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Research Article

Bacterial Response as Determinant of Oxidative Stress by Heavy Metals and Antibiotic

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Abstract

The present study deals with Bacterial response as determinant of heavy metals stress and antibiotics by isolation, identification and characterization of heavy metal resistant bacteria which was isolated from sewage effluent collected in three different places of Ganga river in Allahabad, The two isolates were selected based on high concentration of heavy metal and antibiotic resistances. Morphological and biochemical analysis revealed that, the isolates were identified as *Escherichia coli* (Isolate-1), *Pseudomonas* (Isolate-2). The identify isolates were resistant to Cadmium (Cd), Tin (Sn), Chromium (Cr), Mercury (Hg), Arsenium (As) and Lead (Pb). The minimum inhibitory Concentration (MIC) of Sewage effluent isolates against Pb, Cd, As, Sn, Cr, and Hg was determined in broth isolates showed high resistance to heavy metals with Minimum Inhibitor Concentration (MIC) for heavy metals ranging at 10ppm to 150ppm. Both resistant isolates showed tolerances to heavy metals. Isolates showed antibiotic resistances of which were 50% resistant to *E. coli* and 10% resistant to *Pseudomonas*. Heavy metal tolerance test showed maximum microbial tolerance to chromium and minimum tolerance to mercury in mixed broth sample. The identified heavy metal resistant bacteria could be useful for the bioremediation of heavy metal contamination.

Key words: Heavy metals, resistant bacteria, Antibiotic resistance.

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1. Introduction

Heavy metals are main sources of pollution usually linked with severe industry area and high automobiles use. The harmful effects of heavy metal pollution make aquatic environment more susceptible due to close and long duration contact with the soluble metals [29]. The earliest large toxic metal contamination response is inveterate the fact have had extreme levels of metal contamination habitat for several decades still have microbial populations and behavior that are smaller than the microbial populations in uncontaminated habitats. Heavy metals present in earth's crust as natural components are increasingly found in

microbial habitat due to several natural and industrial processes. Oxygen toxicity due to the high level of oxidative stress exceeds the capacity of the cell defense systems. Oxidative stress is strongly concerned in a number of diseases such as rheumatoid arthritis, inflammatory bowel disorders, and atherosclerosis. It is also promising as one of the chief contributing agents of mutagenesis, tumor genesis and aging [7]. Bacteria are usually divided into two main groups gram-positive and gram-negative, based on their Gram stain retention property; this classification system is indistinct as it refers to three distinct aspects (staining result, envelope organization and taxonomic group). However, although Gram staining response is an experimental criterion; differences in the ultrastructure and chemical composition of the bacterial cell wall, marked by the absence or presence of an outer lipid membrane. Lipids are main targets during oxidative stress. Free radicals can harass directly polyunsaturated fatty acids in membranes and begin lipid peroxidation. A most important consequence of lipid peroxidation is a decline in membrane flexibility, which alters membrane properties and can disrupt membrane-bound proteins radically. This effect acts as an amplifier, formed more free radicals and polyunsaturated fatty acids are degraded to a variety of yield. Some of them, such as aldehydes, are very reactive and can damage molecules such as proteins and DNA [19]. However, in the presence of heavy metal tolerate mechanism involve in microbes either by efflux, complexation or reduction of metal ions or to use metals ion in anaerobic respiration as terminal electron acceptors [21]. Different types of transport system found in bacterial membrane such as CBA transporters are compound of three-component protein that move around whole cell wall of Gram-negative bacteria. An RND protein is the most important component of the transporter that is located in the inner membrane [17,11]. The RND protein family is a related group of bacterial transport proteins involved in heavy metal resistance, nodulation and cell division. P-type ATPases form transport proteins that transport ions against the concentration gradient using energy provided by ATP hydrolysis. The term “P-type” refers to the formation of a phosphoenzyme intermediate in the reaction cycle. The energy released by the removal of the phosphate from ATP is coupled to the translocation of an ion across biological membranes. Substrates are inorganic cations such as H^+, Na^+, K^+, Cd^{2+} and Pb^{2+} etc efflux transporters that play a key role in heavy metal homeostasis and resistance [24]. The cation diffusion facilitator family (CDF) comprises of a group of transporters which can catalyze either influx or efflux of heavy metals. Members of the family have been found from both prokaryotes and eukaryotes [11]. The toughest challenges of living cells to maintain appropriate concentrations of essential and toxic metals such as copper, while rejecting toxic metals like lead and cadmium [10]. Some cations at trace level at the same time to decrease cytoplasmic concentrations from potential toxic levels. Resistant bacterial strains solve these problems by a careful regulation that results from the interaction between chromosomally determined cation transport systems and metal resistance systems that are mostly determined by plasmids [5]. Trace elements, which are toxic to living organisms at excessive concentrations but some trace elements like Cu, Mn and Zn etc., at low concentrations used in the redox reaction, regulation of the osmotic pressure and also enzyme components which are

