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Selection of multi-drug resistant bacteria from water treatment plants

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ABSTRACT

Water disinfection using chlorine is an important step in purification that prevents dissemination of pathogens in drinking water. However, water treatment has become a challenging operation these days as persistence of pathogens after final step of chlorination was evident in many cases. The mechanism of chlorine tolerance is not yet clear. The occurrence may be due to cellular modification of the pathogens. In the present study sensitivity of microorganisms towards chlorine and various antibiotics were determined to find whether chlorine resistance supports pathogen to attain antibiotics resistance or not. *Staph. aureus, Micrococcus, Enterobacter, Morganella morganii, Hafnia alvei, Rahnella aquatilis, Klebsiella* and *Pseudomonas aeruginosa* were isolated from chlorine treated water ($p > 0.05$) using Membrane Filter technique. In- vitro chlorine resistance and antibiotic resistance study reveals that chlorination at sub- optimal dosage ($0.5 \leq 1.5$ ppm) greatly enhances survival ability of *Pseudomonas aeruginosa* and *Enterobacter.* Bacteria from chlorine treated water were found to be more resistant to residual free chlorine and to most of the antibiotics compared to isolates obtained from the un-chlorinated pond water, leading to a conclusion that chlorination might have induced some changes in bacterial cell that ended in acquisition of chlorine tolerance and antibiotics resistance.

Keywords: Chlorine tolerance; Water treatment plant; Residual free chlorine; Sub- optimal dosage; Multi-drug resistant; Emerging pathogens

1. Introduction

Aquatic environment is a good source of acquisition for spread of resistance. Due to extensive genetic exchange in the environment the opportunistic pathogens (commonly found in free-living communities) may develop resistance by acquiring one of these proposed mechanisms: (i) modification of cell surface structures (ii) microbial adhesion to suspended particulate matter (iii) extrusion of protective extracellular capsule or slime layers (iv) formation of resistant spores. A great number of bacteria have developed resistance against different disinfectants used for treatment of water, including chlorination [1–8]. Chlorine is powerful oxidizing agent which eliminates pathogens from water. Chlorination successfully reduces the risk of waterborne diseases. Chlorine is used in many water treatment plants in the form of chlorine gas, sodium or calcium hypochlorite. It is the most commonly used drinking water disinfectant until now [9]. Residual free chlorine (RFC), in the form of unionized hypochlorous acid (HOCl) in aqueous environment, acts as an extremely potent bactericidal agent, even at concentrations of less than 0.1 mg/ litre. For, more than a century the practice of chlorination was believed to impart safety in drinking water supplies. Despite the fact, many researchers have reported the presence of different pathogenic strains from chlorine treated water [1–6]. The addition of chlorine in water at sub- optimal concentration imposes a selective pressure that results in death of susceptible bacteria while favouring resistant strains [10–13]. Bacteria may gain tolerance towards chlorine and become inherently tolerant to many drugs and broad spectrum antibiotics [7,8,11–13]. Chlorine tolerant bacterial species may follow the same pattern of resistance as the microorganisms which show enhanced antimicrobial resistance [13,14]. Armstrong et al. [11,12] suggested, without specifying the mechanisms, that stress-tolerant strains

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selected as a result of chlorination becomes more antibiotic resistant. It is not yet clear whether chlorination selects or induces changes in antibiotic resistance in bacterial populations or not. Murray et al. [13] indicated that chlorination lowered the total number of bacteria in effluent, but substantially increased the proportions of antibiotic-resistant strains that are potentially pathogenic organisms. Chlorination may help in the transfer of antibiotic resistance plasmids to the surviving population of bacteria, which needs an experimental investigation. If such chlorine tolerant antibiotic resistant form survive and perpetuate then it may prove to be a threat to the general public health. The antibiotic resistant bacterial species in the environment has led us to consider that these pathogens are emerging bio-pollutants and their dissemination in the environment should be controlled. The ESKAPE group (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* species) have become worrisome as they are known to develop multi drug resistance rapidly [15–17]. *P. aeruginosa* is an opportunistic pathogen known to cause numerous diseases like skin infection and cystic fibrosis [18–20]. It is evident in municipal water supplies where residual free chlorine is insufficient [14,15,21]. It is reported that chlorine used in potable water may selectively promote the survival of antibiotic resistant bacteria [4,6,12,13]. For instance, drinking water with suboptimal levels of chlorine selectively supported the survival of multi drug resistant *Pseudomonas aeruginosa* [14,17,18,21]. The present study was undertaken to examine the spectrum of bacteria present in the chlorine treated water of a municipal water treatment plant in West Bengal.

