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RESEARCH ARTICLE

Effect of Sodium Hypochlorite on Biofilm-Producing Organisms Isolated from A Hospital Drinking Water

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ABSTRACT

Objectives: Providing safe drinking water is essential for maintaining healthcare quality. The presence of biofilms in the water supply protects the organism from the antimicrobial effects of disinfectants leading to the formation of the MDR pathogen pool. Therefore, this study was taken up to determine the prevalence of biofilm formation in the bacteria isolated from the water system of a tertiary health care setup and study the effect of disinfectants on biofilms.

Methods: Thirty-four drinking water samples were collected in sterile glass stopper bottles and transported to the lab. Standard bacteriological procedures identified isolates. Biofilm detection was carried out by the tissue culture plate (TCP) method. The effect of disinfectant (sodium hypochlorite) at various concentrations (0, 0.25, 0.5, 1, 2, and 4 %) on biofilm-producing organism were studied for 30 minutes and analyzed.

Results: The culture positivity was 76.4% (26/34). Twenty samples showed monomicrobial growth, while only six samples showed polymicrobial growth of organisms. The most common organism isolated was *Pseudomonas aeruginosa*. Biofilm production was seen more in polymicrobial organisms, 91.66 % (11/12). A high level of resistance to chlorine compounds was seen in biofilm-producing microorganisms, especially those that produced robust biofilms.

Conclusion: Resistance of biofilms against high levels of chlorine has implications for the delivery of safe drinking water. Drug resistance was seen in these organisms, which can be transmitted from drinking water sources to humans. Therefore, it is recommended that biofilm production should be evaluated in drinking water samples regularly.*J Microbiol Infect Dis 2022; 12(4):17-24*.

Keywords: *Biofilm, sodium hypochlorite, drinking water*

INTRODUCTION

Providing safe drinking water is an essential prerequisite for maintaining health care quality. The overall tendency of water to promote microbial growth is measured by measuring the biological stability of the water. Biological stability refers to the concept of maintaining microbe-free water from the point of production to its consumption. As a result, water is more safely drinkable with fewer nutrients, making it less prone to microbial contamination [1,2].

Measuring the nutrients in drinking water is usually a crude method of estimating its quality. The biomass quantification or the bacterial load of the water is a better estimate for assessing the microbial quality of drinking water. This is most commonly done by heterotrophic plate count (HPC), also known as standard plate count. This is an internationally accepted test for measuring the cultivable microorganisms in drinking water [2]. Drinking water is prone to contamination at multiple points during the storage and distribution of water. It has been observed that chemical disinfectants such as monochloramines, chlorine dioxide, ozone, and others used to treat water are usually active against the planktonic form of microorganisms found in drinking water [3].

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The presence of biofilm is a primary concern in ensuring water quality. The microorganisms within the biofilms are heterogeneous and can respond to disinfectants differently.

The multispecies provide profitable niches to the organisms making them more tolerant to the effect of disinfectants. Moreover, the extracellular polymeric substance (EPS), one of the significant constituents of biofilms, protects the bacterial cells within the biofilms from the effect of disinfectants by reducing the permeability of disinfectants into the deeper layers [4].

The current biofilm containment strategies employ mechanical cleaning of the drinking water distribution system. There are no clear guidelines for removing biofilms, so the next important step to understand is cleaning biofilms using chlorine-based disinfection methods [5,6]. Hence this study was planned to assess the prevalence of biofilm formation in the bacteria isolated from the drinking water in a healthcare setting and to evaluate the effect of chlorination on biofilm-producing organisms.

METHODS

A total of 34 water samples were collected in sterile glass stopper bottles, transported, and received in the Department of Microbiology of a super specialty hospital in Northern India through a person appointed for the microbiological examination of the quality of water collected from various RO systems installed within the hospital premises.

