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International Journal of Current Research
Vol. 8, Issue, 02, pp.25961-25965, February, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

THE PRESENCE OF BACTERIA FORMING BIOFILM IN WATER PIPES COMMONLY USED AT PUEBLA, MÉXICO

***[1]Flores-Encarnación, M, [1]Jaramillo-Rodríguez, J.B., [2]Xicohtencatl-Cortés, J., [1]Amador-Bravo, D., [3]Aguilar-Gutiérrez, G.R., [4]Cabrera-Maldonado, C., [4]León-Tello, G., [4]Ruíz-Tagle, A., [4]García-López, A. and [4]Meneses-Sánchez, M.C.**

[1]Laboratorio de Microbiología Molecular y Celular. Edif. 323G. Biomedicina, Facultad de Medicina, Benemérita Universidad Autónoma de Puebla, Puebla, México
[2]Hospital Infantil de México Federico Gómez, México, D.F.
[3]Centro de Investigación sobre Enfermedades Infecciosas. INSP. Cuernavaca, Morelos
[4]Depto. de Microbiología. Facultad de Ciencias Químicas, Benemérita Universidad Autónoma de Puebla, Puebla, México

ARTICLE INFO

Article History:
Received 19th November, 2015
Received in revised form
22nd December, 2015
Accepted 15th January, 2016
Published online 14th February, 2016

Key words:

Bacteria, Biofilm,
Pipes, Water,
Drinking.

ABSTRACT

A common problem in water distribution networks is the formation of bacterial biofilms inside them. The biofilm is a community of microorganisms which is embedded in a solid surface and it is the natural state in which the most bacteria are in the environment or infectious processes The biofilm formation is a dynamic and continuous process and it forms a complex microenvironment in the pipes. This process depends on several factors including materials from which pipes are made. So the drinking water has lost quality along its passage by supply pipes. The deterioration of water quality results in altering the taste and odor and sometimes the turbidity. In this study, the presence of bacteria forming biofilm in water pipes was determinated.

Citation: **Flores-Encarnación, M, Jaramillo-Rodríguez, J.B., Xicohtencatl-Cortés, J., Amador-Bravo, D., Aguilar-Gutiérrez, G.R., Cabrera-Maldonado, C. *et al.* 2016.** "The presence of bacteria forming biofilm in water pipes commonly used at Puebla, México", *International Journal of Current Research,* 8, (02), 25961-25965.

INTRODUCTION

In recent years, it has been shown that drinking water has lost quality along its passage by supply networks (pipes). This has been attributed to the action microorganisms which are adhered to the walls of the pipes (Donlan and Pipes, 1988; Hryniszyn *et al*., 2015; Knobelsdorf and Mujeriego, 1997; Mahapatra *et al*., 2015). The deterioration of water quality results in altering the taste and odor and sometimes the turbidity (Characklis and Marshall, 1990; Knobelsdorf and Mujeriego, 1997).

****Corresponding author: Flores-Encarnación, M,***
Laboratorio de Microbiología Molecular y Celular. Edif. 323G. Biomedicina, Facultad de Medicina, Benemérita Universidad Autónoma de Puebla, Puebla, México.

Bacterial growth in the water pipes has been of interest in many countries, especially because the pathogenic microorganisms to humans can grow forming biofilm in them (Chaves-Simões *et al*., 2013; Costerton, 1999; De Leon *et al*., 1993; Mahapatra *et al*., 2015). Bacteria are associated forming biofilms, which are complex communities of bacteria surrounded by a exopolymer matrix; they form colonies adherent to inert surfaces, for example: water storage tanks and pipes. It has reported that a biofilm consist of bacteria, water, exopolisacaride matrix, proteins, nucleic acids and bacterial lysis products (Costerton, 1999; Costerton *et al*., 1995; Flores-Encarnación *et al*., 2014; Hall-Stoodley *et al*., 2008). The bacteria forming biofilm are more effective to absorb nutrients and it has been observed that they are more resistant to environmental stress, eg nutrient limitation and even the action of sanitizers and antimicrobials (Colbourne *et al*., 1988;

Bjarnsholt, 2013; Nazar, 2007; Stewart and Costerton, 2001; Wang *et al.*, 2014). Previously, it was believed that the water of supply systems was unchanged until it reaches the end consumer, however it is not (Ashbolt e*t al.*, 2015; Knobelsdorf and Mujeriego, 1997). In water pipes, bacteria can reproduce from available organic matter and the factors that contribute significantly to the development of these microorganisms are: ineffective concentration of sanitizers, the temperatuta and pH of the water, the water residence time in tanks and pipes, building material pipe (Ashbolt, 2015; Colbourne *et al*., 1988). It described that supply networks of water represent an ideal via by bacterial growth because they offer a continuous flow of nutrients and bacteria and pipe walls are used as the adhesion surfaces (Knobelsdorf and Mujeriego, 1997). Therefore, the present study aimed to seek evidence the occurrence of bacteria forming biofilm in water pipes commonly used at Puebla, México.

