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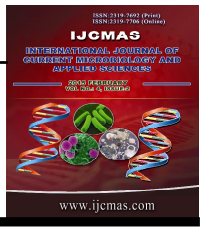

Original Research Article

An Investigation on Metal Tolerance and Antibiotic Resistance Properties of Bacterial Strains Isolated From Two Different Drinking Water Sources

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A B S T R A C T

Bacterial isolates from two different drinking water sources were examined to assess the pattern of antibiotic resistance and metal tolerance. Plasmid curing was carried out for specific antibiotic and metal resistances to ascertain plasmid borne transfer of resistance genes. Positive correlation between tolerance to Cu, Zn, Cd and Cr and double antibiotic resistance were noted among bacteria from distribution system water and not among the bacteria from raw waters. Plasmid curing results showed the loss of antibiotic resistance profile in distribution system strain and confirms a relationship of antibiotic resistance with plasmid. We concluded that the selective factors operating in the aquatic environment of a water treatment facility can act to increase the proportion of antibiotic and metal resistant members of bacterial population in distribution system water.

Keywords

Antibiotic resistance, Disc diffusion, Drinking water, Metal tolerance, Plasmid curing.

Introduction

The environment is changing rapidly due to human intervention. It is getting polluted day by day. But microorganisms have great power of adaptation. They manage to survive even in presence of toxic substances such as metals, organic compounds etc. (Gadd Geoffrey and Allen, 1978). Some metals are necessary for growth of the microorganisms like Cu, Fe, Mo and Mg (Beveridge *et al.,* 1982). They act as enzyme cofactors and also take part in oxidation reduction reactions. Essential for growth but are needed in trace amounts and when present in excess they show harmful effects (Ehrlich, 1997). Microorganisms have developed ways to escape from these harmful effects. Some of the ways include efflux of metal ions, complexation or reduction of metal ions (Sa'ídi Majid, 2010). It was observed in many studies that metal tolerance and antibiotic resistance properties coexist in microbial populations (Sabry *et al.,* 1997; Allen *et al.,* 1977). Present study intends to know patterns of metal tolerance and antibiotic resistance in microbes found in natural habitat like various drinking water sources. This includes raw water which was directly collected from a water source and water from distribution system where the water from same source undergoes different treatments for purification.

Isolates from two different sources (raw water and water from distribution system)

were randomly selected and characterised according to Bergey's Manual of Determinative Bacteriology, 9th edition (Bergey and Breed, 2000). Different staining procedures, morphology, sugar fermentation properties were considered for characterisation of isolated bacterial strains. Metal tolerance profile was studied to check metal tolerance for all the isolates with four different metals. A simple media compiled with metal salts was used. Antibiotic resistance profile for 5 different antibiotics was studied. Kirby Bauer disk diffusion test was used for this purpose.

To know whether phenomenon of tolerance is of plasmid origin or not, plasmid isolation and plasmid curing were undertaken.

Materials and Methods

Sampling

Raw and distribution system water were chosen for sampling (Calomiris Jon *et al.,* 1984). Raw water was untreated while water sample from distribution system undergone numbers of treatments like addition of lime, filtration, chlorination, etc.

Site for collection of raw water was pumping station, 12 Bungalow, Canada corner, Nasik, Maharashtra. The site for collection of distribution water was located 5 km away from raw water source. Distribution system water was household water which was collected from Kashiko nagar, Bhujbal farm, Nasik, Maharashtra.

Standard methodology was used for sample collection. Water samples were collected in sterile glass bottles. Samples were brought to laboratory on ice and were analysed within 6 h of collection (American Public Health Association, 1980; Armstrong *et al.,* 1981).

Isolation of bacteria

Serial dilution technique was followed for isolation of bacteria and plates were incubated at 37^0C for 24 h. Colonies were picked randomly and pure cultures were maintained on nutrient agar slants. Further characterization of bacteria was carried out.

Characterization

Isolates were identified by Bergy's Manual of Determinative Bacteriology, 9th edition. Isolates were placed into genera on the basis of cell and colonial morphology, Gram's stain, endospore staining, acid fast staining, catalase test, oxidase test and mannitol fermentation.