The number of coliforms used to diagnose total bacteriological contamination was based on the multiple tube fermentation method to estimate the most likely number of coliform organisms in 100 ml of water. The test was carried out by inoculation (for 48 hours at 37 ^{0}C) of measured quantities of sample water (5, 10, 50 mL) into tubes of double and single-strength Mac Conkeys lactose bile salt broth with bromocresol purple as an indicator. The tubes showing gas formation were considered to be presumptive coliform positive. The results of MPN were interpreted based on McCrady probability tables from the number of tubes showing acid and gas (fermentation by the coliform organism) to define the sample as excellent, satisfactory, or unsatisfactory [7].

Differential coliform count (Eijkman's test) was performed by incubating subcultures from the positive presumptive tests at 44 ^{0}C and 37 ^{0}C in lactose bile broth and the other subculture at 44°C in tryptophan broth. The production of gas confirmed the presence of coliform bacilli from lactose at 37 ^{0}C, and that of E. coli was confirmed by the production of gas from lactose and indole from tryptophan at 44 ^{0}C, followed by subculture on MacConkey agar.[7] All the media and reagents were procured from Hi-media Pvt Ltd. Mumbai, India. Further, the coliforms and other organisms were analyzed by subculture on MacConkey agar, biochemical reactions, and other identification tests. Conventional biochemical methods identified colonies from these plates according to standard microbiological techniques.

Antimicrobial susceptibility testing has been done by Kirby-Bauer disc diffusion technique as per CLSI guidelines [8]. The following antibiotics were tested: cephalosporins and other beta-lactams, aminoglycosides, fluoroquinolones, polymyxins, and carbapenems (Hi-Media). The control strains used were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213. The automated Vitek II Compact system confirmed the identification and drug susceptibility of the bacterial isolates. All these bacterial isolates were preserved at -20 ^{0}C till further processed.

Biofilm detection

Biofilm detection was performed by the Tissue Culture Plate (TCP) method as described by Christensen et al. (1995), which is considered the standard gold method [9]. The bacteria were revived in brain heart infusion broth (BHIB), and 200 microlitres of this BHIB was inoculated in polystyrene tissue culture plates and incubated at 37 ^{0}C for 24 hours. The biofilm-forming organisms attached to the polystyrene plate and the planktonic cells were removed by washing the microtitre plate with phosphate-buffered saline. The plates were air-dried, and biofilms were fixed with sodium acetate (2%) and stained with crystal violet dye (0.1% w/v). The dye binds to the cells in the biofilm but not the slime layer. The dye was then eluted with 95% ethanol, and the plates were read by measuring the optical density (OD) at 570 nm using an ELISA reader. The experiment was performed in triplicate and repeated three times. The OD value of the uninoculated BHIB was used as negative control and was put in triplicate with each experiment. The biofilm production was interpreted according to

Stepanovic et al.'s criteria [10]. The cut-off (ODc) was defined as three standard deviations above the mean OD of the negative control. The biofilm formation was categorized as no biofilm producer (OD of test strain ≤ ODc),.weak (ODc < OD of test strain ≤.2x ODc), moderate (2x ODc< OD of test strain ≤4x ODc), or firm (4x ODc <OD of test strain) biofilm production.

Disinfectant (sodium hypochlorite) treatment

The content of each well was removed, and the wells were washed with 200 µ L of sterile distilled water twice to remove reversibly adherent bacteria. All experiments were performed in triplicate. The remaining attached bacteria on the inner walls of the wells were exposed to disinfectant solutions (free chlorine) at various concentrations (0, 0.25, 0.5, 1, 2, and 4%). The sodium hypochlorite solutions remained in contact with the biofilms for 30 min.[11] After treatment, the disinfectant solutions were removed. Sodium thiosulfate solution at 0.5% (weight/volume) in sterile distilled water was used to quench the activity of the disinfectant. The biofilms were then analyzed in terms of biomass.

