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Heavy metal tolerance and multiple drug resistance of heterotrophic bacterial isolates from metal contaminated soil

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Abstract

The development of multiple metal/antibiotic resistances among the bacterial population causes a potential risk to human health. Metal contamination in natural environments could have an important role in the maintenance and proliferation of antibiotic resistance. In the present study, a total of 46 heterotrophic bacterial isolates from metal contaminated soil were tested for their sensitivity to 10 widely used antibiotics such as ampicillin, erythromycin, gentamicin, nalidixic acid, penicillin, amikacin, lincomycin, novobiocin, vancomycin and tetracycline. Metal tolerant ability of these isolates against five heavy metals such as lead, zinc, copper, cadmium and nickel were also determined. The results revealed that most of the bacterial isolates were resistant to one or more heavy metals/ antibiotics against which they are tested. Tolerance to heavy metal showed the following pattern; lead > zinc > nickel > copper > cadmium. Resistance to ampicillin (73.91%), penicillin (60.8%), lincomycin (43.47%) and nalidixic acid (21.73%) were encountered frequently. None of the isolates were resistant to amikacin, while resistance to gentamicin and tetracycline were low (2.17%). Out of the 46 bacterial isolates, 36 isolates showed multiple metal and antibiotic resistances. Isolate LOC 10 showed significantly high tolerance (100-300ug/mL) to all the metals and was resistant to 6 antibiotics.
***Keywords**: soil, bacteria, heavy metal tolerance, antibiotic resistance*

1. Introduction

In an environment where resources such as nutrients are inadequate, a bacterium can produce an antibiotic to eliminate or inhibit competing bacteria, thereby limiting struggle for the scant resources. In order to become successful in this event, the bacteria producing the antibiotic should be capable of enduring the antibioitc it has produced by possessing mechanisms of resistance. These mechanisms can be transferred to other bacteria, which have led to a mounting risk to global public health by confounding treatment of infections caused by virtually all major pathogens (Canton *et al.*, 2008; Goossens 2009; Allen *et al.*, 2010). However, it has become evident that antibiotic resistance is also widely prevalent among environmental bacteria (Chopra and Roberts, 2001). This observation assumes significance as bacteria in natural environments can serve as a pool for resistance genes that could ultimately be transmitted to pathogenic species (Alonso *et al.*, 2001). Hence it is important to develop further understanding of the incidence and diversity of antibiotic resistance in a broad range of environments.

Contamination of soils with heavy metals is becoming one of the most severe environmental and human health hazards. Soils contaminated by heavy metals and metalloids through emissions from the rapidly expanding industrial areas, mine tailings, disposal of high metal wastes, leaded gasoline and paints, land application of fertilizers, animal manures, sewage sludge, pesticides, wastewater irrigation, coal combustion residues, spillage of petrochemicals, and atmospheric deposition (Khan *et al.*, 2008; Zhang *et al.*, 2010). Metals play an essential role in the metabolic processes of the biota. Some of the heavy metals are essential and are required by the organisms as micro nutrients (cobalt, chromium, nickel, iron manganese and zinc etc.) and are known as 'trace elements' (Bruins *et al.*, 2000). Elevated levels of heavy metals not only decrease soil microbial action and crop production, but also threaten human health by biomagnification through the food chain (Mclaughlin *et al.*, 1999). However, at high levels both of the essential and non essential metals become toxic to the organisms. These heavy metals influence the microbial population by affecting their growth, morphology, biochemical activities and ultimately resulting in decreased biomass and diversity (Roane and Pepper, 2000). Due to the selective pressure from the metal in the growth environment, microorganisms have evolved various mechanisms to resist the heavy metal stress. Heavy metal contamination in soils from industrial zones of south India and other locations in India have been reported (Krishna and Govil, 2004, 2007, 2008).

Significantly large number of reports proposes that metal contamination in natural environments could have a central role in the preservation and proliferation of antibiotic resistance (Baker-Austin *et al.*, 2006; Berg *et al.*, 2010). This is of increasing concern as anthropogenic activity has resulted in increasing levels of heavy metals in the soil which are

several orders greater than levels of antibiotics (Stepanauskas *et al.* 2005). Different from antibiotics, metals are not subjected to degradation and can offer long-term selection pressure (Stepanauskas *et al.*, 2005). Hence there are concerns about the possibility of metal contaminated sites in soils acting as favourable sites for drug resistant bacteria and there by a reservoir for antibioitc resistant genes in both natural and clinical settings. Therefore in the present study an attempt was made to study the relationship between multiple antibiotic resistance and heavy metal tolerance of heterotrophic bacterial isolates from soils that are not affected by clinical waste, but subjected to probable heavy metal contamination.