2.Materials and methodology

2.1. Collection of samples

This study was conducted at School of Water Resources Engineering, Jadavpur University from May 2015 to June 2016. The pond water is received by the treatment plant that passes through Horizontal roughing filter, activated carbon filter and finally the water is chlorinated using 40% sodium hypochlorite with 65% free available chlorine. The Residual Free Chlorine (RFC) of 0.5 ≤ 1.5 mg/L is usually maintained in the final water which is stored in the overhead storage tank. A standard contact time of 30 min is given for sufficient action of the RFC to take place. The water is sampled aseptically from the pond and the storage tank on each instance. An average of 200 water samples was randomly collected from treatment and distribution plant. The sampling was done maintaining an aseptic condition from each and every point in duplicate sterile containers that contains 3% (w/v) sodium thiosulfate solution. Two types of sample were collected i.e. pre-chlorinated water sample (raw pond water which is not treated yet) and final water or post chlorinated water sample (water treated and stored in overhead tank). All water samples were kept inside ice box and were taken to the laboratory for immediate tests within 4 h.

2.2. Microbiological sampling and taxonomic identification

Membrane filtration technique was followed for microbiological testing of the water samples. All the water samples were filtered through a Millipore Filter Assembly with a sterile nitrocellulose 0.45-mm membrane filter disk. The bacteria present in 250 ml water sample were retained on the surface of the membrane filter disk which was aseptically removed by a sterile forceps, and placed on duplicate Petri plates containing the following differential media: Chromocult Coliform agar (Merck) and MacConkey agar. The plates were then incubated at 37°C for 24–48 h [22–25]. Thereafter, colony morphology and cultural characteristics of each isolates were carried out. The suspected colonies were selected and each colony was streaked on Nutrient agar plates. Gram-staining and biochemical identification test kit with Bio ID Reader (Hi- Media) was used, for identification of the isolates [9]. The biochemical characteristics were identified by stab culturing on KB strips (Merck) that were incubated for 48 h inside the incubator at 35ºC. The strips were then placed inside the Bio-ID reader (Merck) for the species level identification of the strains.

2.3. In vitro resistance to chlorine

The bacterial isolates were suspended in diluted range of chlorine solution (0, 0.5, 1.0, 1.5, 2.0, 2.5 ppm). Sodium hypochlorite solution 40% was used as a stock for preparation of different diluents. Bacteria were grown overnight in nutrient broth and kept in an orbital shaker at 25ºC. The cells were harvested by centrifugation at 3,500 × g for 15 min and were washed twice in sterile ice-cold 50 mM mono basic potassium phosphate buffer (pH 7.0). The cultures were maintained in Nutrient broth with optical density of 0.5 at 600 nm and then 10 ml of the inoculum (cell volume 160×10^2–280×10^3 CFU/ml) was added in 10 ml of the chlorine solution. A contact time of 30–60 min was given for elimination of pathogens. The reaction was stopped by adding 1 ml of 1.0 M sodium thiosulphate solution. A blank was set in a similar way without chlorine. All the cultures were subjected to pour platting in nutrient agar and incubated 37ºC for 24–48 h. Growth shown after incubation was considered a positive result [26].

2.4. Susceptibility of chlorine resistance bacteria towards antibiotics

Bacteria resistant to chlorine were taken for checking their susceptibility towards different antibiotics, which was determined by the Kirby Bauer disk diffusion method. For this assay commercially available Himedia G-2 minus strips containing set of 12 antimicrobial agents - OF-ofloxacin, AK-amikacin, AMC-augmentin, CTX-cafotaxime, GEN-gentamicin, CIP-ciprofloxacin, NET-netillin, CPZ-cefoparazone, PF-pefloxacin, CAZ-ceptazidime, CXM-cefuroxime, LOM-lomefloxacin was chosen as the application of such antimicrobials in medical/clinical practices are frequent. Each strain that was chlorine resistant were picked from the culture plate with the help of a sterile cotton swab and inoculated on Muller Hinton agar (Himedia) plates by swabbing. The culture seeded plates were incubated in bacteriological incubator for 24 h at 37ºC. Thereafter the plates with lawn culture obtained after 24 h were further impregnated with the antibiotic disks. All the disks were placed aseptically onto the surface of the lawn culture by using a sterile forceps. Again the plates were kept for incubation