The biofilms were stained with crystal violet (CV), and the dye was dissolved in ethanol. Each well's optical density (OD) was then measured at 570 nm using a microtiter plate reader (name), and biofilm biomass was presented as OD570. The colony-forming units (CFU) of attached bacteria were enumerated using gradient dilution and spread plate methods. The sodium hypochlorite effectiveness (removal and inactivation) was assessed based on the absorbance values of the blank, the control experiment, and the treated biofilm: biofilm removal/inactivation (%)={[(C-B)-(T-B)]/(C-B)}x100.B indicates the average absorbance for the blank wells (without bacteria), C indicates the average absorbance for the control wells (untreated biofilms), and T indicates the average absorbance for the sodium hypochlorite effectiveness -treated wells [11].

RESULTS

Thirty-four water samples from various sources in and around the hospital were received and processed in the Department of Microbiology from August 2021 to October 2021. Out of which, eight showed no growth of any organism, and 26 samples showed growth of various organisms. Twenty samples showed monomicrobial growth, while only six samples showed polymicrobial growth of organisms.

The most common isolate was *Pseudomonas aeruginosa* (44.1%), followed by *Klebsiella spp.* (20.58%). The results of the antimicrobial susceptibility testing of monomicrobial and polymicrobial organisms are shown in Table 1.

Three-forth (24/32) of the isolates were biofilm producers. Sixteen were weak producers, four were moderate, and four were strong.

Although biofilm production was higher in isolates recovered as polymicrobial flora than in monomicrobial flora, the difference was not statistically significant (Table 2).

On assessing the percentage reduction of biofilm-producing organisms to various concentrations of chlorine exposed for half an hour, it was observed that 75% of the organisms produced strong biofilms and 12.5% produced weak biofilms were chlorine tolerant even at 4% concentration when exposed for half an hour. On the other hand, around 25% of the microbial organism producing moderate biofilms were resistant to 1% residual chlorine concentration (Table 3).

Increasing the percentage of chlorination did eliminate the microbial organisms producing weak biofilms. However, for the organisms producing moderate to strong biofilms, the maximum reduction was seen at 0.5 % concentration of chlorination (Table 3).

DISCUSSION

The availability of safe drinking water (DW) is of utmost importance in the healthcare system to maintain the safety of patients, their attendants, other visitors, and healthcare staff. The drinking water distribution systems (DWDS) usually protect the water from microbial contamination using treatment methods such as filtration, sedimentation, disinfection, and other methods [2,3]. The modern-day drinking water distribution systems mostly use a chlorine-based disinfectant with residual properties to prevent microbial contamination during the distribution system [3,12,13]. In this region, generally, chlorine-based disinfectants are used to maintain the biological stability of DW. Hence, we evaluated the effect of chlorination on microbial flora from drinking water.

Though chlorination has proved to be the most effective water disinfecting strategy owing to its

low cost, ease of application, and a broad range of activity, some studies have shown that highly chlorinated water alters the taste of drinking water and also leads to lowered microbial diversity [13-15]. Further, it has been observed that chlorine reacts with organic matter present in DW to form disinfection by-products (DBP) [2,16,17]. The adverse health effects of DBPs have led many countries to supply DW without any disinfectants [12,14,15]. Some studies have observed that reduced chlorination may benefit the drinking water as the diverse microbial flora existing as stable biofilms can prevent the intrusion of enteric pathogens and other microbial organisms that may gain entry if the drinking water distribution system gets contaminated at some point [13,14]. The policies for the use of disinfectants for DW differ from place to place as per the local regulations. In cases of water supplied without disinfectant, monitoring of water to ensure biological stability of water needs to be done.

The heterotrophic plate count (HPC) employed traditionally only assesses the culturable and metabolically active microorganisms [3]. The use of metagenomics using high throughput sequencing is gaining popularity. However, it detects the complete microbiome, including the non-pathogenic organisms [15]. Culture-dependent methods such as HPC can identify most organisms of human significance and are economical. In the present study, the conventional HPC method was used to assess the microbial flora of the drinking water. More than two third of the drinking sample, 76.4% (26/34), showed the growth of microbial isolates in the present study. Uncontrolled growth of microorganisms is associated with other problems, such as alteration of taste, odor, or color of DW, besides causing corrosion of water pipelines [2].