2. Materials and Methods

2.1 Collection of Soil Sample

The soil samples were collected from six selected sites of Kottayam, South India (9º39'28"N and 76º 32'10"E). Soil samples from a depth of 15 to 20 cm from the surface were collected after removing the top layer. The sites were relatively free of contamination from clinical waste, though subjected to moderate levels of chemical contamination. For each of the sampling sites, sub-samples of soil were collected from different locations, pooled together and homogenized so as to obtain representative sample. Samples were collected using a spade that is thoroughly cleaned and disinfected between sampling so as to prevent cross-contamination.

2.2 Isolation of Heterotrophic Bacteria

Ten grams of soil was aseptically transferred to 90 mL sterile distilled water and agitated vigorously. Different aqueous dilutions, 10^{-1} to 10^{-7} of the suspensions were prepared and 0.2 mL of the inoculum was spread plated on Nutrient Agar. Plates were incubated at room temperature for 48 hours and morphologically different bacterial colonies were isolated using a sterile inoculation needle. The isolates were restreaked to ensure purity and maintained as pure cultures on nutrient agar vials for further study.

2.3 Antibiotic Sensitivity Testing

Bacterial isolates were tested for antibiotic resistance according to the method of Bauer *et al*. (1966). Isolates were enriched in nutrient broth for 16-18 h at 35°C. Using a sterile swab the broth cultures of the bacteria were swabbed evenly onto the surface of sterile surface dried Mueller-Hinton agar plates. Antibiotic impregnated paper discs were placed on the surface of the agar using a sterile forceps and the plates were incubated at 35°C for 24 h. After incubation, the inhibition zones around the antibiotics were measured to the nearest millimeter. Inhibition zones were indicated by a lack of microbial growth due to inhibitory concentrations of antibiotic diffused into semisolid culture media (agar) beneath the antibiotic-impregnated disc. Antibiotics and their doses tested were as follows: ampicillin (10 mcg), erythromycin (15 mcg), gentamicin (10 mcg), nalidixic acid (30 mcg), penicillin (10 units), lincomycin (2 mcg), vancomycin (30 mcg), novobiocin (30 mcg), amikacin (30 mcg), and tetracycline (30 mcg). Antibiotic resistance was determined by comparing the diameter of inhibition zone around each antibiotic disk with zone size interpretive chart supplied by Hi-media laboratories, Bombay.

2.4 Multiple Antibiotic Resistance (MAR) Indexing of the Isolates

The MAR index when applied to a single isolate is defined as a/b where 'a' represents the number of antibiotics to which the isolate was resistant and 'b' represents the total number of antibiotics to which the isolate was exposed. Isolates with a MAR index value higher than 0.2 is considered to have originated from high-risk source of contamination such as human, commercial poultry farms, swine and dairy cattle where antibiotics are often used.

2.5 Heavy metal tolerance analysis

Tolerance of the bacterial isolates to varying concentrations of heavy metals such as lead, cadmium, copper, nickel and zinc were determined by agar dilution method (Lui *et al.*, 1983). Fresh overnight cultures of the isolates grown in peptone water were aseptically inoculated in to nutrient agar plates, which were supplemented with increasing concentration of the aforesaid metals individually (5µg/mL to 4mg/mL). The plates were incubated at 35°C for 24 hours and observed for bacterial growth. The lowest concentration of heavy metals at which no growth occurred was considered as the minimal inhibitory concentration (MIC). All metal salts were added to the medium after autoclaving and cooling to 45-50°C, from filter-sterilized stock solutions. The metal salts used for the study includes Lead nitrate ($Pb(NO_3)_2$), Zinc sulphate ($ZnSO_4.6H_2O$), Nickel sulphate ($NiSO_4.6H_2O$), Copper sulphate ($CuSO_4.5H_2O$) and Cadmium nitrate ($Cd(NO_3)_2.4H_2O$).