at 37°C for 24–48 h. Subsequently, diameters of the zone of inhibition surrounding the disks were recorded. For further reference, the plates were again incubated at 37°C for 18 h. Halos surrounding the discs were measured as an indication of inhibition of growth. This assay was repeated two times in duplicate for confirmation of the results. An antibiogram was prepared based on the result obtained from the test [26].

3. Result and discussion

The results were obtained after thorough out monitoring of the water samples collected within the duration of May 2015 to June 2016. The reports were recorded after calculating the mean findings and the standard deviation of the observed data. Statistical evaluation of the data were done using Standard deviation and Student's t- test for a good fit considering significance at P value < 0.05. The mean findings of the experimental investigation were summarized below:

3.1. Bacteriological profile of the water from water treatment plant

The treatment plant harboured many pathogens despite of regular chlorination of the water. A summary of bacteriological data is represented (Table: 1), which shows predominance of certain strains of bacteria in raw untreated pond water (Source water) and chlorine treated final water (supply water). All the isolated strains from pond and chlorine treated water were analyzed based on the cultural, morphology and biochemical tests. The species level identification was done following Bergey s Manual of Systematic Bacteriology [27]. The isolated bacterial strains were subjected to in- vitro chlorination. Sixteen different strains were isolated from the pond water out of which four species (*Enterobacter, S. aureus, Klebsiella* and *P.aeruginosa)* belonged to the ESKAPE group [16]. Under certain circumstances 60–65% of the bacterial flora was probably arrested in the treatment units. Conversely, the annual survey from May 2015 to June 2016 reports survival of 62.5% bacteria, after treating with the strong oxidizing agent like free chlorine. Few other opportunistic bacteria like *Micrococcus, Morganella morganii* sub sp. *siboni, Rahnella aquatili, Hafnia alvei*) were also isolated after chlorination. This occurrence was evident on several occasions that led eventually to in depth research.

3.2. Chlorine resistant pattern of isolates from treated water

The Arithmetic mean of the data composed from May 2015 to June 2016 signifies that after chlorination the total count of bacteria reduced considerably. However, chlorination could not efficiently reduce certain resistant bacteria like *Pseudomonas*. The result obtained signifies that all the eight strains were sensitive to RFC when a contact time of < 1 h was prevailing. Although, given a contact time of 30 min; *Klebsiella, Enterobacter, Staphylococcus aureus* and *Pseudomonas aeruginosa* showed rather unique pattern of resistance. At sub-optimal dosage of chlorine (0.5 ppm) the isolates reduced in count at a considerable rate but the growth pattern altered once higher concentration of dosage was allowed (i.e., 1 ppm) [2,4,8]. The difference in the colony count was significantly higher for these four resistant strains ($P \leq 0.01$) at higher concentrations of chlorine. Few isolates like *Micrococcus, M. morganii count* was significantly lower with increase in concentration of chlorine. *H. alvei* and *R. aqualtilis* are the rare gram negative bacteria that showed growth in presence of 0.5 ppm chlorine. Although, they could not sustain 1 ppm chlorine but the fact may not be considered negligible as it may imply on some sort of horizontal gene transfer mechanism or a sort of cellular modification, that otherwise turned these non-pathogenic sensitive strains to become resistant to 0.5 ppm chlorine [11–13]. From above results, it is suggested that these isolated strains of bacteria from chlorinated water might have developed specific mechanisms for their survival at high chlorine concentration (Fig. 1) [7,8,11–13,28].