Table 1. Drug resistance of organisms isolated from drinking water.

Name of bacteria	Microbial	Cephalosporins, n (%)	Aminoglycoside, n (%)	Carbapenems, n (%)	Beta-Lactams, n (%)	Fluroquinolones, n (%)	Colistin/Tigecycline, n (%)
Pseudomonas aeruginosa, (n=15)	Monomicrobial n=11	6 (54.5)	0	2 (18.2)	6 (54.5)	0	0
	Polymicrobial n=4	1 (25)	0	1 (25)	1 (25)	0	1 (25)
E.coli, (n=3)	Monomicrobial n=2	1 (50)	0	1 (50)	0	0	0
	Polymicrobial n=1	0	0	0	1 (100)	0	0
Acinetobacter species, (n=2)	Monomicrobial n=2	1 (50)	0	0	1 (50)	0	0
	Polymicrobial n=0	-	-	-	-	-	-
Klebsiella species, (n=7)	Monomicrobial n=1	0	0	0	0	0	0
	Polymicrobial n=6	2 (33.3)	1 (16.7)	1 (16.7)	3 (50)	2 (33.3)	1 (16.7)
Enterobacter cloacae, (n=1)	Monomicrobial n=0	-	-	-	-	-	-
	Polymicrobial n=1	1 (100)	0	1 (100)	1 (100)	0	1 (100)

Table 2: Comparison of biofilm production in monomicrobial vs polymicrobial isolation.

Organism isolated (number)	Microbial	Biofilm Present (24)	Biofilm absent (8)
Pseudomonas aeruginosa (15)	Monomicrobial n=11	10	1
	Polymicrobial n=4	4	0
E. coli (3)	Monomicrobial n=2	2	0
	Polymicrobial n=1	1	0
Acinetobacter species (2)	Monomicrobial n=2	1	1
	Polymicrobial n=0	-	-
Klebsiella species (7)	Monomicrobial n=1	0	1
	Polymicrobial n=6	6	0
Enterobacter cloaca (1)	Monomicrobial n=0	-	-
	Polymicrobial n=1	0	1
Others (4)	Monomicrobial n=4	0	4
	Polymicrobial n=0	-	-

Table 3. Effect of various concentration of hypochlorite on biofilm producers.

Biofilm production (n=24)	0.25%	0.5%	1%	2%	4%	No Effect
Weak (16)	5	4	3	2	0	2
Moderate (4)	0	3	1	0	0	0
Strong (4)	0	1	0	0	0	3

The viable bacteria present in drinking water post-disinfection may be resistant to chlorination. This could have been the reason for the higher culture positivity in this study, or the chlorine concentration might have been suboptimal in the distribution system, as other authors also see [14,18,19].

Chlorine disinfection of DW produces more homogenous flora in DW dominated by Pseudomonas, an opportunistic pathogen. The present study also showed that.*Pseudomonas aeruginosa* was the most common microorganism present in the DW.

Other opportunistic pathogens that may be recovered by the HPC method include *Acinetobacter spp., Aeromonas spp., Klebsiella pneumoniae, Legionella*, and others [2,14].

In the present study, Acinetobacter spp and *Klebsiella spp.* were recovered in around 28% of the water samples either alone or in association with other microorganisms. Acinetobacter can help in the coaggregation of other bacterial organisms found in DWDS and thus promote biofilm formation [13].

It has been observed that apart from fecal coliforms, no other organisms cause an adverse effect on human health. There was

fecal contamination of DW in 3/32 samples in this study. This could be because of contamination of the DW line with sewage water or the presence of these organisms as biofilms. This is because the residual chlorine present in DW cannot eliminate microbial regrowth and biofilm formation.