3. Results and Discussion

3.1 Antibiotics Resistance of the Isolates

Forty six bacterial strains were isolated from the study area and tested for their sensitivity to 10 widely used antibiotics. Of the 46 bacterial isolates 93.4% were resistant to at least one antibiotic. Resistance to ampicillin (73.91%), penicillin (60.8%), lincomycin (43.47%) and nalidixic acid (21.73%) was observed frequently (Figure 1). While none of the isolates were resistant to amikacin (0%), relatively lower level of resistance was recorded against gentamicin (2.17%) and tetracycline (2.17%). Antibiotic resistant bacteria are generally higher in regions that are affected by

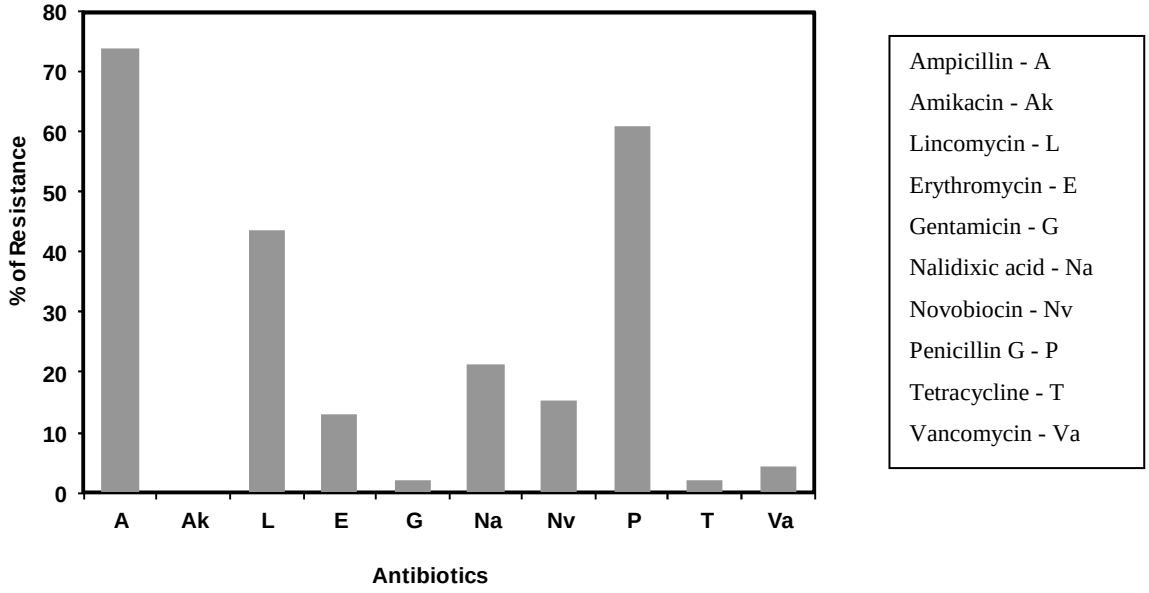

Figure 1. Antibiotic resistance of heterotrophic bacteria isolated from metal contaminated soil samples.

pollution or agriculture. It is reported that in Norway, fields that were without antibiotic application for 10 years nevertheless had high levels of resistant organisms including resistance to chloramphenicol, tetracycline, ampicillin, and streptomycin (Bronstad *et al*., 1996). The study area is more or less unpolluted from clinical wastes. However, there are unaltered areas that might also contain high levels of antibiotic resistant bacteria as well, perhaps from natural production of antibiotics by soil bacteria.

Pseudomonas aeruginosa isolated from various soils in Spain were resistant to many antibiotics and had higher levels of resistance than isolates from nearby surface waters. These isolates were resistant to ampicillin, chloramphenicol, kanamycin, nalidixic acid, tetracycline (Marques *et al*., 1979). Results of our study reveales similarity in resistance levels of some of the antibiotics however, differ in case of antibiotics such as tetracycline as most of our isolates were sensitive to this antibiotic. None of our isolates were resistant to amikacin and most of them were sensitive to gentamycin and vancomycin. This is a possible reflection of less selection pressure for these human derived antibiotic residues in the soils of study area. Naturally resistant strains to these antibiotics also seem to be very low.

Analysis of MAR index and resistance patern revealed 18 resistance patterns were observed among the isolates from the soil (Table 1). Numerous isolates had acquired multiple drug resistance (MDR). The most frequent (30.4%) resistant pattern was PA (penicillin-ampicillin). Diverse antibiotic resistance patterns of bacterial isolates may be due to the disparity in the selection pressure for the resistant mutants. The results of the present study in concordance with those of other works in which varying frequencies of antibiotic resistance patters were reported among bacteria isolated from natural

Table 1. MAR index and percentage occurrence of different resistance patterns among the heterotrophic bacterial isolates from metal contaminated soil.

