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Metal tolerance analysis of Gram negative bacteria from hospital effluents of Northern India

Manzar Alam*, Mohd Imran

Department of Biosciences, Integral University, Lucknow, India.

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ABSTRACT

Effluents from different hospitals were analysed to Nickel, Chromium, Cobalt, Copper, Mercury, Cadmium and Zinc resistance among Gram negative bacteria. The resistance among the Gram negative bacterial population varied considerably in different metal and water sampling sites. Gram negative bacteria showed lower metal resistant viable count range 4.01 x10^4-1.3 x10^3 at 50-100 µg/ml in site-IV as compared to 11.03x105-1.03x10^4, 12.02x10^5-1.4x10^3 and 12.33x10^5-2.7x10^3 in site-I, II and III against all metal tested, respectively. Viable counts of Gram negative bacterial population were recorded higher against Nickel and Zinc from sampling site-III as compared to other sites tested. Lower viable counts of Gram negative bacteria were recorded against Mercury in all sites tested. All the isolates of Gram negative bacteria showed their tolerance level (Minimum inhibitory concentration) in the range of 50-1600µg/ml against all the metal tested. Of 88%, 76% and 86% isolates exhibited their MIC at 50-100 µg/ml against Mercury, Cadmium and Cobalt in all the sites tested, respectively. Maximum 60% and 32% of the isolates demonstrated their MIC at 1200-1600 µg/ml against Cr^{2+} and Cu^{2+} from the entire site tested, respectively. All Gram negative bacterial isolates also observed multiple resistance patterns (2-7 metal) in different combination of metals. The Multi metal resistance Index (MMR) index ranges were found (0.03-0.71) indicating the high risk of environmental contamination and emergence of metal resistance which may promote the development of resistance to antibiotics among the pathogens.

INTRODUCTION

Wastewater released from hospitals could be loaded with antimicrobial resistant micro-organism and toxic chemicals. Improperly treated hospital wastewater is hazardous for reuse or for releasing into natural water source (Rutala and Mayhall, 1992; Blumenthal *et al.*, 2001). Metal pollution remains a major challenge in environmental biotechnology. Some industrial processes results in the discharge of metals into aquatic systems. The concentration of metal pollutants in the environment is usually low excluding in specific areas, which are polluted by various hospitals and industrial wastes. The concentration of heavy metals is very high in ore containing and mining areas (Roane *et al.*, 1996). This has led to growing concern about the consequence of toxic metals as environmental pollutants. This kind of contamination presents a challenge, as the presence of metals in soils and aqueous effluents leads to severe trouble because they cannot be biodegraded. Unlike many other pollutants, metals are complicated to remove from the environment (Ren *et al.*, 2009). Some heavy metals such as nickel, iron, copper and zinc are necessary to metabolic reactions and are required as trace elements by the organisms. Others like mercury, silver and cadmium have no biological role and are injurious to the organisms, even at very low concentrations (Hughes *et al.*, 1989). Many bacteria have precise genetic mechanisms of resistance to toxic metals (Silver and Misra, 1988; Mindlin *et al.*, 2001). In the environment metals, may select these resistant variants in a manner similar to the selection of antibiotic resistant strains. Indeed, it is relatively frequent the association of metal and antimicrobial resistance, since both resistance genes are commonly located on the same mobile genetic elements (Foster, 1983; McIntosh *et al.*, 2008). Accordingly, it can be assumed that the selective pressure exerted by heavy metals contribute to the indirect co-selection of antibiotic resistance, particularly in environments polluted with the two elements.

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* *Corresponding Author*
Email: manzar.160 @ gmail.com

Microorganisms resistant to both antibiotics and metals have been isolated commonly from the environments, and this has led to proposition that the combined expression of antibiotic resistance & metal resistance is caused by selection, consequential from metals present in particular environment (Bell *et al.,* 1983; Viti and Giovannetti, 2003). The occurrence of antibiotic-resistant bacteria in the natural habitats can pose a public health risk (Nies, 1999). The study was aimed to explore the threat of wastewater generated from hospitals to the public. Wastewater treatment requires suitable methods and constant monitoring to ensure that the treated effluent be not hazardous to the environment.