Antibiotic resistance testing

Kirby-Bauer Disc Diffusion method was used to determine antibiotic resistance pattern among isolates (Bauer *et al.,* 1966). Different antibiotic disks used were penicillin G (10 units), ciprofloxacin (5 mcg), levofloxacin (5 mcg), tetracycline (30 mcg), gentamycin (10 mcg) (CLSI, 2011). Using a sterile inoculating loop, four or five isolated colonies of the isolates to be tested were picked and were suspended in 2 ml of sterile saline. The suspension in the saline tube was whirled in the vortex machine to create a smooth suspension. The turbidity of this suspension was adjusted by adding more organism if the suspension was too light or diluting with sterile saline if the suspension was too heavy. The correct density was verified by measuring absorbance using a spectrophotometer. The absorbance at 625 nm was adjusted to 0.08 to 0.13. This suspension was used within 15 minutes of preparation. Sterile swab was dipped into the inoculum tube. The swab was rotated against the side of the tube (above the fluid level) using firm pressure, to remove excess

fluid. The dried surface of a Muller - Hinton agar plate was inoculated by streaking the swab three times over the entire agar surface and the plate was rotated approximately 60 degrees each time to ensure an even distribution of the inoculums. The plate was rimmed with the swab to pick up any excess liquid. The swab was discarded into an appropriate container. The plate was allowed to sit at room temperature at least 3 to 5 minutes. The appropriate antimicrobial disks were placed on the surface of the agar, using forceps to dispense each antimicrobial disk one at a time. After all disks were in place, the plates were inverted and incubated at 35°C for 16 to 18 h.

Profiling metal tolerance

Nutrient agar incorporated with salts of metals was used for selective isolation of metal resistant bacteria. Metal salts used were copper chloride ($CuCl_2$), zinc chloride ($ZnCl_2$), potassium dichromate ($K_2Cr_2O_7$) and cadmium chloride ($CdCl_2$). The concentration of each heavy metal was maintained as follows (Samanta *et al.,* 2012; Calomiris Jon, 1984):

Copper chloride ($CuCl_2$): 0.3, 0.6,1.2,2.4,3.2mg/ml

Zinc chloride ($ZnCl_2$): 0.3, 0.6,1.2,2.4,3.2mg/ml

Potassium dichromate ($K_2Cr_2O_7$): 0.25, 0.5, 0.75, 1.0, 1.25, 1.5mg/ml

Cadmium chloride ($CdCl_2$): 0.25, 0.5, 0.75, 1.0, 1.25, 1.5mg/ml

Plasmid isolation and Curing

Alkaline lysis method of plasmid isolation was used to isolate plasmid DNA which was characterised by agarose gel electrophoresis (Sambrook and Russell, 2007; Patwardhan *et al.,* 2008). Plasmid curing was carried out to determine the likelihood of plasmid-borne resistance pattern (Nageswaran Natasha *et al.,* 2012; Zaman *et al.,* 2010). Curing agent used for plasmid curing was Acridine orange (Salisbury Vyvyan *et al.,* 1972). 200 µg/ml of acridine orange solution was prepared in distilled water. 9 ml of peptone water and 10 ml acridine orange solution were autoclaved separately. 9 ml of peptone water was mixed with 1 ml acridine orange solution. In another tube, 5 ml of peptone water was inoculated with culture at 37°C. After about 7-8 h, 0.1 ml of this broth was transferred to a fresh 10 ml solution of acridine orange. The cultures were then incubated at 37º C in an orbital shaker at 150 rpm for 48 h. The cultures were plated on Luria Agar Medium. After 24 h incubation at 37º C, the plates were observed for growth. From this plate culture some well isolated colonies were randomly selected and checked for metal tolerance and antibiotic resistance. The cured plasmid cells were detected comparing the development of bacterial colonies on metal and antibiotic containing plates with that of the normal plate (without metal and antibiotic). The samples that showed colonies on normal agar but failed to grow on agar supplemented with metal and antibiotic were the possible cured isolates.