MAR Index	Resistance Pattern	No. of isolates showing the pattern	% of occurrence of pattern
0.2	PA	14	30.4
0.1	L	5	10.86
0.2	PANa	2	4.34
0.2	ANa	3	6.52
0.3	PLA	3	6.52
0.3	PANv	2	4.34
0.7	TPLVaANvE	1	2.17
0.6	PLVaANvE	1	2.17
0.3	GAE	1	2.17
0.4	PANaVa	1	2.17
0.1	Na	1	2.17
0.2	NaE	1	2.17
0.2	LA	1	2.17
0.5	PLANaE	1	2.17
0.5	PLANaNv	1	2.17
0.1	P	1	2.17
0.3	TAE	1	2.17
0.2	LNv	1	2.17

Ampicillin - A, Amikacin - Ak, Lincomycin - L, Erythromycin - E, Gentamicin - G, Nalidixic acid - Na, Novobiocin - Nv, Penicillin G - P, Tetracycline - T, Vancomycin - Va

environments including soils (D'Costa *et al.*, 2006; Schmitt *et al.*, 2006; Patterson *et al.*, 2007).

Analysis of multiple antibiotic resistance (MAR) index and resistance patterns of each isolates, revealed prevalence of MAR among the isolates encountered in the study area. This reveals the possibility of resistant mutants in natural environments which may not be subjected to high selection pressure for resistant mutants. Varying frequencies of MAR bacteria have been reported in soils (D'Costa *et al.*, 2006). The prevalence of MAR strains at the soil of study area may be in part due to the probable use of antibiotics in the nearby farming villages, which might have reached the study area through land run-off. The use of antibiotics in animal feeds is supposed to boost resistance among bacteria in surface soils and other environments (Patterson *et al.*, 2007). There has been substantial anxiety about environments containing antibiotics due to the possibility of antibiotic-resistant strains becoming dominant in the bacterial communities in such ecosystems (Wittwer *et al*. 2005; Heuer and Smalla, 2007).

3.2 Metal Resistance of the Isolates

Most of the isolates showed resistance to one or more heavy metals selected, however, the patterns of tolerance among the 46 isolates were varied (Table. 2). Sampling environments that contain elevated concentrations of heavy metals are potential source of toxic-metal-tolerant bacteria (Clausen, 2000). In the present study bacterial strains were collected from the soils of which might have undergone a possible chemical contamination, though not exposed to domestic/ clinical wastes. Among the various isolates LOC 10 showed significant tolerance to various heavy metals tested in this investigation. The pattern of tolerance was as follows: Pb>Zn>Ni>Cu>Cd.

Tolerance to lead was comparatively high followed by zinc, nickel, copper and cadmium. About 33% of the isolates showed very high tolerance (>4000µg/mL) to lead. The resistance towards such high concentration of metal may be due to unavailability of the metal to the bacteria, as there is a possibility of precipitation of metals in nutrient rich media such as nutrient agar, though specific studies were not conducted in this research to determine the same. While tolerance to cadmium was rather low (<100 µg/mL), resistance to zinc ranged between 100µg/mL - 1000µg/mL, with most of the isolates showing resistance range between 200-500 µg/mL. Resistance to nickel was in between 100µg/mL - 1000µg/mL and the majority of them shows resistance in between 300µg/mL - 400µg/mL. Resistance to copper was in between 100µg/mL - 500µg/mL and a good number of them showed resistance in the range between 300µg/mL - 400µg/mL. Differences in the bacterial resistance to different heavy metal are mainly due to the differential concentration of different heavy metal ions in the environment. The high levels of resistance and the widespread tolerance that was found among the isolates is probably due to the metal contamination in the soil (Abou-Shanab *et al.*, 2007).

3.3 Association Between Metal Tolerance and Antibiotic Resistance

Out of the 46 bacterial isolates 36 isolates showed multiple metal and antibiotic resistances. One bacterial isolate (LOC 10) showed multiple antibiotic resistances against 6 antibiotics tested and could tolerate high concentrations (100-300 µg/mL) of all the heavy metals studied. The use of antibiotics in medicine and agriculture clearly stimulates the proliferation of antibiotic resistance (Neu, 1992). Heavy metals and other toxicants have also been suggested to play an important role (Summers, 2002). Multiple genes encoding for metal and antibiotic resistance are usually found on the same plasmids and/or transposons, conferring coresistance (Summers, 2002). In certain cases, single enzymes play the role as efflux pumps for multiple metals and antibiotics, which is defined as cross-resistance (Hayashi *et al*., 2000). It is observed in the present study that copper-resistant strains were significantly more resistant to ampicillin and sulfonamide than copper-sensitive isolates, which strengthened the argument that the traits are co-selected. Berg *et al.* (2005) found that soil microbes isolated from a copper-amended field were more resistant to copper and antibiotics than strains isolated from control plots 21 months after copper amendment.