MATERIALS AND METHODS

Collection of samples

The study samples were collected and examined randomly from domestic water pipes commonly used at the municipality of Puebla, México, over a period of 6 months from July to December 2015. Keys of water pipes were cleaned externally before sampling. Water was allowed to flow for one minute prior to sample collection. So number of water samples considered in this study was 25. The samples were collected using a sterile hyssop scraping the inner surface of the keys. Then, hyssops was introduced in 1.5-mL Eppendorf tubes containing 1 mL of sterile Luria-Bertani broth (LB). Some 1.5-mL Eppendorf tubes containing hyssop with biofilm were incubated in batch at 37°C for 24 hours and then the tests of bacterial isolation and identification were performed. Others 1.5-mL Eppendorf tubes containing hyssop with biofilm were centrifuged to 10,000 x g and the cell pellets were recuperated for extraction of DNA from them.

Crystal violet staining

Bacterial biofilm was evidenced using the crystal violet staining. To do this, a sample of biofilm was placed on a glass slide and then the sample was stained with 0.1% crystal violet. It was left at room temperature for 3 min, then a washing was done with distilled water and it observed under a microscope (40x).

Calcofluor white staining

The exopolysaccharide of biofilm was stained with calcofluor white according to methodology described by Ramos *et al.*, (2006). So, a sample of biofilm was placed on a glass slide and then the sample was stained with 0.02% calcofluor white. The glass slide was incubated at room temperature for 20 min in the dark and it was then exposed to UV light. The light emission confirmed the presence of exopolysaccharides in the samples.

Bacterial isolation

The bacterial and fingi isolation from biofilm of water pipes was performed using microbial methods. Thus, biofilm was placed on Petri dishes containing tryptone soy agar and potato dextrose agar (Difco Co). For bacterial isolation the dishes containing tryptone soy agar were incubated at 37^{o}C for 24 hours, while dishes containing potato dextrose agar were incubated at 28 ^{o}C and 37^{o}C for 24-72 hours. For the identification of isolated microorganisms were used the microbial biochemical tests decribed by Fernández *et al.,* (2010).

Extraction of DNA

Genomic DNA was extracted according to methodology described by Ho *et al*., (1991). For this, it was used cell pellet obtained previously which was resuspended with 1000 µL of TE buffer (10 mM Tris-CI pH 7.5, 1 mM EDTA) in a 1.5-mL Eppendorf tube and it was added 30 µL of 10% sodium dodecyl sulfate and 3 µL (2 mg/100 µL) of proteinase K. Eppendorf tube was incubated at 37°C for 1 h. The mixtures were extracted with an equal volume of phenol-chloroform-isoamyl alcohol and centrifuged at 12,000 x g in a microcentrifuge for 3 min; the aqueous layer was transferred to a fresh Eppendorf tube. Two further extractions were performed with equal volumes of phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol. The DNA was precipitated with 0.7 volume of isopropanol at -20°C. The genomic DNA was pelleted by microcentrifugation at 13,000 x g for 5 min, washed with -20°C 70% (vol/vol) ethanol, desiccated for 30 min, and dissolved in 50 µL of molecular biology-grade water. The DNA was quantified spectrophotometry. Genomic extracted DNA was stored at -20°C.

RESULTS

Between July to December 2015 were examined 25 samples of biofilm from water pipes in common use at the municipality of Puebla. The samples were collected using a sterile hyssop. The results shown that method used allowed the recovery of bacterial biofilm (Fig. 1). Fig. 1A shows the biofilm commonly formed inside of water pipes. As can be seen the biofilm was remarkable in view and it presented in most samples were observed. A biofilm sample was spread on a glass slide and stained with crystal violet (Fig. 1B). In this preparation a lot of staining microorganisms were observed. To confirm the presence of bacteria forming biofilm, it was performed other staining using calcofluor white (Fig. 1C). The calcofluor white is a fluorescent dye that binds to exopolysaccharides of biofilm extracellular matrix. As shown in Fig. 1C, the extended sample on a glass slide produced fluorescence when it exposed to UV light.