MATERIAL AND METHODS

Sample collection

Water samples were collected from three sites of hospital wastewater along with King George's Medical University Site -I untreated, Sanjay Gandhi Post Graduate Institute of Medical Sciences Site-II treated, Sanjay Gandhi Post Graduate Institute of Medical Sciences Site-III untreated and Dr. Ram Manohar Lohia Hospital Site -IV untreated at Lucknow city as shown in **Figure 1**. Samples were collected in sterile 250-ml polypropylene bottles, according to internationally recommended methodology (Lösch *et al.,* 2008). Samples were kept at 4ºC until their arrival to laboratory.

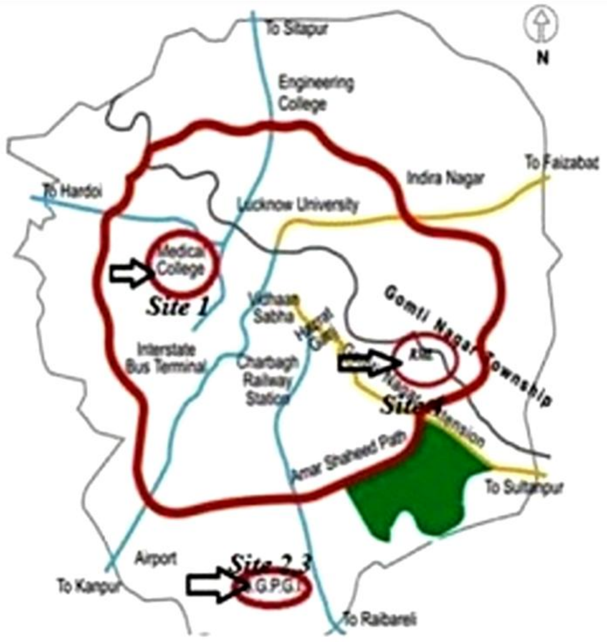

Fig. 1: Sampling sites.

Isolation and identification of metal tolerant Gram negative bacterial population

Isolation of metal resistant Gram negative bacteria from water samples were done on metal amended Mac conky agar plates at varying concentration (25-1200μg/ml). Serial dilutions of the water samples were plated by spreading 0.1 ml on medium for the count of total metal resistant Gram negative bacteria. Plates incubated at 37ºC for 24 hours and Gram negative bacterial counts were expressed as CFU/ml on Mac conky agar medium. The selected isolates were finally identified on the basis of biochemical characterization as described elsewhere (Cappuccino and Sherman, 1995).

Determination of minimum inhibitory concentration (MIC) of metal among different Gram negative isolates

The heavy metal resistance was determined by the minimum inhibitory concentration (MIC) against the test bacterial strain by spot plate method (Malik and Jaiswal, 2000). Nutrient plates of each heavy metal (Chromium K2Cr2O7, Cadmium CdCl2, Cobalt CoCl2, Mercury HgCl2, Copper CuSo4, Zinc ZnCl2 and Nickel NiCl2) of different concentrations (50μg/ml to 1600 μg/ml) were prepared. Inoculums of test strain (3x106 CFU/ml) were spotted on heavy metal amended plates and control plates in duplicate with the help of platinum loop of 5mm diameter.

The plates were incubated at 37 ºC for 24 hr to observe the growth of bacterial strain on the spotted area. The MIC was defined as the minimum inhibitory concentration of the heavy metal that inhibits the visible growth of test strain. Metal concentration range bellow MIC was considered as sub MIC of the isolates.

Multiple metal resistances (MMR) indexing

The MMR index profile based on isolate was evaluated to access the health risk of the environment. MMR index for test isolates was calculated according to the formula: No. of metal to which all isolates were resistant/No. of metal tested x No. of isolates.