Table 1
Bacteriological analysis of the water from treatment plant

Bacterial isolates of pond water (raw or untreated water)	Bacterial isolates of chlorinated water (final or treated water)
Staphylococcus aureus	*Staphylococcus aureus*^{R}
Micrococcus spp.	*Micrococcus* spp.
Bacillus subtilis & Bacillus cereus	*Enterobacter* spp.^{R}
Enterobacter spp.	*Pseudomonas aeruginosa*^{R}
Pseudomonas aeruginosa	*Klebsiella pneumonia*
Citrobacter spp.	*Morganella morganii* sub sp.
Serratia odoriferae	*sibonii*
Serratia entomophila	*Rahnella aquatilis*^{S}
Serratia plymuthica & Sr. marcescens	*Hafnia alvei*^{S}
Morganella morganii sub sp. *sibonii*	
Rahnella aquatilis	
Klebsiella oxytoca & Klebsiella pneumonia	
Hafnia alvei	

*R = Strains that are resistant, S = Strains that are sensitive

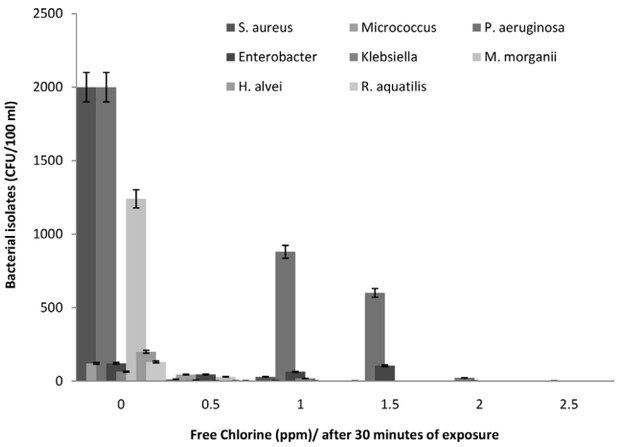

Fig. 1. Isolation of eight different strains from treated water.

3.3. Multi- drug resistance profile

On certain events it was observed that certain strains like *Pseudomonas, Enterobacter* and *S. aureus* strain isolated from the pond water were sensitive to chlorine treatment and to some of the clinically useful antibiotics like ofloxacin, ciprofloxacin, cefuroxime, netilin, amikacin, and gentamicin. Whereas the same strains when isolated from the chlorinated water was completely resistant to few of the above antibiotics. These clinically relevant antibiotics are widely used for medical treatment of diseases caused by Gram negative and other opportunistic pathogens. Hence, it was chosen for the experimental study [5,26]. A comparative report was shown in Table 2 that indicates differences in the resistance patterns of few of the isolates taken from two different sites (pond and chlorine treated water). The statistical data suggests that bacterial isolates from chlorinated water were sufficiently induced to develop specific

Table 2
Inactivation rate of isolates against residual free chlorine

Source	Isolates	Initial inoculum (log10 CFU)	RFC (mg/L)	Viable cells after exposure of 30 min (Log10 CFU)	% Inactivation	C·t (mg·min/L) 3 log
Chlorinated water	*P. aerugunosa*	2.3	0	–	–	–
			0.5	0.8	83.4	0.01
			1.0	1.2	79.2	0.02
			1.5	0.2	99.99	–
			2.0	0.1	99.99	–
Un-chlorinated water	*P. aerugunosa*	3.3	0	–	–	–
			0	0.1	99.99	–
			0	0	99.99	–
			5	0	99.99	–
			0	0	99.99	–
Chlorinated water	*S. aureus*	3.0	0	–	–	–
			0.5	0.4	85.0	0.01
			1.0	0.4	99.99	–
			1.5	2.5	99.95	–
			2.0	0.2	99.99	–
Un-chlorinated water	*S. aureus*	2.7	0	–	–	–
			0	0.01	99.99	–
			0	0	99.99	–
			0	0	99.99	–
			0	0	99.99	–
Chlorinated water	*Enterobacter*	2.9	0	–	–	–
			0.5	1.2	84.97	0.01
			1.0	2.2	84.34	0.01
			1.5	0	–	–
			2.0	0	–	–
Un-chlorinated water	*Enterobacter*	3.0	0	–	–	–
			0	0	–	–
			0	0	–	–
			0	0	–	–
			0	0	–	–

Table 3
Antibiotic susceptibility of different strains isolated from final chlorine treated water

