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*Journal homepage: <http://www.journalijar.com>***INTERNATIONAL JOURNAL
OF ADVANCED RESEARCH****RESEARCH ARTICLE****Determination of Antibiotic and Metal Resistance Patterns in Bacteria Isolated from Potable Water from Karachi, Pakistan****Maqsood Ali Ansari, Samina Imtiaz, Erum Shoeb*, Uzma Badar and Obaid Yousuf Khan**

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Key words:Antibiotics,
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Water bodies with bacteria having antibiotic and metal resistance genes constitute a significant public concern. Excessive use of antibiotics in medicine, agriculture and industrial effluent contamination are suspected to be one of the major causes of increase antibiotic and metal resistance. This study was carried out to determine the antibiotic resistance and metal tolerance in bacteria isolated from potable water from different areas of the city of Karachi. Out of total 3721 bacterial isolates, 4.48% showed the resistance against all the eight antibiotics tested similarly 20.50% showed the resistance against all the four metals tested. More than 50% of the bacterial isolates showed resistance against the antibiotics, ampicillin, streptomycin, erythromycin, rifampicin and metranidazole. The percentage of metal tolerant organisms was also found to be variable ranging from 57.99% showing tolerance to 0.5mM copper to 31.98% showing tolerance to 0.5 mM cobalt. The plasmid profiles of various representative organisms although showed no correlation between the number and size of the plasmid to the level of antibiotic resistance or heavy metal tolerance, however the presence of similar size plasmids among different bacteria isolated from different locations of the city was observed. The finding suggests that potable water is an important source for the spread of antibiotic resistant and metal tolerant bacteria in Karachi. Research also indicates that there is probably a significant amount of antibiotics and metal in the environment of Karachi that exerts high selection pressure on the organisms to maintain antibiotic resistance and heavy metal tolerant genes in their genome.

*Copy Right, IJAR, 2014. All rights reserved.***Introduction**

Water resources are always under the threat of pollution by human and animal wastes. Municipal sewage and industrial effluent contamination is one of the major reason of increasing the pathogenic microorganisms and toxic heavy metal in river water (Pathak et al., 2005). This human and animal wastes adds nutrients like phosphorous, Nitrogen etc. to the receiving waters (Carpenter et al., 1998; Tabbara, 2003) making it a useful medium for the growth of microorganisms including the pathogenic bacteria. Water is also one of the important source of spread of antibiotic resistant organisms among humans and animals and also the source of introduction of resistant genes in bacterial environment (Baquero et al., 2008). Antibiotic resistant bacteria and genes for antibiotic resistance are found in surface waters, river beds etc. possibly due to excessive use of antibiotics along with improper wastewater treatment (Alzahrani et al., 2011). Antibiotics are used extensively for human and animal therapy as well as in agriculture, therefore this could also be the major reasons for the growth and spread of antibiotic resistant organisms. Compounds used by the patients as medicine are metabolized partially and thus pass into the municipal wastewater and can enter in the environment specially water compartment (Kümmerer et al., 2004).

Metals pose a long-term selection pressure as they are not degraded in the environment (Stepanauskas et al., 2005). Antibiotic and metal resistance is frequently associated with each other and often strongly correlated (Bell et al., 1983). Antibiotic resistant and metal tolerant organisms seem to be present due to the exposure to environment

contaminated with metals that causes co-incident selection for antibiotics and heavy metals resistance factors (Ramteke et al., 1997). Mobile genetic elements often contain antibiotic and metal resistant genes closely associated and it is quite likely that presence of one stressor select the others (Knapp et al., 2008). Resistance genes can not only be harboured by the bacteria in the environment but their gene pool frequency can also be increased by the continuous exposure to metals (Knapp et al., 2008). Metals like cadmium, nickel and lead enters the water bodies via wastewater (Ajmal, et al., 1998) that creates the selection pressure.

There are various ways by which the bacteria can achieve the resistance including acquiring resistance genes by horizontal transfer, recombination of foreign DNA into the chromosome, or mutations in different chromosomal loci (Davies et al., 1997). Resistance may be transferred from the resistant bacteria to the previously non-resistant pathogenic bacteria thus making conventional antimicrobial therapies ineffective in humans infected by these organisms (Khachatourians et al., 1998).