RESULTS

In this study, metal tolerant population of gram negative bacteria from the hospital waste water was observed against seven heavy metal (Hg^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+} and Cr^{3+}) at their varying concentrations (25-1200 μg/ml). Viable (CFU/ml) count of gram negative bacteria was observed higher in (non-metal supplemented) control plate than metal supplemented plates in all the sites tested. The viable count of Gram negative bacteria in different concentrations of metal ranged from $11.03x10^5$-$8.0x10^2$, $12.02x10^5$-$1.0x10^2$, $12.33x10^5$-$5.0x10^2$ and $4.01x10^4$-$1.0\ x10^2$ cfu/ml of water in site I, II, III and IV, respectively. Maximum viable count was observed against Cu^{2+} and Cr^{3+} in sampling site-I, while the same was found against Co^{2+} and Ni^{2+} in site II, III at 25μg/ml respectively. In site IV maximum viable count were observed against Zn^{2+}. Minimum viable count was observed against Hg^{2+} and Cd^{2+} at 25μg/ml in sites I, II and IV, respectively. While in site-III minimum viable counts were observed Hg^{2+} and Cu^{2+} at 25 μg/ml concentration. Maximum number of viable count was recorded at higher concentration against Cu^{2+} ($1.06\ x10^4$) and Zn^2+ ($4.35\ x10^4$) in site II and III, respectively (Table1).

Table 1: Viable count of metal tolerant Gram negative bacteria from different wastewater sampling sites.