On the other hand, the DNA quantification in each of the samples was performed. From DNA quantifications it can be concluded that biofilm samples from water pipes were positive for the presence of microorganisms (for example: bacteria) and that different concentrations of DNA should be in proportion to the amount of microorganisms in them (Table 1). As shown in Table 1, all analysed biofilm samples contained different concentrations of DNA from 22.5 to 165 ng/µL of DNA. The DNA detection was indicative of the presence of microorganisms. Subsequently, it is proceeded to perform isolation and identification of bacteria from biofilm in water

pipes. The results were shown in Table 1. As shown in Table 1, all samples were positive for the presence of bacteria. So, it was possible to isolate bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa, Enterobacter aerogenes, E. cloacae, E. gergoviae, Citrobacter freundii, Staphylococcus* sp., *Klebsiella oxytoca, Bacillus* sp., and some fungi such as *Penicillum* sp. and *Paecilomyces* sp.

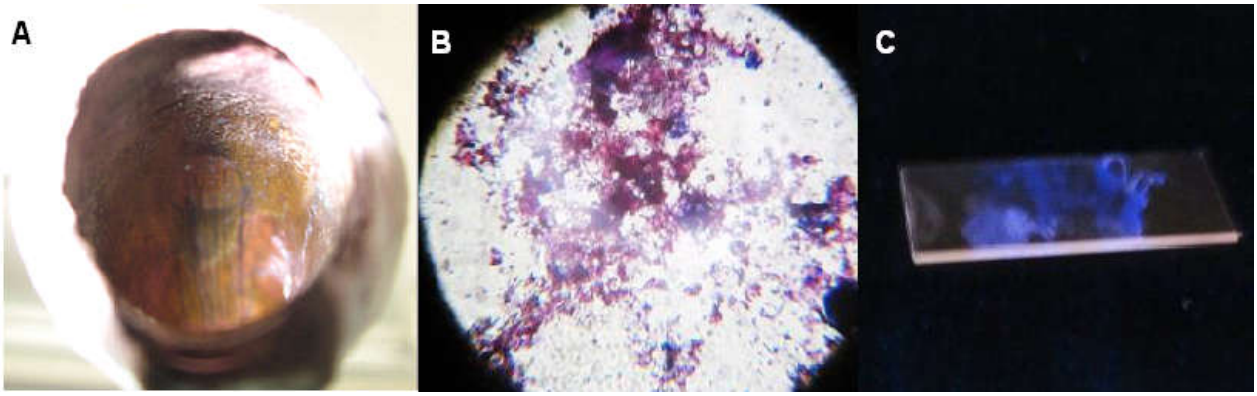

Fig. 1. The bacterial biofilm of water pipes. A. Appearance of the interior of a water pipe commonly used; B. Biofilm stained with crystal violet dye (40x); C. Bacterial extracellular matrix stained with calcofluor white dye.

Table 1. DNA concentrations and bacteria isolated from biofilm of water pipes.

Samples	DNA concentrations (µg/µL)	Bacteria
1	72.5	*Pseudomonas aeruginosa/ Escherichia coli*
2	45.0	*Escherichia coli/ Citrobacter freundii*
3	42.5	*Pseudomonas aeruginosa*
4	50.0	*Enterobacter aerogenes*
5	62.5	*Enterobacter aerogenes/ Enterobacter cloacae*
6	37.5	*Citrobacter freundii/ Enterobacter aerogenes*
7	42.5	*Pseudomonas aeruginosa*
8	52.5	*Pseudomonas aeruginosa*
9	57.2	*Staphylococcus* sp.
10	22.5	*Pseudomonas aeruginosa*
11	35.0	*Pseudomonas aeruginosa*
12	22.5	*Staphylococcus* sp.
13	55.0	*Pseudomonas aeruginosa*
14	27.0	*Enterobacter aerogenes*
15	30.0	*Enterobacter gergoviae/ Klebsiella* sp.
16	40.0	*Pseudomonas* sp./ *Penicillum* sp.
17	17.5	*Klebsiella oxytoca*
18	25.0	*Pseudomonas aeruginosa*
19	50.0	*Klebsiella oxytoca/ Staphylococcus* sp./ *Penicillum* sp.
20	30.0	*Enterobacter aerogenes/ Paecilomyces* sp
21	110.0	*Enterobacter aerogenes*
22	65.0	*Enterobacter aerogenes/ Bacillus* sp.
23	37.5	*Enterobacter aerogenes*
24	165.0	*Enterobacter aerogenes/ Pseudomonas* sp./ *Penicillum* sp.
25	75.0	*Enterobacter aerogenes/ Paecilomyces* sp.

