

Insignificant difference between biofilm forming isolates of filtered water and non-filtered water

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Abstract

Aim: Biofilm formation is a developmental process with intercellular signals that regulate growth. Biofilms contaminate catheters, ventilators, and medical implants; they act as a source of disease for humans, animals, and plants. In this study we have done a quantitative assessment of biofilm formation in bacterial isolates, associated with the drinking water distribution system, in tryptic soya broth, with different incubation times. **Materials and Methods:** The study was carried out on 104 samples of water from different systems. The bacterial isolates were processed as per the microtiter plate method with only tryptic soya broth, and with varying concentrations of glucose, and were observed in response to time. **Results:** Out of the total of 104 samples of water, 63 were found to be bacteriologically positive and 24 of them were biofilm formers. *Enterobacter* spp. and *Pseudomonas* were maximally found to be biofilm producing. **Conclusion:** Biofilm formation depends on adherence of bacteria to various surfaces. Overhead plastic tanks and metal taps were the most common sources being infected. We must have a regular policy to get them clean so as to have affordable and safe drinking water.

Key words: Biofilm, bacteria, drinking water system, microtiter plate method

INTRODUCTION

Biofilms grow easily on surfaces of artificial materials used for water storage. Drinking-water systems are known to harbor 'BIOFILMS', even though these environments are oligotrophic and often contain a disinfectant. The interaction of pathogens with the existing biofilms has predominantly been a concern with man-made water systems, particularly drinking-water distribution systems. Obviously microorganisms face a diversity of habitats with distinct physicochemical and nutritional conditions during treatment, storage, and distribution of drinking

water. Bacteria are affected not only by the environment they live in, but also by a variety of other species that are present. Conducting this study on Biofilm production in drinking water bacterial isolates might help to improve our understanding of the persistence of biofilms and the associated pathogens in the drinking-water distribution systems.^[1-3] There is evidence that the biofilm community diversity can affect disinfection efficacy and allow pathogens to survive within the biofilms. The knowledge of biofilm formation dynamics is fundamental for the understanding of the processes running in the biofilm layer.^[4,5]

The aim of this study is to assess the purity of the filtered water system, biofilm formation, and characterization of the bacterial isolates.

MATERIALS AND METHODS

Permission was obtained from the Institutional Review Board. It was a prospective study on different water sources, for the period of six months. The unfiltered water

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samples were collected from taps, tanks, pots, and plastic bottles, and filtered water was collected from taps, pots, and bottles. The time period of the water storage was 24 hours or more, and was included for selection of source. One hundred and four samples of filtered and unfiltered water were included in the study.

Water sample collection method

Water samples were collected from sources like taps, tanks and filter systems. The samples were collected using a standard sterile aseptic technique.^[1] Sufficient amount (100 ml) was to be collected to make out colony counts.

Water filtering procedure

Filtration was done with the help of sterile 10 mL syringes and filters. The samples were filtered with the help of syringe filter assembly. The membrane filter (0.2 µm) from the assembly was taken out and cultured upside down onto the blood agar plates for the growth of the bacteria present in particular sample.^[6-8]

Identification and colony count

Colony count was done from the media according to the sample volume, per 100 ml of water. Further identification was carried out and the isolates were stored in 15% glycerol broth for the purpose of future processing. The organisms were identified using the standard methods.^[4,9,10]

The isolates derived later from the clinical laboratory, for the purpose of our study, were frozen in nutrient broth with 15% glycerol, at -20°C. The samples retrieved for the study were grown on blood agar plates and were processed as described herewith.

Cultures retrieved from the frozen material retained the same biochemical reactions, confirming that no alteration had occurred in the bacterial isolate because of storage and processing.

Biofilm formation and quantification of activity against biofilms

Preparation of inoculum

Three different media were taken; tryptic soya broth, tryptic soya broth with 0.25% glucose, and tryptic soya broth with 0.5% glucose, for culture. Isolated colonies were inoculated and incubated for 24 hours in these media, and then the cultures were diluted 1 : 200 with the respective fresh media.

Control

Biofilm-producing reference strains of *Acinetobacter baumannii* (ATCC 19606) and *Pseudomonas aeruginosa* (ATCC 27853) and non-biofilm forming reference strains of *Staphylococcus aureus* (ATCC 25923) and *E.coli* (ATCC 25922) were evaluated.^[9]

Microtiter plate assay: Biofilm formation was induced in 96-well, flat-bottomed polystyrene Microtiter plates. An aliquot of 200 µl of diluted bacterial suspension was added to each well and incubated for 16 hours, 20 hours, and 24 hours, at 37°C. At the end of incubation period, the wells were carefully aspirated and washed twice with 300 µl of phosphate-buffered saline (PBS, pH, 7.2), to remove the planktonic bacteria. The wells were emptied and dried by staining before biomass quantification of the biofilms was performed. The staining was done with 200 µl of 0.1% Safranin and 0.1% Crystal Violet (CV) in the respective wells, for 45 minutes. At the end of that time, the wells were carefully washed twice with distilled water to remove the excess stain. After staining, 200 µl ethanol / acetone (90 : 10) was added to each well to dissolve the remaining stain from the wells. The optical density was then recorded at 492 nm with a 630 nm reference filter using an ELISA reader.^[3,10-13]

Wells originally containing uninoculated medium, non-biofilm producing bacteria, and known biofilm producing bacteria were used as controls for cut-off, negative controls and positive controls, respectively. The test was carried out in quadruplicate, and the results were averaged and standard deviations were calculated.