Most of the studies published were mainly focused on antibiotic and metal resistance in bacteria in the large water reservoirs such as rivers, lakes and ponds etc. In the present study we tried to show the occurrence and spread of antibiotic resistant and metal tolerant bacteria via potable water in a large metropolitan city of Karachi.

Material and Methods

Collection of water samples

Drinking water samples were collected during February to April 2011 from various locations of Karachi. The map of Karachi was divided into 42 sectors of equal sizes as shown in the Figure 1. At least two water samples were taken from each of the sector where population was present. Samples were collected in 500 ml sterile glass bottles containing 0.5 ml of 10% sodium thiosulfate to neutralize free residual chlorine. The samples were brought to the laboratory on ice and were analyzed within 4 hours of collection.

Enumeration of Standard Plate Count (SPC) bacteria

25 ml of water from each sample was filtered through the GN-6 Gelman gridded filters (pore size 0.45 μ m) according to Armstrong et al., (1981). The filters were then placed on SPC agar (Oxoid) and after incubation at 35°C for 48 hours, colonies were counted.

Preparation of Master plates

Procedure of Armstrong et al., (1981) was followed with some modifications for the preparations of the Master plates. Briefly from each of the sample 50 colonies were picked with the help of sterile toothpicks, starting from the upper left square and moving to right, continuing row to row till achieving the desired number in order to get random samples. All colonies were selected where the water samples had less than 50 colonies. Colonies selected were arranged on three replica master plates containing SPC agar and the plates were incubated for 48 hours at 35°C.

Antibiotic resistance determination

In order to perform antibiotic resistance, testing procedure of Osterblad et al. (1995) was followed with some modifications. From the two master SPC agar plates impression of the colonies were made with the help of sterile toothpick on the Mueller-Hinton agar plates (Merck) supplemented with the following antibiotic concentrations from Sigma-aldrich, ampicillin 20 μ g/ml, chloramphenicol 25 μ g/ml, streptomycin 10 μ g/ml, erythromycin 10 μ g/ml, Nalidixic acid 30 μ g/ml, Rifampicin 5 μ g/ml, tetracycline 30 μ g/ml, metranidazole 5 μ g/ml. From each master plate bacteria from colonies were transferred onto four plates with different antibiotics plus a fifth control plate having Mueller-Hinton agar without any antibiotic in the last. Only colonies that showed growth on the control plate were selected from the antibiotic plates for enumeration of antibiotic resistant or sensitive bacteria. Most of the antibiotics used in the studies were of Sigma.

Heavy metal tolerance

From the third master SPC agar plate impression of the colonies as mentioned above were taken on the Tris minimal media agar plates (Mergey et al., 1985) supplemented with one of the metals salts Cadmium Chloride, Cobalt Chloride, Copper Sulphate and Nickel Chloride with the concentrations of 0.5 mM. A control agar plate having Tris minimal media without any metal was used in the last for the transfer. Only colonies that showed growth on the control plate were selected from the metal plates for enumeration of metal tolerant or sensitive bacteria. The colonies that showed tolerance to the concentration of 0.5 mM of the metals were then used to have the impression onto the plates with metal concentrations of 1mM and 2 mM respectively along with the control as above. Heavy metal salts used were of BDH.

Plasmid DNA extraction of selected Isolates

120 isolates were selected randomly representing various tested antibiotic resistant and metal tolerant isolates belonging to different sectors of the city. 18-20 hours old Mueller-Hinton broth cultures grown in the presence of

respective antibiotic or metal were used for the plasmid DNA extraction by the GeneJET™ plasmid miniprep kit from Fermentas following manufacturer's protocol.

Plasmid profiling

Plasmid DNA extracted earlier was run on 0.8% agarose (Amresco USA) gel prepared in Tris-Borate-EDTA buffer, pH 8.0 stained with ethidium bromide along with GeneRuler™ 1 Kb Plus DNA Ladder (Fermentas) as a marker. Agarose gels were observed and photographs were taken under UV transilluminator (SCIE-PLAS).

Results

The Enumeration of Standard Plate Count (SPC) bacteria

A total of 3721 SPC bacteria were screened from 72 water samples obtained from various sectors of the city of Karachi. The number of bacteria in different sectors were found to be in the range of 2 to >350 per 25 ml water. None of the water sample was found to be free from bacteria.

