for the inhibition zones, diameters of which were measured by using Vernier Calipers.

RESULTS & DISCUSSION:

In the present study, ten water samples were collected from ten different Effluent treatment plants located in Delhi. These samples were analyzed for the presence of *E.coli, S.aureus, Bacillus cereus and Pseudomonas aeruginosa.* During the study, *E. coli* was isolated from all ten sampling locations; *S. aureus* were isolated from seven locations except ETPW 03, ETPW 05 & ETPW 06. Eight strains of *B.cereus* were isolated from water sample except ETPW 05 & ETPW 06. Out of ten locations *Pseudomonas aeruginosa* were isolated from nine except ETPW 06. These isolates were biochemically characterized and then evaluated for their % of resistance patterns against Zinc, Lead & Cadmium by using primary screening technique as well as their susceptibility patterns also evaluated against ten commonly prescribed

clinically significant antibiotics by using agar well diffusion assay. Table 2 demonstrated the % of resistance patterns of bacterial isolates against three heavy metals used in our study.

Data revealed that all *E.coli, S.aureus* & *B.cereus* were shown resistance (100%) towards Lead. Out of nine *Pseudomonas* strains six were resistant against i.e. 67% Lead. Due to high toxicity all bacterial strains isolated was found to be highly susceptible against Cadmium. All *S.aureus* & *B.cereus* strains isolated here were shown 100% susceptible towards Zinc. On the other hand only 30% *E.coli* and 33% *P. aeruginosa* were resistant against Zinc. The most vulnerable metal was found to be Lead against which all bacterial isolates shows the resistance. Many bacterial species isolated from industrial zones had been shown to develop resistance to heavy metals [28 and 3]. Resistance patterns of above four isolates against three heavy metals used for our study have been shown in Fig 1.

Table 2: Heavy metal resistance incidence of isolated in percentage (%)

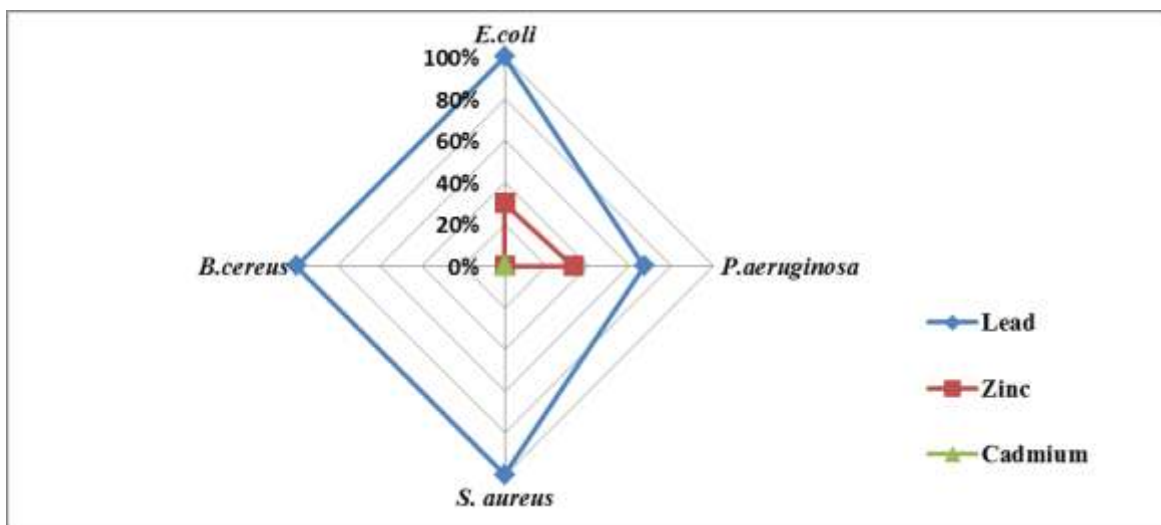| Heavy metals	% of Resistance			
	E.coli	*P aeruginosa*	*S.aureus*	*B. cereus*
Lead	100%	67%	100%	100%
Zinc	30%	33%	0%	0%
Cadmium	0%	0%	0%	0%

Figure 1: Percentage of resistance against Heavy metals

In the polluted sites bacteria are continuously exposed to different heavy metals, thus giving rise to survival of the metal tolerant. Even most of the strains which are not metal tolerant becomes tolerant due to mutations. Thus these strains assist in natural transformation leading to the increased incidence of metal tolerant strains in such

environment and also dissemination to atmosphere. Association between resistance to antibiotics and heavy metals has been reported [4] earlier. The combined expression of metal tolerance and antibiotic resistance is caused by selection resulting from metals present in the environment [26]. Earlier bacterial strains resistant to

Gentamycin and Penicillin were also resistant against to heavy metals [4 and 15]. In this study it is clearly seen that the bacterial isolates also show non vulnerability to different antibiotics.