essential for the normal healthy growth and reproduction by living organisms. At high concentrations, these micronutrients damage DNA and membrane as well as loss of functions of enzyme. However, heavy metals like Cd, Hg and Pb cause oxidative stress, lipid peroxidation, carcinogenesis and mutagenesis humans, animals and plants at low concentrations [12]. Gram-negative bacterium not only needs to defend the cytoplasm but also has to translocate metals across the outer membrane to protect the vital periplasmic compartment from metal-induced damage. Cadmium toxicity in microorganisms, no defined mechanisms of action has been highlighted. The presences of nonbiodegradable heavy metals in such effluents are responsible for bacterial perseverance in the food chain. Microorganisms have acquired a variety of mechanisms for adaptation to the presence of toxic heavy metals. Bacteria have various adaptation mechanisms, metal sorption, uptake and accumulation, mineralization, extracellular precipitation and enzymatic oxidation or reduction to a less toxic form, and efflux of heavy metals from the cell. However, all heavy metals are toxic in micromolar or millimolar concentrations, yet certain bacteria are capable of growing in metal contaminated areas.

2. Materials and Methods

Sample collection: The water samples were collected in sterile glass bottle from three places of Ganga's river Fafamau, Mahadauri and Daraganj in Allahabad near sewage water flow area and transport to laboratory for bacteriological analysis.

Selected heavy metals: The heavy metals are compounds of $K_2Cr_2O_7$, $HgCl_2$, $CdCl_2$, $PbCl_2$, $SnCl_2$ and $NaAsO_2$. These heavy-metal compounds were obtained from chemical laboratory.

Treatment of samples: The water samples was tested by serial dilution till (10^{-5}) using spread plate technique used for total plate count on Tryptic soya agar, Pseudomonas agar and mfc plates and plates were incubated at 37°C for 24 h [4].

Isolation of pure cultures: Obtained bacterial colony was subsequently sub-cultured on fresh media of Tryptic soya agar, Pseudomonas agar and mfc plates by quadrant streaking and was incubated at 37°C for 24 h to obtain pure cultures and then stored in the refrigerator for further use.

Identification of bacteria: The bacteria isolates was subjected for the morphological, microscopic and biochemical identification tests such as oxidase, citrate utilization, catalase, indole production, methyl red, Voges Proskeur and sugar fermentation tests.

Heavy metal resistance tests: Nutrient agar plates were containing varying concentrations (10ppm, 30ppm, 60ppm, 90ppm, 120ppm and 150ppm respectively) of the different heavy metal compounds (Hg^{2+}, Cd^{2+}, Pb^{2+}, As^{2+}, Sn^{2+} and Cr^{2+}) was prepared. A loop full pure isolate directly streaked on the surface of the heavy metal incorporated medium. The process was repeated for each microorganism on the media incorporated with the selected heavy metals and incubated at 37°C for 24-48h. After the incubation period, the plates were observed for any kind of growth. The isolated colonies subcultured repeatedly on the same media for purification. The control experiment was carried out by inoculating the pure isolates on nutrient

agar medium without the heavy metals [20].