Metal	Conc.	Site1	Site2	Site3	Site4
Control	No metal	$12.5\times10^{5}\pm0.56$	$13.00\times10^{5}\pm1.19$	$13.43\times10^{5}\pm1.19$	$8.13\times10^{4}\pm0.65$
Hg^{2+}	25	$2.9\times10^{3}\pm0.01$	$1.4\times10^{3}\pm0.11$	$2.7\times10^{3}\pm0.4$	$1.3\times10^{3}\pm0.13$
	50	$2.0\times10^{3}\pm0.02$	$4\times10^{2}\pm0.41$	$2.2\times10^{3}\pm0.15$	$7\times10^{2}\pm0.55$
	100	$6.0\times10^{2}\pm0.011$	$3\times10^{2}\pm0.32$	$1.9\times10^{3}\pm0.09$	$1\times10^{2}\pm0.11$
	200	ND	ND	ND	ND
	400	ND	ND	ND	ND
	800	ND	ND	ND	ND
Cd^{2+}	25	$3.9\times10^{3}\pm0.02$	$1.13\times10^{4}\pm0.08$	$5.22\times10^{4}\pm0.56$	$1.09\times10^{4}\pm0.42$
	50	$2.7\times10^{3}\pm0.04$	$8.3\times10^{3}\pm0.03$	$4.32\times10^{4}\pm0.32$	$6.3\times10^{3}\pm0.83$
	100	$1.2\times10^{3}\pm0.09$	$3.4\times10^{3}\pm0.12$	$4.09\times10^{4}\pm0.09$	$2.3\times10^{3}\pm0.11$
	200	ND	$1.8\times10^{3}\pm0.18$	$3.89\times10^{4}\pm0.17$	$5\times10^{2}\pm0.54$
	400	ND	ND	$1.69\times10^{4}\pm0.12$	$1\times10^{2}\pm0.09$
	800	ND	ND	$6\times10^{2}\pm0.65$	ND
	1200	ND	ND	ND	ND
Co^{2+}	25	$2.65\times10^{4}\pm0.02$	$11.03\times10^{5}\pm1.19$	$11.22\times10^{5}\pm2.12$	$3.23\times10^{4}\pm0.13$
	50	$1.52\times10^{4}\pm0.01$	$9.35\times10^{4}\pm0,.59$	$10.13\times10^{5}\pm1.97$	$3.00\times10^{4}\pm0.19$
	100	$9\times10^{2}\pm0.09$	$5.03\times10^{4}\pm0.67$	$9.15\times10^{4}\pm0.57$	$1.88\times10^{4}\pm0.09$
	200	ND	$4.17\times10^{4}\pm0.31$	$8.23\times10^{4}\pm0.69$	$1.50\times10^{4}\pm0.05$
	400	ND	$1.09\times10^{4}\pm0.03$	$7.93\times10^{4}\pm0.54$	$1.21\times10^{4}\pm0.05$
	800	ND	ND	$5.02\times10^{4}\pm0.65$	$8.9\times10^{3}\pm0.92$
	1200	ND	ND	$5\times10^{2}\pm0.01$	ND
Ni^{3+}	25	$1.55\times10^{4}\pm0.12$	$12.02\times10^{5}\pm1.4$	$12.33\times10^{5}\pm1.09$	$2.27\times10^{4}\pm0.14$
	50	$1.23\times10^{4}\pm0.14$	$11.12\times10^{5}\pm1.01$	$12.01\times10^{5}\pm1.09$	$2.03\times10^{4}\pm0.32$
	100	$9\times10^{2}\pm0.19$	$2.93\times10^{4}\pm0.03$	$9.53\times10^{4}\pm0.97$	$4.9\times10^{3}\pm0.29$
	200	ND	$1.42\times10^{4}\pm0.09$	$8.97\times10^{4}\pm0.45$	$3.7\times10^{3}\pm0.13$
	400	ND	$4.0\times10^{3}\pm0.52$	$6.13\times10^{4}\pm0.32$	$3\times10^{2}\pm0.15$
	800	ND	ND	$4.22\times10^{4}\pm0.54$	$1\times10^{2}\pm0.09$
	1200	ND	ND	$1.65\times10^{4}\pm0.08$	ND
Zn^{2+}	25	$1.03\times10^{4}\pm0.02$	$3.23\times10^{4}\pm0.31$	$12.8\times10^{5}\pm1.29$	$4.01\times10^{4}\pm0.32$
	50	$8.3\times10^{3}\pm0.13$	$2.93\times10^{4}\pm0.21$	$11.93\times10^{5}\pm1.00$	$3.86\times10^{4}\pm0.12$
	100	$6.9\times10^{3}\pm0.09$	$2.9\times10^{3}\pm0.12$	$11.13\times10^{5}\pm1.41$	$2.09\times10^{4}\pm0.45$
	200	$4.5\times10^{3}\pm0.06$	$2.0\times10^{3}\pm0.09$	$8.65\times10^{4}\pm0.80$	$1.00\times10^{4}\pm0.11$
	400	$1.3\times10^{3}\pm0.02$	$2\times10^{2}\pm0.11$	$8.48\times10^{4}\pm0.66$	$6.5\times10^{3}\pm0.59$
	800	ND	$1\times10^{2}\pm0.01$	$6.15\times10^{4}\pm0.50$	$2.6\times10^{3}\pm0.16$
	1200	ND	ND	$4.35\times10^{4}\pm0.95$	$1.8\times10^{3}\pm0.09$
Cr^{6+}	25	$9.35\times10^{4}\pm0.11$	$2.22\times10^{4}\pm0.21$	$5.23\times10^{4}\pm0.39$	$3.81\times10^{4}\pm0.15$
	50	$3.48\times10^{4}\pm0.05$	$1.40\times10^{4}\pm0.31$	$4.07\times10^{4}\pm0.14$	$3.04\times10^{4}\pm0.32$
	100	$2.09\times10^{4}\pm0.01$	$1.10\times10^{4}\pm0.071$	$3.69\times10^{4}\pm0.12$	$9.6\times10^{3}\pm0.96$
	200	ND	$9.8\times10^{3}\pm0.95$	$1.93\times10^{4}\pm0.12$	$2.3\times10^{3}\pm0.14$
	400	ND	$7\times10^{2}\pm0.829$	$9.2\times10^{3}\pm0.96$	$1.0\times10^{3}\pm0.01$
	800	ND	ND	ND	ND
	1200	ND	ND	ND	ND
Cu^{2+}	25	$11.03\times10^{5}\pm0.15$	$5.38\times10^{4}\pm0.57$	$4.05\times10^{4}\pm0.03$	$1.29\times10^{4}\pm0.4$
	50	$3.09\times10^{4}\pm0.11$	$4.13\times10^{4}\pm1.01$	$3.06\times10^{4}\pm0.42$	$1.13\times10^{4}\pm0.19$
	100	$2.35\times10^{4}\pm0.09$	$3.67\times10^{4}\pm0.75$	$2.11\times10^{4}\pm0.12$	$9.5\times10^{3}\pm0.87$
	200	$1.88\times10^{4}\pm0.03$	$2.91\times10^{4}\pm0.02$	$2.03\times10^{4}\pm0.11$	$8.8\times10^{3}\pm0.34$
	400	$1.03\times10^{4}\pm0.01$	$2.07\times10^{4}\pm0.11$	$1.67\times10^{4}\pm0.22$	$6.0\times10^{3}\pm0.96$
	800	$8\times10^{2}\pm0.29$	$1.69\times10^{4}\pm0.10$	$1.39\times10^{4}\pm0.02$	$2.9\times10^{3}\pm0.14$
	1200	ND	$1.06\times10^{4}\pm0.09$	$1.26\times10^{4}\pm0.07$	ND

Table 2: Multi-metal resistance pattern in 50 gram negative bacterial isolates from KGMU hospital (Untreated).

