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Original Article

In -Vitro Antibiotic Resistance and Heavy Metal Tolerance Patterns of Gram-Positive and Gram-Negative Bacteria Isolated From Effluent Treated Water of Delhi, India.

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Abstract

Heavy metal contamination and antibiotic resistance is a global problem. Bacterial populations in metal polluted environment adapt to toxic concentration of heavy metals and become metal resistant. In the present study several samples were collected in gamma sterilized bottles for evaluating the heavy metal tolerance and antibiotic resistance pattern of isolated bacterial strains. Isolation and identification of bacterial strains were done by using IS guidelines. These bacterial strains were isolated from different effluent treatment plants located in community of Delhi. All the samples were analyzed for the presence of Gram-positive bacteria like *S.aureus*, and for Gram-negative bacteria like *P.aeruginosa, E. coli*. After isolation, biochemical and morphological methods were followed for the identification of strains. Primary screening methods were applied to isolate heavy metals resistant organisms. Further susceptibility patterns of these isolates were studied towards Several Antibiotics such as Amoxicillin, Ampicillin, Cloxacillin, Cephalosporin, Doxycycline, Gentamycin, Meropenem, Streptomycin and Tetracycline. All isolates were found to have 100% resistance against lead except *P.aeruginosa* (67%). Gram-positive bacteria i.e. *S.aureus* was found susceptible when checked against all selected antibiotic except Doxcyline where no zone of inhibition was observed. Organisms such as *E.coli and P. aeruginosa showed variable resistance pattern* These heavy metal resistant organisms could be a potential agent for bioremediation of heavy metals polluted environment.

Keywords: Gram-Positive Bacteria, Gram-Negative Bacteria, Heavy metals, Effluent treatment water, Antibiotics.

1. Introduction

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.

Pollution in source water is a problem in developing as well as in developed countries (American Society for Microbiology (ASM) Colloquium Report, 1999). 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

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[33]. Heavy metals are recognized to be powerful inhibitors of biodegradation activities [14]. 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 [15]. 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 [44] and cadmium has high toxicity at low concentrations [25]. Most heavy metals are metabolically poisonous in low concentration. They inhibit the activities of certain enzymes involved in the metabolic processes [27]. 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-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 [34]. 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 megaplasmid) 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 [41]. The mechanism of heavy metal resistance is found to enhance the antibiotic resistance ability of microorganisms [16]. 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 [35, 28 and 42] and its spread between different bacterial strains in different habitats has also been demonstrated [43, 37 and 36]. 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 [38]. Bacterial species had been isolated from drinking water that was tolerant to metals and antibiotics [10]. 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, 13, 26, and 17]. 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 [30, 39]. 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, P.aeruginosa*, from water samples. (c) Evaluation the % of resistance against Lead (Pb) and Zinc (Zn) 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) and to correlate metal and antibiotic resistance for the benefit of human welfare by increasing general awareness among the people.

Materials and Methods

Method used for the Collection of water samples

On the basis of literature, samples locations were selected and collection plan was prepared. 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 Water samples from different effluent treatment plant were collected in Gamma irradiated clean and sterilized bottles (1lt capacity). All the samples were marked according to the locations and other essential information including date of collection. All the samples brought to Laboratory in ice box and were analyzed within 6 h of collection.

Selected Sites with Sample code

The samples collected from Delhi are coded as ETPD-1(BDP), ETPD-2 (BDP), ETPD-3(OHE), ETPD-4(DNDH), ETPD-5(NETP), ETPD-6(IP), ETPD-7(RP), ETPD-8(WH) and ETPD-9(NI). The code has been given to the collected samples not to disclose the contaminated site as to maintain the confidentiality of research findings.

Procedure used for 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 MacConkey 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 [21].

Interpretation: On the basis of characteristics colonies as observed on selective media and biochemical evaluation, results are recorded as *E. coli* 'Present or Absent/250ml of Water Sample'

Quality Control: During the experiment, quality control is achieved by running the pure cultures obtained from Culture Collection Centers. During this experiment, *E. coli* as 'Positive Control' and *S. aureus* as 'Negative Control' are used.

Procedure used for Isolation and Identification of *S.aureus*: 250 ml of each in 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 [24].

Interpretation: On the basis of characteristics colonies and biochemical test, results are recorded as '*S. aureus* present or Absent/250ml of Water Sample'.

Quality Control: During the experiment, quality control is achieved by running simultaneously, the pure cultures of *S. aureus* as 'Positive Control' and *E. coli* as 'Negative Control'.

Procedure used for 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 [22].

Interpretation: On the basis of characteristics colonies and biochemical test, results are recorded as '*Pseudomonas aeruginosa* present or Absent/250ml of Water Sample'.

Quality Control: During the experiment, quality control is achieved by running simultaneously, *Pseudomonas aeruginosa* as 'Positive Control' and *E. coli* as 'Negative Control'.

Heavy metals and their Primary Screening: 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 37o C for 24 hrs. After incubation % of resistance against three heavy metals was evaluated for microbial cultures used for this study

Antibiotic Solutions

All these antibiotics were obtained from local pharmacy store and working solution having 10mg/ml concentration of each antibiotic was used for the study.

Preparation of Inoculum

Both Gram-positive and Gram-negative bacteria species 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 x 108 cfu/ml.

Cup Plate Method for Antibacterial Activity: Antibiotic susceptibility and resistance were evaluated by agar well diffusion assay [9, 10, 11, 12]. 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 37o C. Following incubation, petri-plates were observed for the inhibition zones, diameters of which were measured by using Vernier Calipers.