Determination of Minimum Inhibitory Concentration (MIC): Minimum Inhibitory Concentration (MIC) of the heavy metal resistant bacteria isolates were determined by gradually increasing the concentration of the heavy metals start from 2ppm in the nutrient agar broth until the strains failed to give colonies on the agar plate. Minimal inhibitory concentration noted when the isolates failed to grow on the plate at 37°C for one week after incubation [27].

Antibiotic susceptibility/resistance: Heavy metal resistant isolates were assayed according to the Kirby-Bauer disc diffusion method [14]. The antibiotics resistances of the isolates were determined with the following disks containing Chloromphenicol 30µg, Streptomycin 25 µg, Ofloxacin 10 µg, Ciprofloxacin 10 µg, Gentamicin 10 µg and Ampicillin 10µg respectively. A standard inoculums of each isolate from overnight culture on nutrient agar made by inoculating a discrete colony in 10 ml of sterile peptone water and incubating for 3 h at 37°C. Thereafter, 0.1 ml culture spread evenly on the surface of solid nutrient agar medium. A sterile forceps used to place multi-antibiotic discs containing the selected drugs over the surface of each inoculated plate. This procedure is repeated for all the isolates and incubated at 37°C for 24 h after which they examined for growth inhibition. The zone (diameter) of growth inhibition for each antibiotic on the different isolates was measured in millimeters.

Statistical Analysis: Data recorded from the study were statistically analyzed using two way analysis of variance technique and critical difference test. In which we can analyze (application with hypothesis) two-way analysis of variance (ANOVA) tests (also called two-factor analysis of variance) measure the effects of two factors simultaneously [24]**.**

3. Results

Isolation of heavy metal resistance bacteria

Bacterial strains were isolated from sewage effluent. Six bacterial colonies were screened from initial level of heavy metal supplemented Tropic soy agar, Pseudomonas agar and eosin methylene blue. Out of six bacterial strains, three bacterial isolates were Escherichia coli (Isolate 1) and three species of *Pseudomonas* (Isolate2). Table 1.

Microbial resistance to heavy metals concentration

The resistance test indicated that among six experimented heavy metals, maximum resistance was shown to lead showing the growth of *E. Coli* up to 120ppm and minimum resistance to mercury showing no growth above 30ppm in compare to *pseudomonas* which showed maximum resistance in five heavy metals at 150ppm and minimum resistance in mercury not above than 60ppm. The microbial resistance at each concentration of heavy metal was depicted by the microbial load on TSA plate expressed as C.F.U/ml. (Table 2).

Minimal inhibitory concentration of heavy metals resistance bacteria

The minimum inhibitory concentration of heavy metal was studied against heavy metal resistance bacteria. MIC of heavy metal has shown in table3. *E. coli* showed high resistance of lead, Arsenium and chromium have MIC value at 90ppm while Cadmium and Tin have 80ppm and minimum resistance showed in mercury

Morphological		
Colony color	White (isolates 1)	Light brown (isolates2)
Gram nature	Negative	Negative
Cell morphology	Rod	Rod
Biochemical		
Catalase	+	+
Oxidase	-	-
Indole	+	-
VP	-	-
MR	+	-
Citrate	+	+
Pseudomonas isolation agar	-	+
Lactose	Acid and gas	-
Dextrose	Acid and gas	-
Sucrose	Acid and gas	-

Table 1. Characteristics of bacterial isolates from sewage effluent

Microorganism	Heavy metals	10ppm	30ppm	60ppm	90ppm	120ppm	150ppm
		50 µl	150 µl	300 µl	450 µl	600 µl	750 µl
E.*coli*	Pb	+	+	+	+	+	-
	Cd	+	+	-	-	-	-
	Hg	+	+	-	-	-	-
	As	+	+	+	+	-	-
	Cr	+	+	+	-	-	-
	Sn	+	+	+	-	-	-
Pseudomonas	Pb	+	+	+	+	+	+
	Cd	+	+	+	+	+	+
	Hg	+	+	+	+	+	+
	As	+	+	+	+	+	+
	Cr	+	+	+	+	+	+
	Sn	+	+	+	-	-	-