Joint expression of antibiotic resistance and heavy metal resistance may not be accidental. Nakahara *et al.* (1977) have reported that the joint expressions of antibiotic resistance and metal tolerance may be caused by selection resulting from metals present in an environment. Many reports suggest that metal contamination in natural ecosystems could have key role in the maintenance and proliferation of antibiotic resistance (Summers *et al.*, 1993; Alonso *et al.*, 2001; Summers, 2002). This is of particular concern considering that anthropogenic levels of heavy metals are currently several orders of magnitude greater than levels of antibiotics (Stepanauskas *et al.*, 2005). Elevated frequencies of microbial resistance to various antibiotics have been observed in metal-contaminated freshwater streams (McArthur and Tuckfield, 2000), coastal areas (Rasmussen and Sorensen, 1998) and metal contaminated ash settling basins (Stepanauskas *et al.*, 2005).

4. Conclusion

The present study highlight the prevalence of multiple antibiotic resistant bacteria in soil samples collected from sites that might have undergone possible chemical contamination. Improper release of such chemical wastes in to the environment might increase selection pressure for the emergence of drug

Table 2. Heavy metal resistance patterns of heterotrophic bacterial isolates from metal contaminated soil.

Isolate No.	Tolerance range of various heavy metal concentration (µg/mL)				
	Zn	Ni	Cd	Cu	Pb
MP1	300-400	300-400	<100	300-400	>4000
MP 2	300-400	300-400	<100	400-500	>4000
MP 3	200-300	300-400	<100	300-400	>4000
MP 4	200-300	100-200	<100	200-300	1000-1500
MP 5	200-300	100-200	<100	100-200	1000-1500
MP 6	<100	100-200	<100	<100	>4000
MP 7	200-300	100-200	<100	<100	1500-2000
MP 8	200-300	300-400	<100	<100	1500-2000
CD1	200-300	300-400	<100	<100	1500-2000
CD2	200-300	300-400	200-300	300-400	1500-2000
CD3	900-1000	300-400	<100	300-400	1500-2000
CD4	<100	300-400	<100	<100	<100
CD5	300-400	200-300	<100	<100	2000-3000
CD6	100-200	300-400	<100	100-200	>4000
Gr1	<100	100-200	<100	<100	1000-1500
Gr2	400-500	300-400	<100	100-200	1000-1500
Gr3	900-1000	300-400	100-200	300-400	1000-1500
Gr4	<100	300-400	<100	<100	1000-1500
Gr5	<100	300-400	<100	100-200	1000-1500
Gr6	<100	300-400	<100	<100	2000-3000
Gr7	<100	300-400	<100	<100	1000-1500
WDS1	900-1000	100-200	100-200	300-400	>4000
WDS2	200-300	300-400	300-400	100-200	500-1000
WDS3	500-600	100-200	100-200	300-400	2000-3000
WDS4	900-1000	300-400	100-200	300-400	>4000
WDS5	<100	200-300	100-200	100-200	2000-3000
WDS6	900-1000	300-400	100-200	300-400	1500-2000
WDS7	<100	100-200	100-200	100-200	2000-3000
WDS8	400-500	100-200	300-400	300-400	1500-2000
WDS9	300-400	100-200	200-300	300-400	>4000
LOC1	<100	300-400	100-200	100-200	>4000
LOC2	900-1000	300-400	100-200	300-400	1500-2000
LOC3	500-600	300-400	>500	300-400	2000-3000
LOC4	900-1000	300-400	>500	200-300	>4000
LOC5	200-300	200-300	<100	<100	500-1000
LOC6	100-200	200-300	<100	100-200	500-1000
LOC7	900-1000	300-400	<100	300-400	>4000
LOC8	900-1000	300-400	100-200	200-300	2000-3000
LOC9	500-600	100-200	<100	<100	1000-1500
LOC10	900-1000	300-400	>500	300-400	>4000
LOC11	400-500	300-400	200-300	300-400	>4000
SPAP1	900-1000	300-400	100-200	300-400	>4000
SPAP2	400-500	900-1000	<100	100-200	>4000
SPAP3	900-1000	300-400	100-200	200-300	2000-3000
SPAP4	100-200	100-200	100-200	100-200	2000-3000
SPAP5	900-1000	300-400	100-200	400-500	2000-3000

resistant mutants. These mutants could ultimately find their way to natural waters where they could exchange these resistance features to a harmless microbe or an antibiotic sensitive pathogen, making them a potential health hazard. Further work at molecular level is required to identify specific mechanisms involved in the association between metal tolerance and antibiotic resistance.

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