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Original Article

Microbiology Section

Study of Biofilm in Bacteria from Water Pipelines

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ABSTRACT

Context: A biofilm is a layer of microorganisms contained in a matrix (slime layer), which forms on surfaces in contact with water. Their presence in drinking water pipe networks can be responsible for a wide range of water quality and operational problems.

Aim: To identify the bacterial isolates, obtained from water pipelines of kitchens, to evaluate the water quality & to study the biofilm producing capacity of the bacterial isolates from various sources.

Settings and Design: A prospective study using water samples from aqua guard & pipelines to kitchens of S.C.B Medical College hostels.

Materials and Methods: Standard biochemical procedures for bacterial identification, multiple tube culture & MPN count to evaluate water quality & tissue culture plate (TCP) method for biofilm detection was followed.

Statistical analysis: STATA software version 9.2 from STATA Corporation, College station road, 90 Houston, Texas was used for statistical analysis.

Results: One hundred eighty seven isolates were obtained from 45 water samples cultured. The isolates were *Acinetobacter* spp. (44), *Pseudomonas* spp.(41), *Klebsiella* spp.(36) & others . Biofilm was detected in (37) 19.78 % of the isolates (95% CI 30.08% -43.92%) including *Acinetobacter* spp.-10, *Klebsiella* spp. - 9, *Pseudomonas* spp. - 9, & others, majority (34) of which were from kitchen pipelines.

Conclusion: Water from pipeline sources was unsatisfactory for consumption as the MPN counts were > 10. Most of the biofilm producers were gram negative bacilli & *Pseudomonas & Acinetobacter* spp. were strong (4+) biofilm producers.

Keywords: Bacterial aggregates, Health risk, Microorganisms, Planktonic

INTRODUCTION

According to the World Health Organisation drinking water should be free from any organism that might pose a health risk to the human population [1]. Water is normally disinfected before being distributed to the end point users and its microbial level before leaving the treatment plant should be within limits set by water authorities .Unfortunately by the time it reaches the consumer water quality may differ dramatically from the time of treatment. Furthermore though the process of disinfection substantially reduces the number of microorganisms, it does not sterilise it, allowing the surviving microbes to grow under favourable conditions. This decline in water quality may lead to recovery and subsequent growth of sub lethally damaged bacteria due to system deficiencies such as cross connections, broken water mains and contamination during bulk storage & repairs [2]. Moreover these bacterial cells can attach & form biofilms on the surfaces of piping material from which cells may be released into the flow [2]. The majority of bacteria in the drinking water system occur in biofilms rather than in water phase [3]. Biofilms are defined as bacterial aggregates attached to various biotic and abiotic surfaces which interact with each other to adapt themselves to environmental stressors compared to planktonic existence [4]. The organisms in biofilms tend to become more resistant to antibiotics and disinfectants there by become a reservoir for subsequent spread of pathogenic organisms. In addition they offer increased virulence and resistance that potentially reduces the LD 50 (lethal limit) by increasing the viable organisms to survive and pass through the human stomach and reach the intestine [5]. Moreover biofilm can influence the taste and odour of the water & when developed on ferrous metal surfaces, they may cause corrosion of the pipes and also the release of iron particles into the water [6].

Rationale of the study: As we know water is used throughout the food chain starting from the farm to the kitchen table, hence the quality of water can therefore have significant impact on the quality of food products. Moreover, several food-borne pathogens are waterborne pathogens. The microbial quality of water is therefore essential with respect to food hygiene and food spoilage. The mechanism of biofilm formation has been well-studied in the gram negative marine bacterium *Vibrio fisheri*, where N-Acetyl Homoserinelactone (AHLS) is the specific inducer [7]. But in gram positive bacteria the inducers are oligopeptides. Furanone is present in both gram positive & negative bacteria & acts as an interspecific inducer [8]. These intercellular molecules are responsible to form a quorum i.e. minimum number or density of members necessary to conduct the business i.e. derive benefits like-adaptive plasticity, sporulation, gene exchange, enzyme production, virulence, synthesis of antibiotics & metabolites, etc [9]. Different methods such as Tissue culture plate(TCP), Tube method and Congo red agar (CRA) are followed by various observers for biofilm but TCP was found to be the most sensitive, accurate and reproducible screening method [10]. The present study was conducted with the objectives: To isolate and identify bacteria from water obtained from pipelines to kitchens of S.C.B Medical College hostels, to evaluate the water quality by MPN count & to study the biofilm producing capacity of the bacterial isolates from various sources.