No of Metal	Resistance Pattern	Isolates	Percentage %)	MMR Index
3	Cu, Ni, Zn	1	2	0.42
4	Cr, Cu, Ni ,Zn	1	8	0.14
	Co, Ni, Cd, Zn	1		
	Cu, Ni, Cd, Zn	1		
	Cr, Cu, Cd, Zn	1		
5	Co, Cu, Ni, Cd, Zn	2	38	0.03
	Co. Cr, Cu, Ni, Zn	11		
	Hg, Cr, Cu, Ni, Cd	1		
	Hg, Cr, Cu, Cd, Zn	1		
	Cr, Cu, Ni, Cd, Zn	4		
6	Hg, Co, Cr, Cu, Ni, Zn	4	42	0.04
	Co, Cr, Cu, Ni, Cd, Zn	13		
	Hg, Co, Cu, Ni, Cd, Zn	1		
	Hg, Co, Cr, Cu, Ni, Cd	1		
	Hg, Cr, Cu, Ni, Cd, Zn	2		
7	Hg, Co, Cr, Cu, Ni, Cd, Zn	5	10	0.2

All isolates were identified and characterized on the basis of their biochemical properties. The main genus were identified as '*E.coli, Enterobactor, Pseudomonas, Klebsiella, Salmonella, Serratia, Citrobacter and Proteus*' from the entire sampling sites of hospital wastewater (Table 2) and then tested for their MIC against seven heavy metals (Hg^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, $Co^{2+}$and Cr^{3+}) at varying concentration (50-1200 µg/ml). The Hg2+ showed highest toxicity against all the gram-negative bacterial isolates from the entire sites tested. In site 1, 72% of the total isolates showed their MIC range 50-100µg/ml for Hg2+ followed by 42%, 34%, 10%, 6% and 2% against Cd^{2+}, Co^{2+},Cr^{3+}, Ni^{2+} and Zn^{2+}, respectively. Maximum 30% of the isolates showed their MIC range 1200-1600 µg/ml against Cu^{2+}. No MIC range was recorded at lower concentration (50-100) against Cu^{2+} and at higher concentration (1200-1600) against Hg^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Cr^{3+}, respectively (Fig-2).

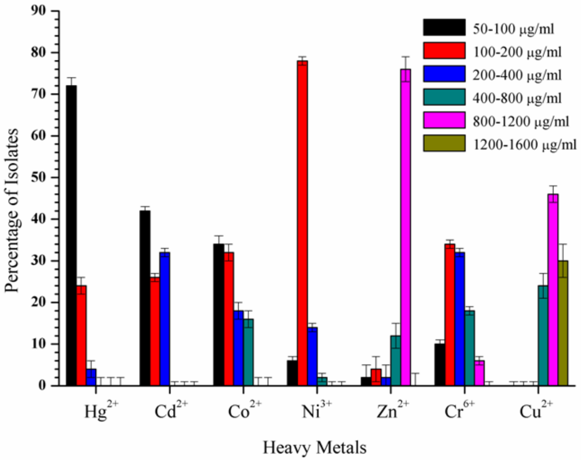

Fig. 2: Gram negative isolates showing various ranges of MIC of metal from site 1).

In site-II maximum 88%, 86% and 42% isolates showed their MIC range 50-100 µg/ml against Hg^{2+}, Co^{2+}, and Cd^{2+}, respectively. 78% of the isolates showed their MIC range 100-200 µg/ml against Zn^{2+} while 60%, of the isolates demonstrated their MIC 1200-1600 µg/ml against Cr^{3+}. The MIC was not detected at range 100-200 and, 400-800 µg/ml against Cr^{3+}, and Hg^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} respectively (Fig-3).