Table 2. Heavy metals resistance bacteria

Microorganism	Heavy metals	2 (ppm)	6	12	18	25	30	60	80	90	100	120	140	150	Control
E. *coli*	Pb	1.37	1.29	1.20	1.14	1.11	1.09	1.07	1.00	0.91	0.88	.79	.67	0.54	1.41
	Cd	1.21	1.13	1.09	1.05	1.04	1.02	0.96	0.86	0.80	0.69	0.56	0.54	0.39	1.39
	Hg	1.08	1.04	0.80	0.69	0.65	0.58	0.28	0.17	0.16	0.11	0.08	0.06	0.03	1.37
	As	1.16	1.11	0.96	0.89	0.86	0.82	0.79	0.76	0.69	0.63	0.57	0.47	0.39	1.29
	Cr	1.97	1.83	1.60	1.53	1.15	1.13	1.11	1.02	0.89	0.73	0.54	0.49	0.35	1.30
	Sn	1.5	1.27	1.15	1.09	1.03	1.01	0.84	0.77	0.67	0.61	0.57	0.42	0.25	1.34
Pseudomonas	Pb	1.4	1.32	1.22	1.13	1.09	1.03	0.98	0.92	0.90	0.84	0.80	0.67	0.46	1.50
	Cd	1.12	1.10	1.07	1.05	1.00	0.96	0.86	0.61	0.70	0.82	0.68	0.47	0.52	1.53
	Hg	1.2	1.09	0.76	0.71	0.68	0.55	0.40	0.38	0.25	0.10	0.11	0.08	0.04	1.36
	As	1.24	1.17	1.13	1.08	1.04	1.02	1.01	0.97	0.91	0.80	0.78	0.64	0.53	1.40
	Cr	1.32	1.29	1.27	1.23	1.16	1.08	1.03	0.98	0.89	0.83	0.63	0.43	0.29	1.03
	Sn	1.25	1.15	1.05	1.03	1.01	0.94	0.87	0.69	0.53	0.48	0.36	0.23	0.19	1.36

Table 3. Minimal inhibitory concentration of heavy metals resistance bacteria

Microorganism	Heavy matals	Zone of inhibition (mm)					
		10mcg	5mcg	25mcg	10mcg	5mcg	30mcg
		Ampicillin	ciprofloxacin	streptomycin	gentamycin	oflascin	Chloramphenicol
E.*coli*	Original strain	-	-	16	20	-	22
	Pb-450	-	-	13	17	-	20
	Cd-400	-	-	11	13	-	20
	Hg-30	-	-	12	15	-	19
	As-450	-	-	11	18	-	18
	Cr-450	-	-	10	13	-	17
	Sn-400	-	-	10	15	-	18
Pseudomonas	Original strain	-	23	24	21	27	23
	Pb	-	20	14	17	23	20
	Cd	-	19	20	18	25	18
	Hg	-	21	16	15	20	16
	As	-	20	21	20	22	21
	Cr	-	21	15	16	21	20
	Sn	-	18	17	14	18	22