MATERIALS AND METHODS

Study was conducted in the Department of Microbiology, S.C.B. Medical College, Cuttack after approval of Institutional Ethical Committee for a period of two months (July & August 2012). Under aseptic conditions 50 ml of water samples were collected in wide mouthed sterile containers (HiMedia) from 45 sources

Sources	Number of Samples (n=45)	
	Aquaguard	Kitchen
Old gents' hostel	2	5
New gents' hostel	2	2
HS hostel	2	3
Gents PG Hostel	2	5
New Boys' hostel	2	5
Old U.G Girls Hostel	2	2
New U.G Girls Hostel	2	2
Ladies P.G hostel	2	1
Nursing hostel	2	2
Total=45	18	27

[Table/Fig-1]: Distribution of water samples collected from various sources

including pipe lines & commercial purifier systems [Table/Fig-1]. The source of pipeline waters was water tank of Municipal corporation, Cuttack & the supply pipelines were made up of plastic (PVC). The commercial purifier systems were wall mounted Aquaguards (Eureka Forbes) of dimension 355×102×307mm, 27-month-old with two yearly services completed. Before collection, both the inner and the outer mouths of the taps were cleaned with rectified spirit & water was allowed to run for 2-3 min. Single and double strength MaConkey broth were used for culture of water in multiple tubes (1*50 ml, 5*10ml and 5*1ml). Water was added in equivalent amounts to respective tubes & incubated at 37^{0}C for 48 h. All the tubes were subcultured on MaConkey agar and the bacterial isolates were identified using standard biochemical techniques [11]. The presumptive coliform count was calculated by following the McCrady table [12]. Biofilm formation was detected by TCP method [10]. Inoculum was prepared from fresh isolates in brain heart infusion broth with 2% sucrose, incubated for 18 h at 37°C & turbidity adjusted to Mc farland's 0.5. The prepared inoculum was diluted 1in100 with fresh medium and dispensed in 0.2 ml amounts to individual wells of sterile, 96 well-flat bottom tissue culture plates (Tarson), incubated at 37^{0}C for 24 h then broth was aspirated out. The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating 'planktonic' bacteria. Sodium acetate (2%) was added to fix biofilms if formed by adherent 'sessile' bacteria & subsequently stained with crystal violet (0.1%). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. The absorbance of the wells was measured at wavelength 630nm by Micro ELISA auto reader. A blank well with reagent was also measured for absorbance (% t blank). The (%) of transmittance (% t) was subtracted from % t blank to obtain the amount of light blocked. Biofilm production was scored as negative, weak, moderate and strong when % t blank= <5, 5-20, 20-35 & >50 respectively. Experiment was performed in triplicate and the data were then averaged.

RESULTS

Total 187 bacterial isolates were obtained from 45 water samples processed including 9 from commercial purifier system (Aquaguard) & rest 178 from pipe lines. Single bacteria were isolated from 7 samples & rest 38 yielded multiple bacterial combinations. The isolates identified were *Acinetobacter* spp.(44), *Pseudomonas* spp. (41), *Klebsiella* spp.(36), *E .coli* (22), *Staphylococcus aureus* (14), *Aeromonas* spp (2) &. *Enterococcus* spp. (28) [Table/Fig-2]. Biofilm was detected in 19.78 % (37) of the isolates {95% CI 30.08% -43.92%} including *Acinetobacter* spp.(10), *Klebsiella* spp. (9), *Pseudomonas* spp.(9), & others [Table/Fig-3]. Among them, 34 were from pipeline water & 3 from aqua-guard sources. Among the biofilm producers, *Acinetobacter* spp & *Pseudomonas* spp. (OD >.240) were identified as strong, *Klebsiella* spp.(OD .120-.240) moderate & *E .coli*, *Staphylococcus aureus* & *Aeromonas* spp

Bacterial isolates	Source		Total
	Kitchen	Aquqguard	
Acinetobacter spp.	44	0	44
Pseudomonas spp.	37	4	41
Klebsiella spp.	33	3	36
E .coli	22	0	22
Staphylococcus aureus	14	0	14
Aeromonas spp.	0	2	02
Enterococcus spp.	28	0	28
Total	178(95.18%)	9(4.82%)	187

[Table/Fig-2]: Distribution of bacterial isolates from water samples

Bacterial isolates	Source		Number (N=37)
	Kitchen	Aquqguard	
Acinetobacter spp.	10(Strong)	0	10
Pseudomonas spp.	7(Strong)	2 (Weak)	9
Klebsiella spp.	8 (Moderate)	1(Weak)	9
E .coli	5(Weak)	0	5
Staphylococcus aureus	2(Weak)	0	2
Aeromonas spp.	2(Weak)	0	2

[Table/Fig-3]: Distribution of biofilm producers from water samples of various sources

(OD<.120) as weak biofilm producers respectively. The MPN counts were more than 10 in 25 water samples from pipeline sources & within satisfactory range from all aquaguard water.