Most of the water disinfectants used for drinking water focus mainly on planktonic organisms, whereas the majority of the bacterial biomass in the drinking water is present as biofilms [2,4]. In the present study, 75% of isolates were biofilm producers. The complex interactions between the microbes could help maintain higher extra polysaccharide concentration and cell-to-cell connections leading to stronger biofilm production, especially in polymicrobial biofilms. The present study also observed that 91.6% (11/12) of the microbial organisms isolated as polymicrobial growth were biofilm producers compared to 65% (13/20) organisms isolated as monomicrobial flora. This follows the literature showing the chlorine resistance of polymicrobial biofilm to be 5-250 times higher than monomicrobial biofilm [13].

Due to co-selection factors, these bacteria may also have increased resistance to many antibiotics [19-21]. In the present study high degree of antibiotic resistance was observed against beta-lactam antibiotics, including cephalosporins and carbapenems in pseudomonas isolates. Antibiotic resistance was also seen in isolates such as *Enterobacter cloacae*, *Escherichia coli*, and *Klebsiella spp.* There was no significant difference in the degree of resistance between monomicrobial and polymicrobial isolates.

Whether antibiotic-resistant genes can be transferred to humans through drinking is not very clear. However, few authors have observed that antibiotic-resistance genes can be transferred in aquatic environments such as drinking water, which can adversely affect human health [20-22]. This finding highlights the importance of removing all microorganisms, whether present as planktonic or in biofilms, to prevent the dissemination of antibiotic-resistance genes.

The effect of chlorination on biofilm-producing organisms was suboptimal, and almost 21% of the organisms were found to be chlorine tolerant till 4% of concentration when exposed for half an hour. Increasing the percentage of chlorination did eliminate the microbial organisms producing weak biofilms. However, for the organisms producing moderate to strong biofilms, the maximum reduction was seen at 0.5 % concentration of chlorination.

The concentration of residual disinfectant varies across the distribution system, and hence the efficiency of the disinfectant also varies. The suboptimal concentration of disinfectant promotes the selection of antibiotic-resistant and chlorine-tolerant microbes in the system [19,20]. This might have been responsible for chlorine tolerance and higher antibiotic resistance in the present study.

Further, in a hospital setting, drinking water is used for washing tools at many places besides hand scrub and hand hygiene. The increased organics load due to these infectious pathogens may lead to inadequate sterilization and disinfection of the equipment. Though most widely employed for water treatment, it has been observed that chemical disinfection has limitations in its immediate and prolonged effectiveness. This is because of other confounding factors such as extreme pH, temperature variation, high salinity, and the presence of other organic material that may reduce disinfectants' effectiveness and result in the selection of tolerant strains [14,20].

It would be worthwhile to monitor the concentration of residual chlorination at the point of use of another confounding factor, including the production of biofilms, to help formulate or revise the guidelines for drinking water safety in that region.

There has been a change of emphasis from infection control to infection prevention. Consequently, there is a greater interest in the role of healthcare premises as an environment for the proliferation and transmission of pathogens. The institutes must develop consistent strategies to eliminate the hazards and communicate them clearly to clinical and estates staff. Such strategies usually require a behavioral change to prevent microbial transmission effectively.

The importance of regularly servicing a water system is beyond doubt. Equipment used in the supply, storage, and transfer of drinking water must be maintained to ensure that there is no build-up of organic matter, other debris, and biofilm that can facilitate the survival and growth of microbial organisms.

Conclusion

In the present study, it was seen that drinking water from a tertiary care health setting was contaminated with microbial flora, and these organisms were most likely resistant to chlorination due to biofilm production. It was observed that almost 75% of the microbial organisms isolated from drinking water were biofilm producers, and many of them were chlorine tolerant to 4% of concentration when exposed for half an hour.

As a part of routine drinking water surveillance in hospitals, if the heterotrophic plate count shows the presence of microbial organisms despite adequate chlorination, the biofilm-forming ability of the microorganisms should be evaluated. The authors also suggest that alternative methods of water disinfection and, more importantly, mechanical scrubbing should be done to maintain biologically safe drinking water.

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