	Range of antibiotics (µg)											
Isolates	OF	AMC	CTX	GEN	CIP	CXM	NET	CPZ	PF	CAZ	AK	LOM
	(5)	(30)	(30)	(10)	(5)	(30)	(30)	(75)	(5)	(30)	(30)	(10)
I	R	S	R	R	R	R	R	R	S	R	R	R
II	R	S	S	S	S	R	R	S	S	R	S	S
III	S	S	S	S	S	S	S	S	S	S	S	S
IV	S	S	S	S	S	S	S	S	S	S	S	S
V	S	S	S	S	R	R	R	S	S	S	S	S
VI	S	S	S	S	R	R	S	R	S	S	S	S
VII	R	S	S	S	R	R	S	S	S	R	S	R
VIII	S	S	S	S	S	R	S	S	S	R	S	S

Strains: *I- P. aeruginosa, II- Enterobacter, III- Hafnia alvei, IV- R. aquatilis, V- M. morganii sibonii, VI- Klebsiella, VII- S. aureus, VIII- Micrococcus*

*Susceptibility towards antibiotics: (R - resistant, S - sensitive),*Antibiotics: (OF - ofloxacin, AK - amikacin, AMC - augmentin, CTX - cafotaxime, GEN - gentamicin, CIP - ciprofloxacin, NET - netillin, CPZ - cefoparazone, PF - pefloxacin, CAZ - ceptazidime, CXM - cefuroxime, LOM - lomefloxacin)

mechanisms for survival in high chlorine concentrations. Either, unique proteins were synthesized by bacteria as a response to stress, or they have modified their membrane accessory components which led them to achieve enhance resistance against other antimicrobials further signifying the selection of multi drug-resistant Pathogens from chlorinated water [5,11–13].

3.4. Activation of multi- drug resistant bacteria (MDR) by chlorination

The anti bio gram showed in Table 3, indicates that *P. aeruginosa* is one of the potent multi- drug resistant pathogen followed by *S. aureus* > *Enterobacter* > *Klebsiella* > *M. morganii*. Gram negative bacteria like *H. alvei* and *R. aquatilis* were completely susceptible to the 12 set of antibiotics. The experimental data suggest that chlorinated water harbour resistant bacteria which imparts much more resistance to disinfectants like chlorine and to toxic agents like antibiotics. *P. aeruginosa* isolated from chlorinated water was most resistant among all the types and also resistant to multiple drugs. Though, an opportunistic pathogen it has slowly emerged as the most powerful and potentially pathogenic bacteria [13,16,19,29].

4. Conclusion

The supply water harboured a large number of pathogens among which *S. aureus, Enterobacter* and *P. aeruginosa* survived chlorination at suboptimal concentration; these are the ones that imparted fairly good resistance to almost all the clinically useful antibiotics tested. This is the first report of the selection of multi drug-resistant bacteria by chlorine treatment of water in West Bengal. The result obtained showed that the bacteria that survived sub- optimal dosage of chlorination (0.5 ppm) developed resistance towards increased level of chlorine ≤ 1.5 ppm. The chlorine resistant pattern of the isolates obtained was as follows: *Pseudomonas* > *Enterobacter*> *S. aureus*> *Micrococcus*> *Klebsiella*> *Morganella morganii* > *Hafnia alvei* > *Rahnella aquatilis*. The present investigation indicated that residual free chlorine concentration of up to 2 ppm was merely effective in eliminating *P. aeruginosa* isolates (in vitro). Antibiotic resistance gene expression of *P. aeruginosa* gain significant importance as it is one among the emerging pathogens of ESKAPE *(Enterobacter, S. aureus, Klebsiella, Acinetobacter, Pseudomonas and E.coli)* group [4,9,13,15]. Frequent chlorine exposure may increase the expression of efflux pumps and genes, conferring resistance to low level of chlorine and antibiotics that contain quinolones, aminoglycoside and beta-lactams [26]. These observations led to conclusion that the sub- optimal level of residual free chlorine sufficiently induces *P. aeruginosa* in water, thereby drastically increasing the resistance towards clinically applicable antibiotics. At sub-optimal chlorine concentrations the isolates are induced to response to the stress by expressing their efflux pump genes [9,14,16,30]. Furthermore, they may develop resistance by modifying cell wall permeability. *Enterobacter, S. aureus* and *Klebsiella* showed somewhat similar pattern of resistance.

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