Results and Discussion

The results of the present study are summarized in the tables. Nine 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* and *Pseudomonas aeruginosa*. During the study, *E. coli* was isolated from all nine sampling locations; *S. aureus* were isolated from six locations except ETPD-2 (BDP), ETPD-4(DNDH) and ETPD-5(NETP). Out of nine locations *Pseudomonas aeruginosa* were isolated from eight except ETPD-5(NETP). These isolates were biochemically characterized and identified and then evaluated for their % of resistance patterns against Zinc and Lead by using primary screening technique as well as their susceptibility patterns also evaluated against nine commonly prescribed clinically significant antibiotics by using cup-plate zone of inhibition assay. Isolated Bacterial strains were checked against two common heavy metals such as Lead and Zinc. Data revealed that all *E.coli*, *S.aureus* was shown resistance (100%) towards Lead. Out of eight *Pseudomonas strains* five were resistant against i.e. 67% Lead. All *S.aureus* 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 [31, 3].

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 [29]. Earlier bacterial strains resistant to Gentamycin and Penicillin were also resistant against to heavy metals [4 and 18]. In this study it is clearly seen that the bacterial isolates also show non vulnerability to different antibiotics. All isolated bacterial strains were tested for their antibiotic sensitivity patterns. Resistance pattern of bacterial strains are given in the table. Gram-positive bacteria i.e. *S.aureus* was found susceptible when checked against all selected antibiotic except Doxcyline where no zone of inhibition was observed. Organisms such as *E.coli and P. aeruginosa showed variable resistance pattern as shown in the table.*

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 [19]. 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 [40]. 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 [45 and 46]. This shows that there is a close association between metal resistance and antibiotic resistance.

Conclusion

The research data of present study reflect the variable resistant and Heavy metal tolerance patterns when checked against isolated bacterial strains. 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 present study may be help out in the identification of alternate drug to control these multidrug resistant bacterial strains and can be a useful literature as far as Research and Development in this area is concerned.

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Table 1. Biochemical Tests carried out for the Identification of *E. coli.*

S. No.	Name of test	Response of *E. coli*
1.	Gram's Staining	Gram Negative Rods
2.	Motility	Motile
3.	Indole production	Positive
4.	Citrate utilization	Negative
5.	Glucorinidase	Positive
6.	Nitrate reduction	Positive
7.	ONPG	Negative
8.	Lysine utilization	Positive
9.	Lactose	Positive
10.	Glucose	Positive
11.	Sucrose	Positive
12.	Sorbitol	Positive

Table 2. Biochemical tests carried out for further Identification.

S. No.	Name of test	Response of *S. aureus*
1.	Gram's Staining	Gram positive cocci
2.	Coagulase test	Positive

Table 3. Biochemical tests carried out for further Identification.

S.No.	Name of test	Response of *P. aeruginosa*
1.	Gram's Staining	Gram Negative Rods
2.	Catalase test	Positive
3.	Oxidase test	Positive
4.	Hugh Leifson's test	Positive(Oxidative)
5.	Nitrate Reduction	Positive
6.	Gelatin Liquefaction	Positive
7.	Casein hydrolysis	positive
8.	Starch Hydrolysis	Positive
9.	Growth at 42 0C	Positive
10.	Growth at 4 0C	Negative

Table 4. Isolation and Identification of Bacteria from collected water samples.

S. No.	Sample Code	Bacteria Isolated
1.	ETPD-1(BDP)	*E.coli, S. aureus* and *P. aeruginosa*
2.	ETPD-2 (BDP)	*E.coli* and *P. aeruginosa*
3.	ETPD-3(OHE)	*E.coli, S. aureus* and *P. aeruginosa*
4.	ETPD-4(DNDH)	*E.coli* and *P. aeruginosa*
5.	ETPD-5(NETP)	*E.coli*
6.	ETPD-6(IP)	*E.coli, S. aureus* and *P. aeruginosa*
7.	ETPD-7(RP)	*E.coli, S. aureus* and *P. aeruginosa*
8.	ETPD-8(WH)	*E.coli, S. aureus* and *P. aeruginosa*
9.	ETPD-9(NI)	*E.coli, S. aureus* and *P. aeruginosa*

Table 5. Heavy Metals Tolerance Pattern of isolated Bacterial species.

S. No.	Bacterial Isolates	Heavy Metals % Resistance	
		Lead	**Zinc**
1.	*Staphylococcus aureus*	100	0
2.	*Escherichia Coli*	100	30
3.	*Pseudomonas aeruginosa*	67	33

Table 6. *In-Vitro* Antibiotic Resistance pattern of Isolated Gram-positive and Gram-Negative Bacteria.

S. No.	Antibiotics	Zone of Inhibition* Bacteria Isolates		
		Gram-positive	**Gram-negative**	
		Staphylococcus aureus	*Escherichia Coli*	*Pseudomonas aeruginosa*
1.	Amoxicillin	18.12	NZI(0)	NZI(0)
2.	Ampicillin	21.10	NZI(0)	NZI(0)
3.	Cloxacillin	23.10	NZI(0)	22.13
4.	Cephalosporin	22.10	16.13	25.10
5.	Doxycycline	NZI (0)	NZI(0)	NZI(0)
6.	Gentamycin	16.12	13.12	14.12
7.	Meropenem	28.12	21.13	25.15
8.	Streptomycin	20.12	23.32	22.10
9.	Tetracycline	19.19	19.12	17.10

*in mm including the size of well (6.0mm), NZI (0): No Zone of Inhibition Observed

Source of Support: Nil. Conflict of Interest: None declared

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