Result and Discussion

Bacterial species identified in raw water system are shown in Table 1. The predominant organisms in raw water were *Micrococcus sp.* and *Bacillus sp.* The remaining types in the population included *Streptococcus sp.* and *Corynybacterium sp.*

Bacterial species identified in distribution water system are shown in Table 2. The distribution system water was dominated by

Bacillus sp. The remaining type in the population included *Lactobacillus sp.*, *Staphylococcus sp.* and *Enterobacter sp.*

The numbers of isolates in raw water were more than in distribution water

Testing of antibiotic resistance

From raw water system, isolate 1,2,3,8 and 9 were resistant to penicillin G while other isolates were sensitive to the same. All the isolates were sensitive to remaining antibiotics (Table 3). Isolate I and V from distribution water system were doubly antibiotic resistant to penicillin G and tetracycline. Isolate II was resistant to penicillin G. Remaining isolates were sensitive to all other antibiotics (Table 4).

Metal tolerance profile

From raw water system, isolates 1, 2, and 3 showed growth at concentration 0.3mg/ml. Rest of the isolates didn't show growth at minimum concentration. At 1.2 mg/ml concentration only isolate 2 survived. Isolates were sensitive to other metals ($ZnCl_2$-0.3 mg/ml, $CdCl_2$-0.25 mg/ml, $K_2Cr_2O_7$-0. 25mg/ml) even at minimum concentration (Table 5).

Among 5 isolates of distribution system, each isolate was tolerant to at least two metal ions at minimum concentration. To chromium all isolates except isolate IV showed tolerance at minimum concentration. All isolates except isolate V were resistant to $CuCl_2$ at minimum concentration. Similarly, isolates I, IV, V showed tolerance to $ZnCl_2$ (0.3 mg/ml) and $CdCl_2$ has proved fatal to all isolates except IV (Table 6). None of the isolates showed growth at higher concentrations of metals. Hence we can say that as compared to raw water, distribution system showed increased level of metal tolerance.

Plasmid isolation and curing

All metals and antibiotics towards which the isolates showed resistance (for metals at a particular concentration) were subjected to plasmid curing experiment to determine the likelihood of plasmid borne resistance pattern. Among all the 9 raw water isolates, isolate 7 showed the presence of plasmid which was sensitive for all the metals and antibiotics. Hence none of the raw water isolate was plasmid cured.

From the distribution system water isolates, isolate I, II and V showed presence of plasmid. Isolates I, II and V were resistant to antibiotics and metal tolerant, hence plasmid curing of these species was done. After the plasmid curing, isolate I, II and V remained resistant to penicillin G while isolate E had turned from resistant to sensitive for tetracycline. This suggested that, the genes responsible for penicillin G resistance were located on chromosome while genes responsible for tetracycline resistance in isolate V were located on plasmid.

Changes in raw water microbial population upon entering the distribution system were reflected. Percentage of antibiotic resistance in raw water isolate was 30% while in distribution system water isolates it was 50%. These values were comparable to those obtained by Calomiris *et al.* (1984) who found proportion of up to 20.4% in raw water and 36.7% in Distribution water. Also in raw water 60% bacteria were resistant to single metal while in distribution water all the bacteria were resistant to at least one metal at minimum concentration. The observed occurrences apparently reflected water treatment processes.

Table.1 Characterization of Bacteria isolated from raw water

Isolate No.	Organism	Morphology	Gram stain	Catalase test	Mannitol test	Yellow pigment	Spore formation	Strict anaerobe	Acid fast
1	*Micrcoccus sp.*	Cocci	+	+	-	+	…	…	…
2	*Micrcoccus sp.*	Cocci	+	+	-	+	…	…	…
3	*Strepto coccus sp./ Entero coccus sp*	Cocci	+	-	…	…	…	…	…
4	*Bacillus sp.*	Rod	+	…	…	…	+	-	…
5	*Bacillus sp.*	Rod	+	…	…	…	+	-	…
6	*Bacillus sp.*	Rod	+	…	…	…	+	-	…
7	*Corynebacterium sp.*	Rod	+	+	…	…	-	…	-
8	*Micrcoccus sp.*	Cocci	+	+	-	…	…	…	…
9	*Micrcoccus sp.*	Cocci	+	+	-	…	…	…	…