Table 4. Antibiotics susceptibility test of heavy metals resistance bacteria

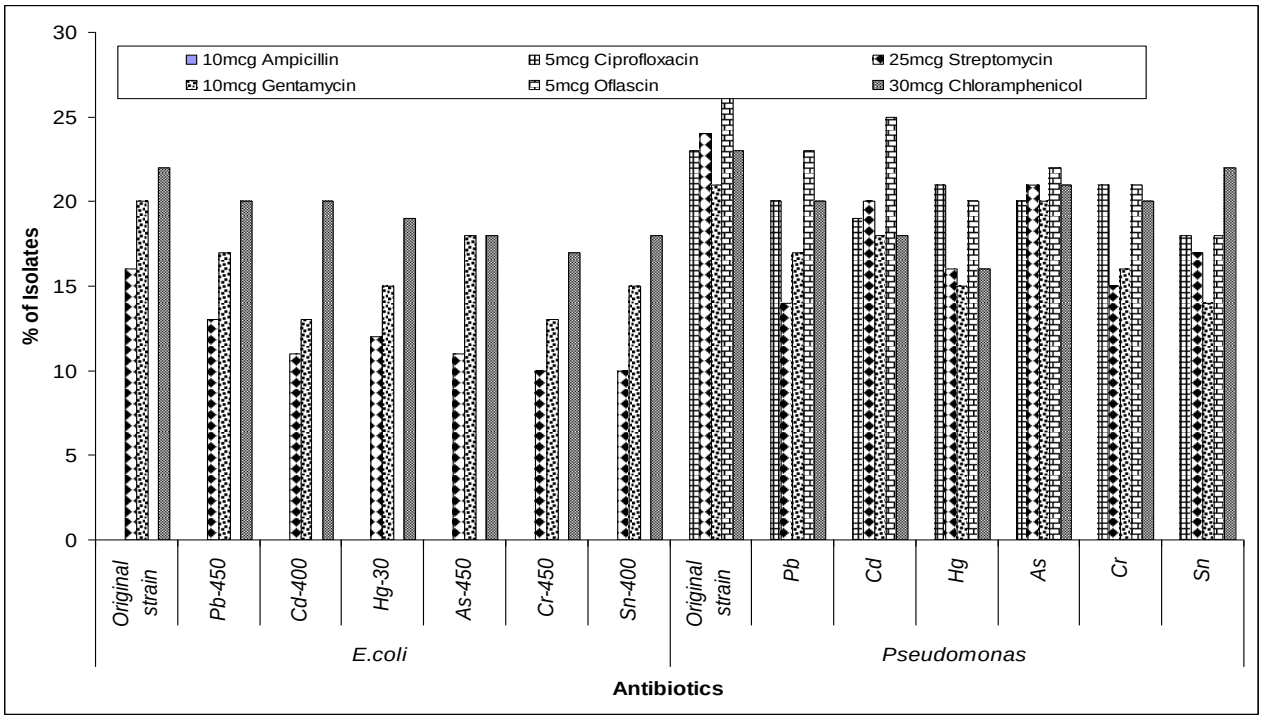

Figure 1. Antibiotics susceptibility test of heavy metals resistance bacteria

at 6ppm. In compare to *E. coli, Pseudomonas* was showing maximum resistance of Arsenium at 90ppm and minimum in mercury at 12ppm. (Table 3).

Antibiotic sensitivity of heavy metals resistant isolates-

The consequences of antibiotics sensitivity tests were performed on both original and heavy metal treated isolates of both organisms as shown in Table 4 showed that the *E. coli* exhibited resistance in three antibiotics like ampicillin, ciprofloxacin and oflascin, very low minimum inhibition in Streptomycin but two antibiotics Gentamycin and chloramphenicol were showing susceptibility on *E. coli.* However, majority of the tested organisms were particularly susceptible to most of the antibiotics as in *Pseudomonas* spp. that was 100% resistant to Ampicillin. These data were deduced from the number of the organisms that survived the effect of the antibiotics.

4. Discussion

The present study was found with the aim of identifying native bacteria having prospective for heavy metal and antibiotic resistance. Two bacteria species (*Pseudomonas* and *Escherichia coli*) were isolated from polluted Ganga's River water. In the study of [30] the five isolates were selected based on high level of heavy metal and antibiotic resistances. On the basis of morphological, biochemical analysis exposed that, the isolates were reliably identified as *Escherichia coli, Bacillus sps, Pseudomonas sps, Flavobacterium sps and Alcaligenes sps.* The identified isolates were resistant to Zinc (Zn), Copper (Cu), Chromium (Cr), Mercury (Hg) and Lead (Pb). The microbial level of resistance of each concentration of heavy metal was obtained by the level of growth on the agar plates. According to Kawane [15] the drinking water and clinical *E. coli* showed more or less equal resistance to antibiotic: metronidazole, penicillin, clindamycin,