Antibiotic resistance determination

Of the total 3721 SPC bacteria screened for the resistance to various antibiotics, 1545 (41.52%) showed no resistance to any of the antibiotic tested. Interestingly none of the bacterial isolate showed resistance to one or two antibiotics i.e. either they were resistant to three or more antibiotics or were sensitive. 2176 (58.47%) bacteria showed resistance to three or more antibiotics making them multiple antibiotic resistant (MAR) bacteria (Figure 2). 4.48% were found to resist all the eight antibiotics tested whereas 4.0% to seven, 31.49% to six, 13.49% to five, 4.48 to four and 0.51% to three antibiotics tested respectively (Table I).

Metal tolerance determination

20.98% of the 3721 bacteria showed tolerance to all the 4 heavy metals tested at a concentration of 0.5mM. Similarly 29.99% showed tolerance to 3 heavy metals, 6.5% to 2 and 1.5% showed tolerance to one of the heavy metals tested respectively (Table II). The frequency of tolerant bacteria changed when the concentration is raised to 1mM and 2.0mM as shown in Figure 3.

Plasmid profiling

Although many bacterial isolates showed similarity in the numbers and sizes of the plasmids as shown in figure 4, however it did not showed any correlation with the pattern of resistance to the antibiotics or heavy metal tolerance (Table III).