In site-III, 76% of the isolates showed their MIC range 50-100 µg/ml against Cd^{2+} followed by 48%, 24%, 22% and 14% against Hg^{2+} ,Zn^{2+}, Cr^{3+} ,Co^{2+} and Cu^{2+}, respectively. Maximum 62%, 38%, 32%, 26%, 20%, 16% and 8% of the isolates showed their MIC (200-400 µg/ml) against Cu^{2+}, Ni^{2+},Zn^{2+}, Cr^{3+}, Co^{2+}, Cd^{2+} and Hg^{2+}, respectively. No MIC was recorded at varying concentration range of the heavy metal tested (Fig-4).

In case of site-IV, no MIC was observed at range 50-100µg/ml against Co^{2+}, Ni^{2+}, Cr^{3+} and Cu^{2+}, respectively. 76% of the isolates showed their MIC at lower concentration (50-100 µg/ml) against Hg^{2+} while 46% of the isolates showed their MIC at 1200-1600 µg/ml against Cr^{3+}. Maximum 72%, 66%, 42%, 38%, 34%, 16% and 12% of the isolates showed their MIC at 200-400 µg/ml against Zn^{2+}, Co^{2+} Cu^{2+}, Cd^{2+}, Ni^{2+}, Hg^{2+} and Cr^{3+}, respectively (Fig-5).

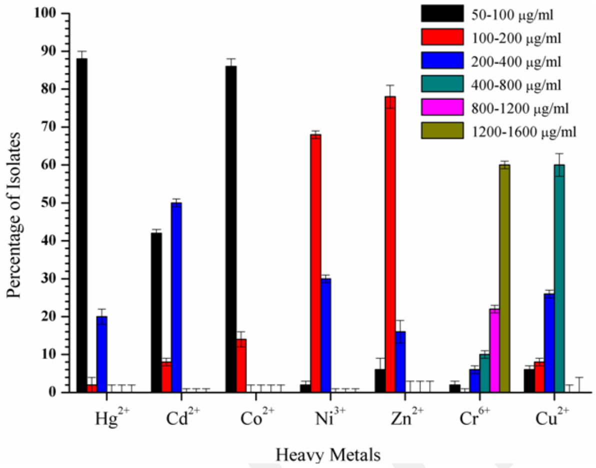

Fig. 3: Gram negative isolates showing various ranges of MIC of metal from site 2).

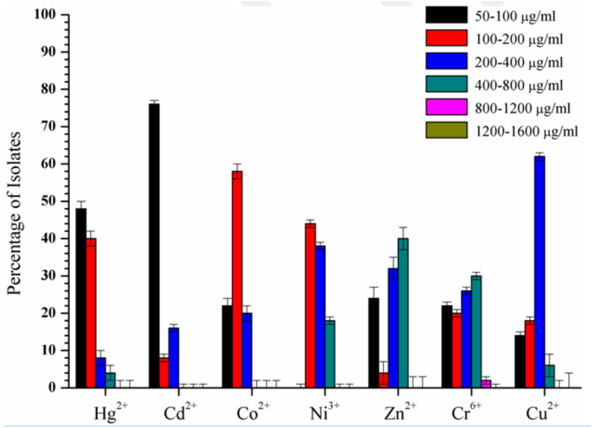

Fig. 4: Gram negative isolates showing various ranges of MIC of metal from site 3).

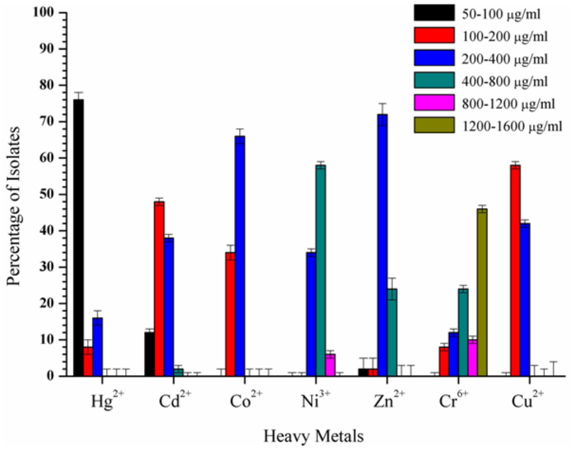

Fig. 5: Gram negative isolates showing various ranges of MIC of metal from site 4).