The cut-off was defined as three standard deviations above the mean ODc.^[14] Each isolate was classified as follows: Weak biofilm producer $OD = 2 \times ODc$, moderate biofilm producer $2 \times ODc < OD = 4 \times ODc$, or strong biofilm producer $OD > 4 \times ODc$.^[9,15,16] A statistical analysis was conducted.

RESULTS

The sources of the samples were plastic water bottle, pot water, overhead tank, and tap water. Out of 104 water samples, 63 were positive for bacterial growth. Forty-nine samples were of nonfiltered sources and 55 were of filtered water sources. All the bacterial isolates were

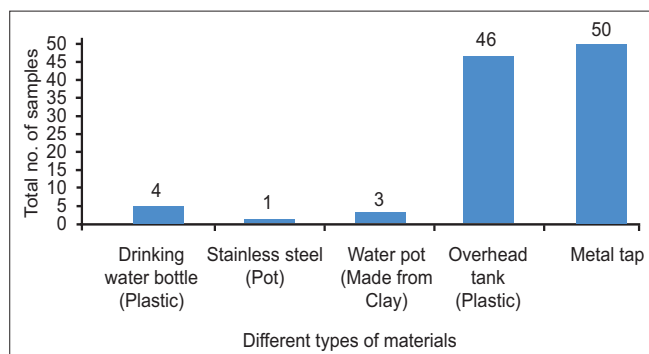


Figure 1: Different source of water samples according to the type of materials

found to be gram negative. Thirty-one (56%) samples of filtered water and 32 (65%) samples of nonfiltered water were found to be positive. Minimum 0 and maximum 430 colony forming units of bacteria were found per 100 ml of water.

Figure 1 describes the different types of materials from which the water samples were collected

Association of different bacterial isolates with the type of water sample can be seen in Table 1.

Maximum isolates were *Pseudomonas* and *Enterobacter* sp. followed by *Klebsiella* sp., *Escherichia* sp. and *Acinetobacter* sp. Similar results were seen in filtered as well as nonfiltered sources of water. The only difference was that the *Enterobacter* sp. were more common in the filtered water system as compared to the nonfiltered water system, as shown in Figure 2. Biofilm quantification was done using the microtiter plate method. Isolates were studied for their ability to form weak, moderate or strong biofilms.

Table 2 describes the quantitative assessment of biofilm production from a filtered source, and also states the different materials used for water storage and the number of biofilm producers, either strong, moderate, weak or negative, in which tap water shows the highest values, whereas, analysis of nonfiltered sources showed maximum biofilm forming strains from tap water, followed by overhead tank, as in Table 3.

Table 1: Different types of bacterial isolates recovered from water sources

Name of bacteria	% (Filtered water)	% (Nonfiltered water)
<i>Enterobacter</i> Sp.	38.7	25.5
<i>Klebsiella</i> Sp.	6.5	13.1
<i>Pseudomonas</i> Sp.	51.6	54.1
<i>Escherichia</i> Sp.	3.2	4.2
<i>Acinetobacter</i> Sp.	0	3.3

DISCUSSION

Water for human consumption is usually disinfected before being distributed to the consumer, to ensure that the level of any potentially harmful microbial agents falls under the defined low levels, for example zero fecal coliforms per 100 ml. In many instances the quality of the water may have deteriorated by the time it reaches the consumer. This is often due to recontamination after treatment, owing to the regrowth of sub-lethally damaged bacteria or contamination from bacteria harbored in the biofilms.^[18,19]

Drinking water in the distribution system is not sterile, regardless of the degree to which the water is treated. The water contains microbes that survive the treatment process or enter the distribution system through the pipe network. Many of these microbes can attach to the pipe wall and become a part of the biofilm. Pathogens may accumulate in the biofilm, and the biofilm may extend the survival of primary pathogens by protecting them from disinfectants. These pathogens may be sloughed from the biofilm into the water column due to changes in the flow rate. The persistence of waterborne disease, or of microbial contamination in a distribution system, long after the cause of the distribution system problem has apparently been corrected, suggests that there may be an isolated pocket of

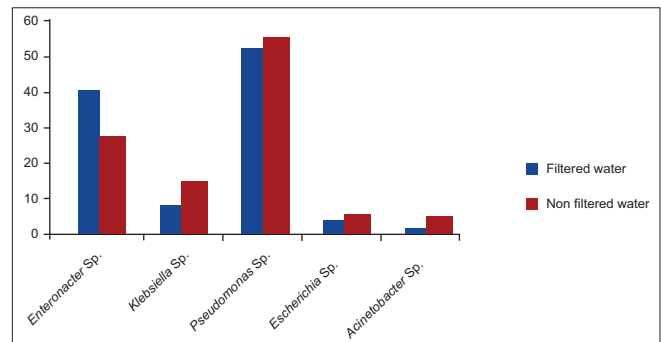


Figure 2: Percentage of bacterial isolates from different water sources

Table 2: Quantitative assessment of the biofilm producer from filtered sources

Name of source	Strong biofilm producer	Moderate biofilm producer	Weak biofilm producer	Non-biofilm producer
Plastic water bottle	0	0	0	1
Pot water	0	0	0	1
Tap water	1	2	10	17