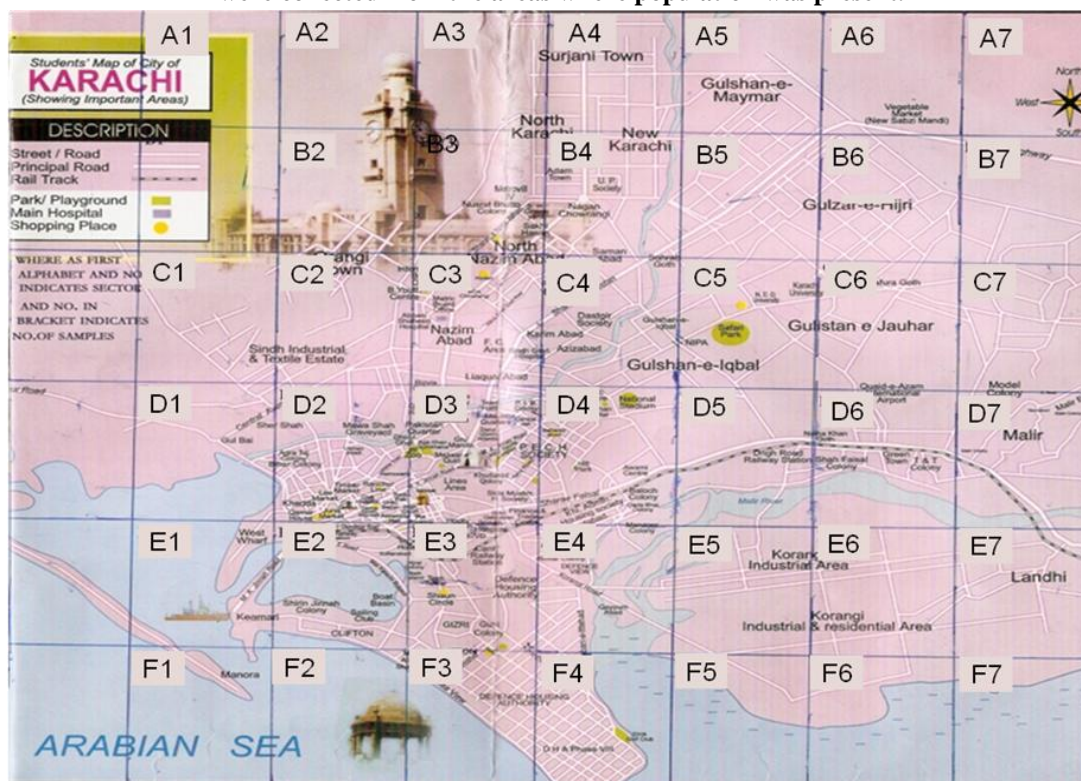
Discussion

Antibiotic resistance in bacteria has raised great concern all over the world. The possible role of metal contamination in the maintenance and spread of antibiotic resistant genes in the environment has raised the concerns even further. This investigation documents the presence of multiple antibiotic and metal tolerant bacteria in potable water in the city of Karachi. The Karachi city map was divided into 42 sectors of equal sizes and two to three potable water samples were collected from each of the sector depending on the population thickness. No samples were collected from the areas without population. Out of the total 3721 bacterial isolates 58.47% showed multiple antibiotic resistance against three or more of the eight antibiotics tested similarly 56.0% showed multiple metal tolerance against two or more of the four heavy metals tested. 41.52% of the bacterial were found sensitive to all the antibiotics tested and 41.49% were sensitive to all the four heavy metals tested. Interestingly no bacterial sample showed the resistance against one or two antibiotics i.e., either there was resistance against three or more antibiotics or no resistance at all. This could be due to the presence of more than two antibiotic resistance genes together on a DNA fragment which is either inherited or horizontally transferred among various bacteria (Shoeb et al., 2012). Many researchers have found metal tolerance and antibiotic resistance in the same bacteria (Summers, 1993; Alonso et al., 2001). Our results also demonstrated the same phenomenon where the bacteria showed the ability of both resisting antibiotics and tolerating heavy metals.

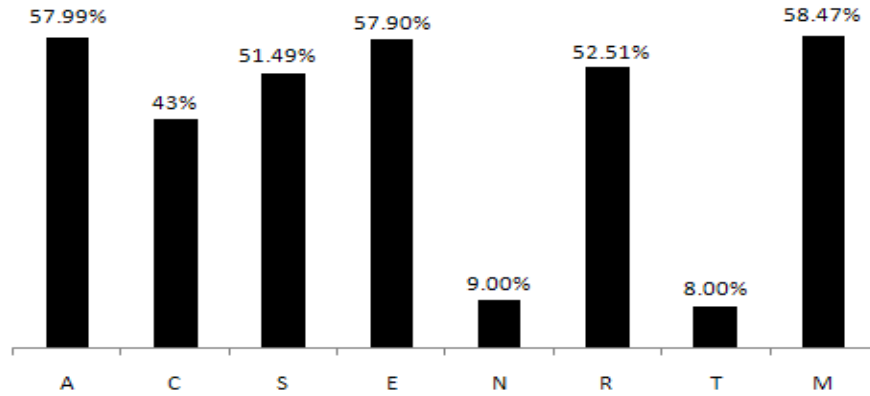
It is believed that bacteria in the water system come across variety of metals which exerts the selection pressure for metal tolerance (Baker-Austin et al., 2006). Ridgway et al., (1981) with the help of X-ray energy-dispersive microanalysis have shown that galvanized iron distribution pipes can accumulate metals especially at the dead ends and allow metal tolerant organisms to survive and eventually these organisms are released in the drinking water system. This may be true as metal pipes are generally used for supplying the water inside the houses. There are many other factors contributing towards the survival and spread of metal tolerance and antibiotic resistance gene in the bacteria. Various studies have shown that chlorine that is used as disinfectant selects antibiotic resistant bacteria (Shrivastava, 2004; Hiraishi et al., 1998). In Karachi, as chlorination is the major way of disinfection used by the Municipal cooperation for treating water, therefore it could be an important factor for the selection of the antibiotic

resistance gene in the bacteria. Over the counter availability of the antibiotics without doctor's prescription, self medication, running of water lines along with the sewerage lines, old city infrastructure and disposal of factory effluents in the environment without proper treatment are probably some other possible reasons of the spread of antibiotic resistant and metal tolerant bacteria in the waters of Karachi city. A study by Calomiris et al. (1984) showed that the bacterial isolates from raw water system were less tolerant to many metals compared to the isolates from drinking water system and the bacterial isolates tolerant to metals were found to be resistant to antibiotics as well. A study by McArthur and Tuckfield, (2000) has concluded that antibiotic resistance is possibly promoted by mercury pollution as they found a positive correlation between resistance of antibiotics in resident sediment bacteria and stream sediment mercury concentration. It may be possible that similarly other metals also play some role in the promotion of some antibiotic resistance in bacteria. Jones et al. (1986) showed that antibiotic resistance and metal tolerance is a phenomenon of natural selection taking place in the environment for survival. Our results showed the highest resistances against ampicillin that was 58.72% and interestingly out of all the resistant bacteria, less than 1% of the bacteria were found to be sensitive to ampicillin. Study of Pathak and Gopal, (2005) also showed the presence of high proportion of bacteria from various river fish organs, resistant to ampicillin, tetracycline, chloramphenicol and streptomycin. This could be due to the presence of antibiotics and heavy metals in the water environment. The finding of our research also suggests that potable water is an important source for the spread of antibiotic resistant and metal tolerant bacteria in the city of Karachi as antibiotic resistant and metal tolerant bacteria were found in all the sectors of the city. Our research also indicates that there is probably a significant amount of stress in the environment of Karachi that exerts high selection pressure on the organisms to maintain the antibiotic resistance and heavy metal tolerant genes in their genome. This highlights the importance of imposing strict regulations for the treatment of factory effluents and disposal in the environment and ban on the selling of antibiotics without the doctor's prescription. Public awareness for the prudent antibiotic use and cautious prescriptions of antibiotics by the medical practitioners can also reduce the spread of antibiotic resistance among bacteria.