The antibiotic resistance patterns in terms of average zones of diameter considering 4 plates for bacterial isolates against each of ten antibiotics of 10mg/ml concentration were calculated and shown in Table 3.

Table 3: Antibiotic resistance patterns of different pathogens

Antibiotics used	Zone of inhibition*			
	E.coli	*P aeruginosa*	*B. cereus*	*S.aureus*
Streptomycin	21	23	17	22
Gentamycin	13	15	14	15
Tetracycline	20	18	19	21
Amoxicillin	0	0	24	21
Ampicillin	0	0	24	18
Cephalosporin	15	26	24	22
Meropenem	22	26	27	29
Vancomycin	0	0	18	15
Doxycycline	0	0	0	0
Cloxacillin	0	26	19	25

*Zone of inhibition in mm. Diameter including well diameter of 6.0 mm

Four pathogens were tested for their antibiotic sensitivity patterns. Resistance rate was high for Gram negative bacteria. *E.coli* was resistant to Amoxicillin, Ampicillin, Vancomycin, Cloxacillin and Doxycycline. *P. aeruginosa* was resistant to Amoxicillin, Ampicillin, Vancomycin and Doxycycline. The most vulnerable antibiotic was found to be Doxycycline against which both Gram positive as well as Gram negative bacteria isolates shows the resistance i.e. 100% resistance. Percentages of susceptibility against antibiotics were demonstrated in Table 4. All ten *E.coli* isolates were shown 100% susceptibility against Streptomycin, Gentamycin, Tetracycline and Meropenem. Intermediate susceptibility i.e. 57% was shown by *E.coli* towards Cephalosporin. In case of seven *S.aureus* isolates 100% susceptible against Streptomycin, Cephalosporin, Meropenem and Vancomycin. Susceptibility range was between 14% to 85% (Table 4) of *S.aureus* towards other antibiotics except Doxycycline.

Table 4: Percentage of pathogens susceptible to antibiotics

Antibiotic used	% Susceptible pathogens			
	E.coli	*P aeruginosa*	*B. cereus*	*S.aureus*
Streptomycin	100	88	84	100
Gentamycin	100	50	89	43
Tetracycline	100	75	84	85
Amoxicillin	0	0	32	43
Ampicillin	0	0	32	29
Cephalosporin	57	100	79	100
Meropenem	100	50	100	100
Vancomycin	0	0	89	100
Doxycycline	0	0	0	0
Cloxacillin	0	38	21	14

However, *P. aeruginosa* showed more susceptibility towards Streptomycin, Tetracycline, Gentamycin and Meropenem. Among nine *P. aeruginosa* isolates all were susceptible (100%) against Cephalosporin and only 38% were susceptible against Cloxacillin. In case of *B.cereus* susceptibility range was vary between 21% to 100% (Table 4). Among eight *B.cereus* isolates all were susceptible towards Meropenem. Multiple antibiotic resistances were shown by two Gram negative bacteria isolates i.e. *E.coli* and *P. aeruginosa.* Meropenem was

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found to be more promising as all four bacterial isolates have shown high level of susceptibility followed by Cephalosporin, Streptomycin, Tetracycline, Gentamycin etc. The cumulative effectiveness of the antibiotics as obtained in this study is Meropenem > Cephalosporin >

Streptomycin > Tetracycline > Gentamycin > Cloxacillin > Amoxicillin > Ampicillin > Vancomycin > Doxycycline. Susceptibility patterns of all four isolates towards antibiotics used in the present study also shown in Fig 2.
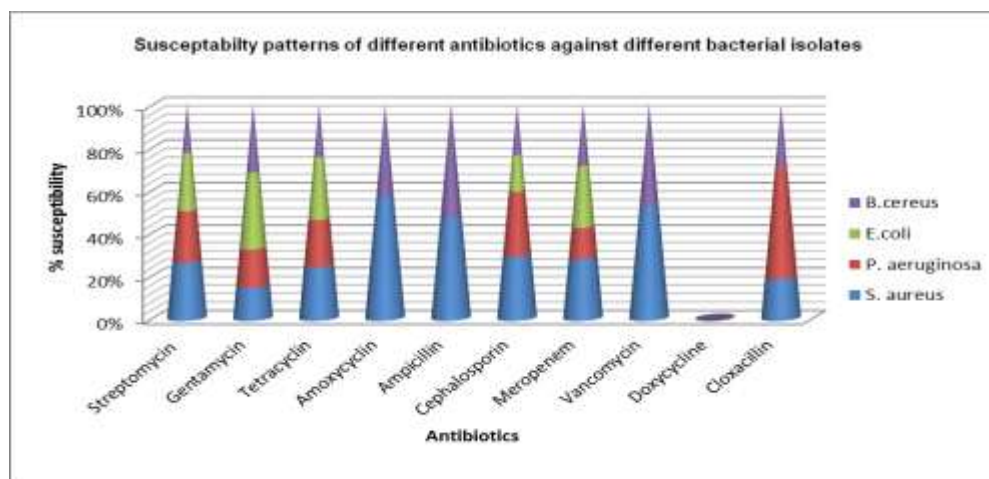