Majority of isolates from all the sites exhibited resistance to multiple metals (Tables 3, 4 and 5). Maximum 42% and 38% of the isolates showed 6 and 5 metal resistance pattern at a time in five different combinations in site-I. Whereas, in site-II 36% and 34% of isolates exhibited resistance pattern among 4 and 6 metal at a time in two and three different combinations.

Table 3: Multi-metal resistance pattern in 50 gram negative bacterial isolates from SGPGI hospital (Treated).

No of Metal	Resistance Pattern	Isolates	Percentage (%)	MMR Index
3	Cu, Ni, Zn	1	8	0.10
	Cr, Cu, Zn	1		
	Cr, Ni, Zn	2		
4	Cr, Cu, Ni ,Zn	16	36	0.03
	Cr, Cu Ni, Cd	2		
5	Cr, Cu, Ni, Cd, Zn	3	22	0.06
	Co. Cr, Cu, Ni, Zn	1		
	Co, Cr, Cu, Ni, Cd	1		
	Cr, Cu, Ni, Cd, Zn	6		
6	Co, Cr, Cu, Ni, Cd, Zn	6	34	0.05
	Hg, Cr, Cu, Ni, Cd, Zn	11		

Table 4: Multi-metal resistance pattern in 50 gram negative bacterial isolates from SGPGI hospital (Untreated).

No of Metal	Resistance Pattern	Isolates	Percentage (%)	MMR Index
2	Cr, Ni	2	6	0.09
	Ni, Zn	1		
3	Cu, Ni, Zn	1	12	0.07
	Cr, Cu, Zn	1		
	Cr, Ni, Zn	1		
	Co, Cu, Ni	1		
	Cr, Cd, Zn	1		
	Cr, Co, Ni	1		
4	Cr, Cu, Ni ,Zn	1	16	0.07
	Cr, Ni, Cd, Zn	1		
	Co, Cu, Ni, Zn	2		
	Hg, Cr, Ni, Zn	1		
	Hg, Co, Cu, Ni	2		
	Co, Cr, Cu. Ni	1		
5	Co. Cr, Cu, Ni, Zn	11	36	0.03
	Hg, Cr, Cu, Ni, Zn	1		
	Hg, Co, Cu, Ni, Zn	2		
	Hg, Co, Cu, Ni, Cd	1		
	Hg, Co, Cr, Cu, Ni	2		
	Co, Cr, Cu, Ni, Cd	1		
6	Hg, Co, Cr, Cu, Ni, Zn	10	26	0.06
	Hg, Co, Cr, Cu, Ni, Cd	1		
	Co, Cr, Cu, Ni, Cd, Zn	2		
7	Hg, Co, Cr, Cu, Ni, Cd, Zn	2	4	0.5

Table 5: Multi-metal resistance pattern in 50 gram negative bacterial isolates from RML hospital (Untreated).

No. of Metal	Resistance Pattern	Isolates	Percentage (%)	MMR index
5	Co, Cu, Ni, Cd, Zn	1	2	0.71
6	Hg, Co, Cr, Cu, Ni, Zn	2	60	0.02
	Co, Cr, Cu, Ni, Cd, Zn	28		
7	Hg, Co, Cr, Cu, Ni, Cd, Zn	19	38	0.05

In addition, of 36% and 26% isolates showed metal resistance pattern among five and six metal at a time in five and three different combinations from site-III while 60% and 38% of the isolates exhibited metal resistance pattern among 6 and 7 metals at a time in two and one combination in site-4, respectively. Resistance potential of the isolates was also evaluated in terms of multiple metal resistance indexes. A varied trend of MMR Index was observed among the isolates from the four different sampling sites. Low and high risk MMR were recorded among the Gram negative bacterial isolates from the hospital wastewater. MMR range 0.03-0.42, 0.03-0.10, 0.03-0.5 and 0.05-0.71 were recorded among the isolates from site I (untreated), site II (treated), site III and site IV (untreated), respectively.