Figure 1 Map of the city of Karachi showing 42 sectors. All sectors were of equal sizes and water samples were collected from the areas where population was present.

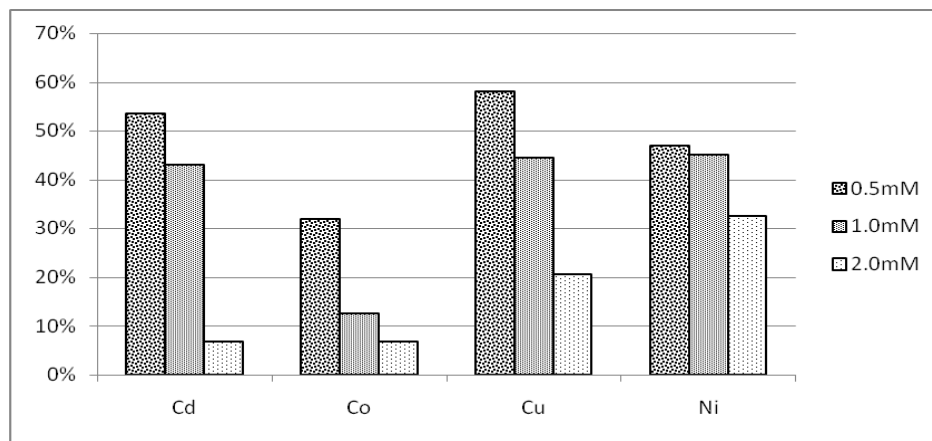


Map of the Karachi city



Frequency of antibiotic resistance among SPC bacteria

Figure 2 The frequency of bacterial isolates resistant to various antibiotics was found to be variable ranging from 8% against Tetracycline to 58.47% against Metronidazole. More than 50% of the bacterial isolates showed resistance against the antibiotics, ampicillin, streptomycin, erythromycin, rifampicin and metranidazole (A – Ampicillin, C – Chloramphenicol, S – Streptomycin, E – Erythromycin, N – Nalidixic acid, R – Rifampicin, T – Tetracycline, M – Metronidazole).



Heavy Metal Tolerance of Isolates

Figure 3 Percentages of bacterial isolates with tolerance against metals at 0.5mM, 1.0mM and 2.0mM concentrations. Maximum percentage was found against 0.5 mM copper, while minimum percentage of isolates tolerated cobalt at 2.0 mM concentration (Cd – Cadmium, Co – Cobalt, Cu – Copper, Ni – Nickel)

Figure 4 Agarose gel photograph showing different plasmids found in different isolates

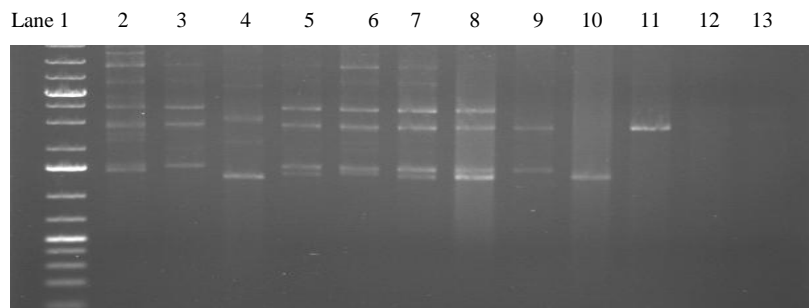


TABLE I: FREQUENCY OF SENSITIVE AND MULTIPLE ANTIBIOTIC RESISTANT PHENOTYPES AMONG ISOLATES.