Table 3: Quantitative assessment of biofilm producer from non-filtered sources

Name of source	Strong biofilm producer	Moderate biofilm producer	Weak biofilm producer	Non-biofilm producer
Plastic water bottle	0	0	0	2
Pot water	0	0	0	1
Overhead tank	1	1	2	9
Tap water	2	1	4	4

static or slow-flowing water, biofilm erosion, or sloughing occurring (i.e., the slow-release mechanism).^[19]

Water distribution system biofilms are largely dominated in number by aerobic Gram-negative bacteria belonging to *Pseudomonas*, *Acinetobacter* and the related genera, of which, the members of the genera *Klebsiella*, *Enterobacter*, and *Citrobacter* are the most successful colonizers in the distribution networks Coliform bacteria, thermotolerant (fecal) coliforms, and *E. coli* have, for almost a century, been used as indicators of the bacterial safety of drinking-water. However, their use in isolation to predict the viral and protozoal safety of drinking-water has been questioned since the 1970s. The failure of these indicators in isolation has been demonstrated by recent outbreaks of waterborne cryptosporidiosis. As a pattern indicator of bacterial enteric pathogens, it appears essential to assess the behavior of these organisms in the freshwater environment and particularly in the water distribution system biofilms. Most health scientists tend to believe that all strains of *E. coli* are incapable of significant growth in the environment. In one extensive review on *E. coli*, various variables that affect its life span in both the natural and laboratory conditions, which could range between four and twelve weeks, in water containing moderate microflora, at a temperature of 15 – 18°C. Survival or growth is determined mainly by the nutrients present, temperature, and chlorination. When most conditions conducive to their growth have been met, the *E. coli* can multiply in experimental studies or in the natural aquatic environment. At a defined dilution rate of river water in a chemostat, various strains, including *E. coli*, *Salmonella*, and *Shigella* spp., grow. The generation time ranges between 3.33 and 90.0 hours for bacteria and pathogens in drinking-water, at 30°C. At temperatures below 30°C, the generation time for all organisms tested, increases, and die-off occurs in most cases at 5°C. *E. coli* are not particularly fastidious in their growth requirements; therefore, presumably the potential exists, as it does with other coliforms, for regrowth in nutrient-rich waters.^[18]

In humans, *Pseudomonas aeruginosa* is an opportunistic pathogen or colonizer, well known in the hospital environment; it seems likely to be the cause of 10 – 20% of nosocomial infections. Its extreme resistance to antibiotics explains why this ubiquitous bacterium has been selected to colonize the skin and mucous membranes of patients. As some *P. aeruginosa* strains are capable of producing enterotoxins, the enteropathogenicity of this species has sometimes been surmised. Many publications have recognized this bacterium as an enteric pathogen and the causative agent of diarrhea in infants and children. However, each of these ‘infections’ was diagnosed before there was an adequate means of precluding a viral or protozoan etiology. Community-acquired *P. aeruginosa*

gastrointestinal disease with sepsis rarely occurs in healthy infants — that is, those who do not have identified underlying immunological or hematological problems. There have been no significant outbreaks reported in recent decades, possibly as a result of better hygienic control measures and diagnostic techniques. *P. aeruginosa* is predominantly an environmental organism, and fresh surface water is an ideal reservoir. It proliferates in the water piping systems and even more in hot water systems and spa pools. As a consequence of the contemporary lifestyle, *P. aeruginosa* reaches relatively high numbers in food and on moist surfaces. Daily, substantial numbers of the species are ingested with our food, particularly with raw vegetables, while our body surfaces also are in continuous contact with the organism.^[17-19]

Placing samples into containers terminates the exchange of cells, nutrients, and metabolites, with the *in situ* surrounding environment. Compressed air is used at virtually all stages of the water bottling process. The microbial quality of the processed air must be of a very high standard. On the other hand, the complexed organic matter present in a low concentration can be dramatically modified through bottling, under the influence of increasing temperature and oxygenation.^[18,19]

CONCLUSIONS

Drinking water from different sources was tested for Biofilm Detection, and out of the 104 water samples tested, 63 showed Gram negative bacterial growth. Among these, the most common was *Pseudomonas* Sp., both from filtered and non-filtered sources, and 55.55% of these bacteria were Biofilm Producers. Overhead plastic tanks and metal taps were the most common sources being infected. We must have a Regular policy to get them clean so as to have affordable and safe drinking water. It is essential to put a comprehensive water safety plan in place to protect the water from the source to the tap. This plan should address multi-barrier treatment and integrity of the water distribution system, to avoid the entrance of pathogens into the system.

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