cephoxithin and heavy metals; copper, mercury and lead, except cadmium metal ions. The microbial load decreased with an increase in the concentration (30 ppm) of heavy metal indicating the toxic effect of the heavy metals on the growth of microorganisms as earlier stated by [26]. Rajbhansi [27] studies chromium resistant *Staphylococcus* spp, *Escherichia coli, Klebsiella* spp; cadmium resistant *Acinetobacter* spp*, Flavobacterium* spp, *Citrobacter* spp; nickel resistant *Staphylococcus* spp, *Bacillus* spp; copper resistant *Pseudomonas* spp; and cobalt resistant *Methylobacterium* spp. that resistance mechanisms do not offer protection at extremely high levels of free metal ions and a lethal toxic effect is observed, However, no visible growth of microorganisms at high concentration. Raja [26] avowed that bacterial Resistance can be used to minimize the effect of heavy metals on total biological activity of the environment. Usually, pollution with a specific metal increases the level of resistance of the bacterial community to that metal.

Minimal Inhibitory Concentration (MIC) is the highest concentration of the heavy metal required to inhibit the growth of microorganisms. Consequently, minimum MIC values designate more toxic metals and maximum MIC values show less toxicity. The minimum inhibitory Concentration (MIC) of tannery effluent isolates against Pb, Cu, Zn, Cr, and Hg was determined in solid media. All the tannery effluent isolates resistant to Pb (50-90%), Cu (30-85%), Zn (50-80%), Cr (30- 70%) and Hg (30-80%)[30]. The resistance test indicated that among the six experimented heavy metals, maximum heavy metal resistance was shown to lead by *Escherichia coli* at 90ppm and *Pseudomonas* for tin at 100ppm and minimum tolerance shown by both isolates to mercury showing no growth above 12ppm of the heavy metal. Qing [25] gave result that two strains ATCC14579 and ATCC25922 showed major resistance to high concentrations of cadmium, and also have great tolerance to copper, lead, zinc and cobalt. MICs were roughly equal in both strains, which obscure that these strains obtainable relatively good resistance against Cu2+, Pb2+ and Zn2+. The order of toxicity of the metals to the two strains was found to be Co>Zn>Cu>Pb>Cd. According to Feriance [8] the characteristics of the cadmium-induced proteins gives some imminent into the latent targets in the cell. Elevated production levels of exodeoxyribonuclease III-endonuclease I1, encoded by *xth* **A,** it seems that cells growing in the presence of cadmium have an increased require for excision repair enzymes. Exodeoxyribonuclease III-endonuclease I1 is involved in 3' phosphatase, *5'* endonuclease and **3'** exonuclease activities, and an xthA mutant is sensitive to killing by agents such as near-UV radiation and H_2O_2**,** which produce oxidative damage of DNA. Rajbhansi [27] studies *Staphylococcus* spp, *Escherichia coli, Klebsiella* spp, *Acinetobacter* spp*, Flavobacterium* spp, *Citrobacter* spp, *Pseudomonas* spp; and *Methylobacterium* spp.isolates all showed high resistance to heavy metals with Minimum Inhibitor Concentration (MIC) for heavy metals ranging from 150 µg/ml to 500 µg/ml. Six resistant isolates showed multiple tolerance to heavy metals. Chromium showing the growth of microorganisms up to 500 µg/ml and minimum tolerance to nickel showing no growth above 200 µg/ml. *Pseudomonas*, *Aeromonas*, *Bacillus*, *Escherichia*, *Micrococcus* and *Proteus species*. All the strains showed resistance against heavy metals with Minimal Inhibitory Concentration (MIC) values ranging from 0.1 to 3.0 mg/L, respectively. Generally, all

the organisms had low MIC values for Pb+ and high MIC value for Zn2+ and Fe2+. This indicates average and low toxicity respectively of the heavy metals to the organisms [20]. Minimal Inhibitory Concentration (MIC) is the highest concentration of the heavy metal required to inhibit the growth of microorganisms. Thus, lower MIC values indicate more toxic metals and higher MIC values indicate less toxicity.