DISCUSSION

Any microbe including primary and opportunistic pathogens present in water may attach or become enmeshed in the biofilm. However, the survival time for many pathogens in biofilms is uncertain and likely varies depending on the organism. Aquatic microbes are well-adapted to the low nutrient level and cool water temperature of the distribution system [13]. Present study yielded 187 bacterial isolates from 45 sources where 7 were monobacterial whereas rest 38 yielded multiple bacterial combinations. This interspecific or nonspecific combinations between gram positive and gram negative as observed in our study is most wide for biofilm formation than intraspecific bacterial population [8]. The MPN counts were more than 10 in 25 samples from pipeline sources indicating the water quality as unsatisfactory for consumption, while that of all aquaguard water were within the satisfactory range. The isolates identified were *Acinetobacter* spp.(44), *Pseudomonas* spp. (41), *Klebsiella* spp. (36), *E.coli* (22), *Staphylococcus aureus* (14), *Aeromonas* spp (2) & *Enterococcus* spp.(28) [Table/Fig-2]. There were no isolates of potential enteric pathogens such as Salmonella, *Shigella* etc. But in a study by Armon et al,. *S.typhimurium* was able to grow at 24^{0}c for a short period in nonsterile tap water [14]. Yet in another study, *Acinetobacter* spp. was detected on the surface layer of a mortar-lined pipe at levels upto 109/cm^2 which correlates well with that of ours [15]. September et al., had reported high number of *Aeromonas*, *Pseudomonas*, *klebsiella* & *Enterococcus* spp. from the biofilms of drinking water distribution systems in South Africa, but no putative *Salmonella* and *Shigella* could be confirmed by him [16]. In the present study only 2 isolates of *Aeromonas* spp. were identified. TCP method was followed in the present study for biofilm detection which is a semiquantitative method & could detect 19.78% (*Acinetobacter* spp.-10, *Klebsiella* spp.- 9, *Pseudomonas* spp.- 9, *E.coli*-5, *S.aureus*-2, *Aeromonas* spp.-2) biofilm producers {95% CI 30.08% -43.92%}. Among them, 34 were positive from pipeline water & 3 from aqua-guard sources. Most of the biofilm producers were either *Acinetobacter* spp, *Klebsiella* spp. or *Pseudomonas* spp. [Table/Fig-3]. *Acinetobacter* & *Pseudomonas*

were strong biofilm producers. Mathur T et al., had reported TCP method to be the most sensitive and specific with high accuracy in terms of discriminating between biofilm producers and non-producers [10]. In tube method strong producers could be easily detected whereas difficult to differentiate between moderate and weak producers and CRA method have very little correlation with the above two methods. In the present study also discrimination between moderate and non-biofilm producers was very clear. In our study none of the *Enterococcus* spp. produced biofilm & only 9.09 % of *Staphylococcus aureus* were identified as biofilm producers which correlate well with the study of Mathur T et al., [10]. Park et al., have noted the presence of *H. pylori* in biofilms of drinking water mains [17]. Opportunistic pathogens including *Pseudomonas aeruginosa*, *Legionella pneumophila* and *Mycobacterium* avium complex (MAC) etc. have also been associated in biofilms [16]. It has been proven beyond doubt that hypochlorite has little effect on biofilms. However, chlorine dioxide dosed at a continuous low level, ozone & UV disinfection were used for removal & prevention of biofilm from water systems. Moreover, quorum quenching or antiquorum sensing molecules like UW85 strain of *Bacillus cereus* can be introduced into the water supply systems to prevent development of biofilms [18].

CONCLUSION

So, to conclude the present study, culture of the aqua-guard water revealed a very low prevalence of bacteria & all were weak biofilm producers, while pipeline sources revealed the presence of strong biofilm producers in high numbers (17.1 %) majority being *Acinetobacter* and *Pseudomonas* spp. along with other potential gram negative pathogens which can cause food spoilage and food borne diseases. Therefore water from pipelines should be screened for MPN count & biofilm before consumption. It is also essential to put a comprehensive water safety plan in place to protect the water from the source to the tap. This plan should include multi-barrier treatment to avoid entry of pathogens to the system. Currently most research in this field of biofilm is being done by smaller biotech firms. Owing to its importance in food and water hygiene, it is to be hoped that big pharma can break out of their current paradigm and purpose for this new approach.

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