Figure 2: Susceptibility patterns of bacterial isolates against antibiotics

A lot of study has previously been done in this area to evaluate the contamination of water bodies and isolation of resistant microorganisms from different environment and clinical samples because the presence of antibiotic resistant bacteria in natural habitat can pose severe public health risk. Resistant bacteria have been isolated from a variety of sources, including domestic sewage, drinking water, rivers, and lakes and it may be due to the selection resulting from heavy metals present in the particular environment. The bacterial isolates from the Antarctic waters from the Indian side were examined for the incidence of metal and antibiotic resistance bacteria during the austral summer along the cruise track extending from 50 degrees South and 18 degrees East to 65 degrees South and 30 degrees East [16]. In the year 2006 one of the study revealed the impact of bacterial resistances to diverse metals and antibiotics that are often genetically linked, suggesting that exposure to toxic metals may select for strains resistant to antibiotics and vice versa [37]. In one of the study the total aerobic heterotrophic and metal-resistant bacterial communities were studied in marine water. The resistance patterns, expressed as MICs, for 81 bacterial isolates to eight heavy metals were surveyed by using the agar dilution method. A great proportion of the isolates were sensitive to Cadmium (99%), Mercury (91%), Zinc (84%) and Cobalt (83%). On the other hand, 94%, 40%, 35% and 22% were resistant to Lead, Nickel, Arsenate and Copper respectively. The majority of the tested strains (95.06%) were multiple metal-resistant, with penta-metal resistance as the major pattern (25.9%). The response of

the isolates to 11 tested antibiotics was tested and ranged from complete resistance to total sensitivity and multiple antibiotic resistances was exhibited by 70.38% of the total isolated population. The highest incidence of metal-antibiotic double resistance existed between Lead and all antibiotics (100%) that of Copper and Penicillin and Nickel and Ampicillin are 95%. A high percentage of the isolates (40%) demonstrated resistance to Nickel. The percentage of resistance for the 81 strains at the standard levels of the eight heavy metals was tested. Nearly all strains exhibited resistance to lead (98.76%). In our study multiple antibiotic resistances with tolerance to Lead were observed among all four isolates both Gram positive as well as Gram negative bacteria. *E.coli* and *P. aeruginosa* both Gram negative bacteria have shown high percentage of multiple antibiotic resistances as well as resistance against both Lead and Zinc.

Based open these study it was found that most of the isolates in the present study showed multiple tolerances to both heavy metals and antibiotics. Since heavy metals are all similar in their toxic mechanism, multiple tolerances are common phenomena among heavy metal resistant bacteria. In wastewater, there are some substances that have the potential to select for antibiotic resistance even though they are not antibiotics themselves. Heavy metals and biocides are two of them. The exposure to heavy metals or biocides results in the selection of bacterial strain also able to resist antibiotics. The genes that code for antibiotic resistance traits and genes that code for metal resistance are often carried on the same plasmid or mobile genetic elements [42 and

43]. This shows that there is a close association between metal resistance and antibiotic resistance.

CONCLUSION:

The industrial effluents are enriched media to grow and spread microbial population. An alarming consequence has been occurred due to widespread emergence of resistance among microorganisms against clinically significant antibiotics as well as different heavy metals. The identification of resistance against different heavy metals may provide a useful tool for the simultaneous monitoring of several toxic pollutants in the environment. It is clearly indicated that domestic waste and industrial waste are responsible for the development of bacterial resistance along with the risk of human health and environment. Thus, this study is highly informative to suggest that the potential impact of metal polluted locations in human life may be much greater than the direct effect of the pollution. Among all the isolates *E.coli* and *Pseudomonas* showed resourceful tolerance against heavy metals used here. Hence these species can be used as a bioremediation tool for the treatment of effluent handling heavy metals like Lead and Zinc. Regular surveillance of the effluent in industrial sites is a must if the risk of disease due to such antibiotic resistant organisms is to be avoided. The results were indicative of very high antimicrobial resistance to Doxycycline among all bacterial isolates involved in our study. Due to the uncontrolled use of this antibiotic led to the generation of multi-drug resistant strains. The fourth generation antibiotic i.e. Meropenem is found to have significant efficacy and can be considered appropriate for empirical treatment of above four bacterial infections. Although the present study can lead to beneficially assist in the identification of alternate drug to control these multi-drug resistant bacterial strains*.

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