Kawane [15] studied that Multiple antibiotic resistance (MAR) indices in the clinical isolates were high as compare to MAR indices of drinking water. *E. coli* isolates showed higher MAR indices to cephalothin, cephoxithin, clindamycin, metronidazole, penicillin and vancomycin indicated its human origin in drinking water. MAR indices were much more reliable indicator to differentiate origin of *E. coli.* The incidence of high level of metal tolerance among bacteria could be attributed to release of metal ions in water bodies due to geochemical processes the resistant develop due to acquiring during environmental adaptability. Regarding to Rajbhansi [27] as well as concentration of heavy metals increased microbial load decreased due to toxicity of the high heavy metals it affects on the growth of bacteria. The existence of minute quantity of antibiotics and heavy metals in the polluted water provoke the emergence of antibiotic and heavy metal resistant bacteria. Most of the isolates showed multiple tolerances to both heavy metals and antibiotics. The heavy metal resistance microorganism is recognized by various resistance mechanism developed by microorganisms such as metal reduction, binding with bacterial cell envelopes, complexation by exopolysaccharides, metal efflux etc. [31] .Microorganism resistance mechanisms are encoded in plasmid genes which facilitate transfer of toxic heavy metal resistance gene from one cell to another cell. Toxic compounds cause multiple tolerances microorganisms that have similar mechanisms underlying their toxicity. Since all heavy metals are correlated in their toxic mechanism, multiple tolerances are common phenomena among heavy metal tolerance microorganism. In polluted water, there are some substances which are not antibiotics themselves but have efficiency to select for antibiotic resistance such as heavy metal and biocides. Heavy metals or biocides exposure causes selection of bacterial strain also able to resist antibiotics. This happens due to heavy metals or biocides encoding gene are located same or alternative antibiotic resistance genes because bacteria have general unspecific resistance mechanism for different substances including biocides, heavy metals and antibiotics [17]. Mgbemena [20] considered that *Pseudomonas*, *Aeromonas*, *Bacillus*, *Escherichia*, bacterial isolates also exhibited high tolerance to most of the antibiotics like Gentamycin (77.7%), Rifampicin (66.0%) and Oflaxacin (57.3%). The ability of these microorganisms to resist the presence of both metals and antibiotics could be causes very serious health implication because of the ability of these microorganisms to pass these resistant genes via R-plasmids to the next cell around will affect a whole bacterial population thereby complicating treatment. Selvi [31] gave the multiple metal resistances of these isolates *Escherichia coli , Bacillus sps, Pseudomonas sps, Flavobacterium sps and Alcaligenes sps* .were also associated with antibiotics Ampicillinm(AM), Ciprofloxacin (C), Cotrimazole (Co), Gentamycin (GM), Kanamycin (K), Nalidixic acid (NA) and Streptomycin (S). Varying microbial resistance levels to heavy metals has been

recognized to a variety of tolerance mechanisms such as differences in uptake and/or transport of the toxic metal while in other cases, the metal may be enzymatically transformed by oxidation, reduction, methylation or demethylation into chemical species which may be less toxic or more volatile than the parent compound, resistance genes to both antibiotics and heavy metals could be closely located on the same plasmid in bacteria. The toxic levels of heavy metals affect structural and permeability properties of inner membranes and organelles, cause inhibition of enzymatic activities, nutrient imbalances, decreases in rates of photosynthesis and transpiration stimulate formation of free radicals and reactive oxygen species resulting in oxidative stress suppress bacterial reproductive development and persuade deleterious anatomical and ultra structural changes in bacteria.

5. Conclusion

Microbes have capability to survive in the excessive heavy metals polluted area and utilized metal constituents as their growth; as such they can be used to clean up metal-contaminated sites. Bacteria exposed to high levels of heavy metals in their environment have adapted to this stress by developing various resistance mechanism. It is important to remember that what we put in the environment can have many effects, not just on humans, but also on the environment and on the microbial community on which all other life depends. Different types of environmental waste are conscientious for the growth of resistance bacteria. According to this study *E. coli* showed more metal resistance than *Pseudomonas*, the identified bacteria can be used to remediate heavy metal polluted water and sewage.

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