DISCUSSION

Metal resistance is a common process in many microorganisms that deal with toxic compounds in their habitats. In the last few years, metal resistance has increased our knowledge about the cellular mechanisms involved in metal resistance. Mercury resistance has been described in a number of bacterial species (Nakamura and Silver, 1994; Mergeary *et al.*, 2003). In Present study, all the isolates were tested for viable count and their resistance against certain metals (Hg^{2+}, Cd^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, $Co^{2+}$and Cr^{3+}). The viable count of gram negative bacteria in different concentrations of metal ranged from $11.03x10^5$-$8.0x10^2$, $12.02x10^5$-$1.0x10^2$, $12.33x10^5$-$5.0x10^2$ and $4.01x10^4$-$1.0\ x10^2$ cfu/ml of water in site I, II, III and IV, respectively. Out of 50 Gram negative bacteria isolated from all sites, 42% were found to be resistant to six metal ions at a time in five different combinations, while only 2.0% of the isolates were resistant to three metal ions at a time. 36% resistance were found in four and five metals at a time in two and five different combinations in both site II and III, respectively. Maximum 60% of the isolates were found to be resistance to six metals at a time in two different combinations and 38% of the isolates were resistance in seven metals at a time in site IV (Table 3, 4, 5). The frequency of metal resistance in the present study is comparable to those reported elsewhere (Malik and Jaiswal, 2000; Ansari *et al.,* 2008; Alam and Imran, 2014). Malik and Aleem, (2010) reported that the majority of bacterial isolates from metal contaminated soil showed resistance to multiple metal ions. 20.8% of the pseudomonas isolates were resistant to eight metal ions at a time, while 12.5% of the isolates from groundwater irrigated soil were resistant to five metal ions at a time in two different combinations. Similar observations have also been reported earlier (Appanna *et al.,* 1996; Malik *et al.,* 2008; Wei *et al.,* 2009). Present results exhibited a high incidence of metal resistance in the isolates from untreated wastewater as compared to treated wastewater (Table 2). Sabry *et al,* (1997) isolated heterotrophic aerobic metal-resistant bacterial communities from marine water and reported that great portion of the isolates were resistant to lead (94%), nickel (40%), arsenate (35%) and copper (22%). Similarly, Shakoori and Muneer, (2002) also reported that bacteria from wastewater origin exhibited resistance against Ag^{2+} (280– 350 μg/ml), Co^{2+} (200–420 μg/ml), Cr^{6+} (280–400 μg/ml), Cd^{2+} (250–350 μg/ml), Hg^{2+} (110–200 μg/ml), Mn^{2+} (300–380 μg/ml), Pb^{2+} (300–400 μg/ml), Sn^{2+} (480–520 μg/ml) and Zn^{2+} (300–450 μg/ml). In the present study, maximum 88%, 76% and 72% bacterial isolates from hospital wastewater exhibited MIC value of 50-100μg/ml for Hg^{2+} while

60% and 30% of the isolates showed their MIC range 1200-1600 μg/ml against Cr3 and Cu2+ in entire site tested respectively. High levels of resistance were found in Cu^{2+}, Zn^{2+} Cr3+, Ni^{2+} and Co^{2+} respectively. This coherent with other reports where author have found that the multi-resistant strains had higher MIC values compare to the sensitive ones (Karbasizaed *et al.*, 2003; Vajiheh and Naser, 2003; Basu *et al.,* 1997). We also determined the MMR index of Gram negative bacterial isolates from all sampling sites. Isolates exhibited a variation in their MMR index based on sampling sites. Low and high risk MMR were recorded among the Gram negative bacterial isolates from the hospital wastewater. MMR index is ranges between 0.03-0.71 among the isolates from entire sites tested.

CONCLUSION

Our observations are contributing to the understanding of metal tolerant among Gram negative bacteria from aquatic environment and underline the importance of describing the succession of bacterial populations indigenously present in such environment due to contamination events. Bacterial resistance to antibiotics and heavy metals is an increasing problem in today's society. Microbes have adapted to tolerate the presence of metals or can even use them to grow. Thus, a number of interactions between microbes and metals have important environmental and health implications. Accordingly, considerable variation in MIC of untreated and treated isolates has been found. Multiple resistances in untreated isolates have also been observed, indicating public health concern. Therefore, there is urgent need of detoxification of untreated sites.

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