Table.2 Characterization of Bacteria isolated from distribution water

Isolate no.	Organism	Morphology	Gram stain	Catalase test	Oxidase	Mannitol test	Yellow Pigment	Spore formation	Strict anaerobe	Acid fast
I	*Bacillus sp.*	Rod	+	…	…	…	…	+	-	…
II	*Lactobacillus sp.*	Rod	+	-	…	…	…	-	…	-
III	*Staphylococcus sp.*	Cocci	+	+	…	-	-	…	…	…
IV	*Enterobacter sp.*	Rod	-	…	-	…	…	…	…	…
V	*Bacillus sp.*	Rod	+	…	…	…	…	+	-	…

Table.3 Antibiotic susceptibility of raw water isolates

Isolate no.	Penicillin G	Tetracycline	Ciprofloxacin	Gentamicin	Levofloxacin
1	R*	S^	S	S	S
2	R	S	S	S	S
3	R	S	S	S	S
4	S	S	S	S	S
5	S	S	S	S	S
6	S	S	S	S	S
7	S	S	S	S	S
8	R	S	S	S	S
9	R	S	S	S	S

*: Resistant ^: Sensitive

Table.4 Antibiotic susceptibility of distribution water isolates

Isolate no.	PenicillinG	Tetracycline	Ciprofloxacin	Gentamicin	Levofloxacin
I	*R	R	S	S	S
II	R	S	S	S	S
III	S	S	S	S	S
IV	S	S	S	S	S
V	R	R	S	S	S

Table.5 Metal tolerance pattern of raw water isolates

Isolate no.	$CuCl_2$ (0.3mg/ml)	$CuCl_2$ (1.2 mg/ml)
1	+	-
2	+	+
3	+	-
4	-	-
5	-	-
6	-	-
7	-	-
8	-	-
9	-	-

Table.6 Metal tolerance pattern of distribution water isolates

Isolate no.	$CuCl_2$ (0.3mg/ml)	$ZnCl_2$ (0.3mg/ml)	$CdCl_2$ (0.25mg/ml)	$K_2Cr_2O_7$ (0.25 mg/ml)
I	+	+	-	+
II	+	-	-	+
III	+	-	-	+
IV	+	+	+	-
V	-	+	-	+

Although results of research suggested an association of chlorine disinfection with selection for antibiotic resistance, additional factors such as metal exposures from distribution water pipe material could indirectly explain the significant increase of antibiotic resistant bacteria in distribution networks (Murray and Kushner, 1984; Armstrong *et al.,* 1982). For this reason, bacterial isolates were studied to determine the manner by which metal tolerance was associated with antibiotic resistance.

Positive correlations were observed between tolerance to Cu, Zn, Cd and Cr and antibiotic resistances. Because tolerance to these metals was expressed by greater proportions of distribution system isolates than raw water isolates, microbial selection phenomenon for metal tolerance must have occurred during treatment or within the distribution system. Since multiple tolerances to Cu, Zn, Cd and Cr is significantly associated with distribution water isolates that are doubly resistant but not those that are antibiotic sensitive, it appears that simultaneous selection for metal and drug resistant bacteria may occur within the drinking water system.

Previous studies have demonstrated the role of plasmids in conferring resistances to both antibiotic and metals (McHugh *et al.,* 1975). Curing results of the study suggested that in isolate V from distribution water tetracycline resistance gene was conferred by plasmid DNA, while Penicillin G resistance gene seems to be encoded by genes of bacterial chromosome.

With drinking water, health quality standards with regard to the presence of antibiotic resistant bacteria have not been established. However, proper maintenance procedures should and can be practiced to minimize the occurrence of high concentrations of bacteria in certain areas of some distribution work systems (Allen *et al.,* 1980).

The present study clearly showed difference in number of bacteria from two different drinking water sources. Also, it was observed that there was difference in the antibiotic and metal resistance patterns for two different sources. Hence we can say that treatments are actually selecting more resistant strains. A similar study showed that chlorination treatment used for water purification is responsible for resistant strain selection (Murray and Kushner, 1984).

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