Types of Multiple Resistance	Number of Isolates	Percentage of Isolates	Pattern of Resistance	Number of Isolates	Percentage of Isolates from Group	Percentage of Isolates from Total (3721)
Group-I (8 AB)	167	4.48	ACESTNMR	167	100	4.48
Group-II (7 AB)	149	4	ACSTNMR	18	12.08	0.48
			AESTNMR	18	12.08	0.48
			ACETNMR	20	13.42	0.53
			ACESTMR	18	12.08	0.48
			ACESTNM	75	50.33	2.01
			Total	149		
Group-III (6 AB)	1172	31.49	ACESNM	18	1.53	0.48
			ACESRM	1154	98.46	31.01
			Total	1172		
Group-IV (5 AB)	502	13.49	AESRM	391	77.88	10.50
			ACERM	56	11.15	1.50
			CESRM	18	3.58	0.48
			ACESM	37	7.37	0.99
			Total	502		
Group-V (4 AB)	167	4.48	AESM	18	10.77	0.48
			AERM	130	77.84	3.49
			ACES	19	11.37	0.51
			Total	167		
Group-VI (3 AB)	19	0.51	AEM	19	100	0.51
Sensitive Strains	1545	41.52	-	-	-	-
Total	3721					

A – Ampicillin; C – Chloramphenicol; E – Erythromycin; S – Streptomycin; T – Tetracycline; N – Nalidixic acid; M – Metronidazole; R – Rifampicin; AB – Antibiotics

TABLE II: Different patterns of heavy metal tolerance of isolates

Number of Heavy Metals	Heavy Metal Concentration					
	Resistance at 0.5 mM		Resistance at 1.0 mM		Resistance at 2.0 mM	
	Number of Isolates	Percentage of Isolates	Number of Isolates	Percentage of Isolates	Number of Isolates	Percentage of Isolates
Four	781	20.98	354	9.51	186	4.99
Three	1116	29.99	465	12.49	372	9.98
Two	242	6.5	986	26.49	688	18.48
One	56	1.5	298	8	0	0

TABLE III: PLASMID PROFILE SHOWING NUMBERS OF PLASMIDS IN DIFFERENT ISOLATES.

	4 Heavy Metals		3 Heavy Metals		2 Heavy Metals		1 Heavy Metals	
	No. of Strains	No. of Plasmids	No. of Strains	No. of Plasmids	No. of Strains	No. of Plasmids	No. of Strains	No. of Plasmids
8 Antibiotics	1	6	1	5	-	-	-	-
	2	3	3	4				
	1	1						
7 Antibiotics	1	7	1	5	-	-	-	-
	1	4	1	4				
	1	4	1	1				
	1	1						
6 Antibiotics	4	6	4	6	2	6	-	-
	2	5	4	5				
	11	4	10	4				
	4	3	9	3				
5 Antibiotics	4	4	7	5	1	6	1	4
	1	3	4	3				
			2	1				
4 Antibiotics	-	-	1	4	1	3	1	4
			1	3				
			1	1				
3 Antibiotics	-	-	1	4	-	-	-	-

References

- Ajmal, M., Mohammad, A., Yousuf ,R.. and Ahmad, A. (1998)** : Adsorption behaviour of cadmium, zinc, nickel and lead from aqueous solution by *Mangifera indica* seed shell. Indian J. Environ. Health., 40:5-26.
- Alonso, A., Sánchez, P. and Martínez, J.L .(2001)**: Environmental selection of antibiotic resistance genes. Environ microbial., 3:1-9.
- AlzahranI, A.M. and Gherbawy, Y.A.(2011)**: Antibiotic resistance in *Escherichia coli* strains isolated from water springs in Al-Ahsa Region. Afr. J. Micro. Res., 5:123-130.
- Armstrong, J.L., Shigeno, D.S., Calomiris, J.J. and Seidler,R.J. (1981)**: Antibiotic-Resistant bacteria in drinking water. Appl. Environ. Microbiol., 42: 277-283.
- Baker-Austin, C., Wright, M.S., Stepanauskas, R. and Mcarthur, J.V. (2006)**: Co-selection of antibiotic and metal resistance. Trends Microbiol., 14: 176-182 .
- Baquero, F., Martinez, J.L. and Canton, R.(2008)**: Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotech., 19: 260-265.
- Bell, J.B., Elliot, G.E. and Smith, D.W. (1983)**: Influence of sewage treatment and urbanization on selection of multiple faecal coliform population2. Appl. Environ. Microbiol., 46: 227–32.
- Calomiris,J.J., Armstrong, J.L. and Seidler, R.J. (1984)**: Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. Appl. Envir. Microbiol., 47: 1238 - 1242.

Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N. and Smith, V.H. (1998): Nonpoint pollution of surface waters with phosphorus and nitrogen. *Ecol Appl.*, 8:559–568.

Davies, J.E. (1997): Origins, acquisition and dissemination of antibiotic resistance determinants. *Ciba Found. Symp.*, 207:15–27.

Hiraishi, A. (1998): Respiratory quinone profiles as tools for identifying different bacterial population in activated sludge. *J. Gen. Appl. Microbiol.*, 34: 39-56.

Jones, J.G., Gardener, S., Simon, B.M. and Pickup, R.W. (1986): Antibiotic resistant bacteria in Windermere and two remote upland tarns in English Lake District. *J. Appl. Bacteriol.*, 6:443-453.

Khachatourians, G.G. (1998): Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. *Can. Med. Assoc. J.*, 159: 1129-1136.

Knapp, C.W., Engemann, C.A., Hanson, M.L., Keen, P.L., Hall, K.J. and Graham, D.W. (2008): Indirect evidence of transposon-mediated selection of antibiotic resistance genes in aquatic systems at low-level oxytetracycline exposures. *Environ. Sc. Tech.*, 42: 5348–5353.

Kümmerer, K. (2004): Resistance in the environment. *J. Antimicrob. Chemother.*, 54: 311–320.

Mcarthur, J.V. and Tuckfield, R.C. (2000): Spatial patterns in antibiotic resistance among stream bacteria: effects of industrial pollution. *Appl. Environ. Microbiol.*, 66:3722-3726.

Mergeay, M., Nies, D.H., Schlegel, H.G., Gerits, J., Charles, P. and Van Gijsegem, F. (1985): *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *J. Bacteriol.*, 162: 328-334.

Osterblad, M., Leistevo, T. and Huovinen, P. (1995): Screening for antimicrobial resistance in fecal samples by the replica plating method. *J. Clin. Microbiol.*, 33:3146-3149.

Pathak, S.P. and Gopal, K. (2005): Occurrence of antibiotic and metal resistance in bacteria from organs of river fish. *Environ. Res.*, 98: 100-103.

Ramteke, P.W. (1997): Plasmid mediated co-transfer of antibiotic resistance and heavy metal tolerance in coli forms. *Indian J. Microbiol.*, 37: 177-181.

Ridgway, H.F., Means, E.G. and Olson, B.H. (1981): Iron bacteria in drinking water distribution systems: elemental analysis of *Gallionella* stalks, using X-ray energy-dispersive microanalysis. *Appl. Environ. Microbiol.*, 41:288–297.

Stepanauskas, R., Glenn, T.C., Jagoe, C.H., Tuckfield, R.C., Lindell, A.H. and Mcarthur, J.V. (2005): Elevated microbial tolerance to metals and antibiotics in metal-contaminated industrial environments. *Environ. Sci. Technol.*, 39:3671–3678.

Shrivastava, R., Upreti, R.K., Jain, S.R., Prasad, K.N., Seth, P.K. and CHATURVEDI, U.C. (2004): Suboptimal chlorine treatment of drinking water leads to selection of multidrug-resistant *Pseudomonas aeruginosa*. *Ecotoxicol., Environ. Saf.*, 58:277-283.

Shoeb, E., Badar, U., Akhter, J., Shams, H., Sultana, M. and Ansari, M.A. (2012): Horizontal Gene Transfer of Stress Resistance Genes through Plasmid Transport. *World J Microbiol Biotechnol.*, 28: 1021–1025.

Summers, A.O., Wireman, J., Vimy, M.J., Lorscheider, F.L., Marshall, B., Levy, S.B., Bennett, S. and Billard, L. (1993): Mercury released from dental "silver" fillings provokes an increase in mercury and antibiotic-resistant bacteria in oral and intestinal floras of primates. *Antimicrob. Agents Chemother.*, 37: 825-834.

Tabbara, H. (2003): Phosphorus loss to runoff water 24 h after application of liquid swine manure or fertilizer. *Journal of Environmental Quality.*, 32: 1044–1052.