DISCUSSION

The biofilm is a community of microorganisms which is embedded in a solid surface and also it can also be found in a gas-liquid interface (Costerton, 1999; Costerton *et al.*, 1995; Characklis and Marshall, 1990; Flores-Encarnación *et al.*, 2014; Hall-Stoodley *et al.*, 2008). The biofilm is the natural state in which the most bacteria are in the environment or infectious processes (Costerton, 1999; Hall-Stoodley *et al.*, 2008). It has been reported that the biofilm allows bacteria to be more resistant to action of antibiotics and even they are able to evade the host immune response (Bjarnsholt, 2013; Nazar, 2007; Stewart and Costerton, 2001). The biofilm consists of an extracellular matrix composed of exopolysaccharides, proteins, DNA, water (97% volume approximately) and bacteria (Nazar, 2007; Sack *et al.*, 2014). The close connection between bacteria forming biofilm favors the constant exchange of genetic information.

The water, nutrients and waste pass through small channels formed between the bacterial extracellular matrix, which resembles a primitive circulatory system (Kalbbach *et al.*, 1997; Nazar *et al*. 2007; Zhang *et al.*, 2012). On the other hand, a common problem in water distribution networks is the formation of bacterial biofilms inside them (Ashbolt, 2015; Chaves-Simoes and Simoes, 2013; Kalbbach *et al.*, 1997; Knobelsdorf and Mujeriego, 1997; Richards *et al.*, 2015; Zhang *et al.*, 2012). The biofilm formation is a dynamic and continuous process and it forms a complex microenvironment in the pipes. This process depends on several factors including materials from which pipes are made (Niquette *et al.*, 2000;

Rozej *et al*., 2015; Sack *et al*., 2014; Shelton *et al*., 2013; Yu *et al*., 2010). In this study, the presence of bacteria forming biofilm in water pipes was determinated according to that reported by other authors (Ashbolt, 2015; Hryniszyn *et al*., 2015; Knobelsdorf and Mujeriego, 1997; Mahapatra *et al*., 2015; Niquette *et al*., 2000; Shelton *et al*., 2013). So Fig. 1 has showed abundant bacterial biofilm attached to water pipes analyzed. Through the crystal violet staining, bacterial organic matter attached to the walls of the water pipes was observed, while the calcofluor white staining confirmed that it is indeed biofilm.

The calcofluor white is a fluorescent dye that binds in the glycosidic linkages β-(1-3) and β-(1-4); it is generally used for to observe the exopolysaccharides in biofilm (Ramos *et al*., 2006). Likewise, it was possible to carry out the extraction of genomic DNA from collected biofilm, which it was a proof that there were microorganisms attached to the walls of water pipes (Table 1). These tests were an evidence of the presence of bacteria inside water pipes. In the present study, all samples of biofilm obtained from water pipes were found to be contaminated with bacteria attached to the walls of water pipes. The major bacterial genera isolated from water pipes were: *E. coli, P. aeruginosa*, *Enterobacter* sp., *Staphylococcus* sp., *Klebsiella* sp., and fungi, for example: *Penicillum* sp. and *Paecilomyces* sp. So, the biofilm inside water pipes contains bacteria that cause serious damage to public health. This results are according to those reported by other authors (Chaves-Simões *et al*., 2015; De Leon *et al*., 1993; Gião *et al*., 2011; Kilvington *et al*., 2004; Knobelsdorf and Mujeriego, 1997; Richards *et al*., 2015; Rozej *et al*., 2015; Yu *et al*., 2010; Zhang *et al*., 2012).

The presence of bacteria in water pipes indicates the poor microbiologic quality of the water used for human, being the water a vehicle for the storage and dissemination of pathogenic bacteria to humans (Bahrami *et al*., 2013). It has reported that microorganisms members of biofilm are more protected from the action of biocides than microorganisms suspended in the water. The attached microorganisms have also a resistance to chlorine 150 times greater than the microorganisms in suspension (Knobelsdorf and Mujeriego, 1997). In this study the isolation of iron-reducing bacteria was not covered, however these microorganisms are important. Microorganisms which colonize interior of water pipes might contribute to corrosion-microbiologically influenced corrosion (Hryniszyn *et al*., 2015).

Conclusion

The purpose of a water distribution system is to reach the consumer, the water with good quality and an acceptable flavor, odor, appearance. In recent years, it has been shown that drinking water has lost quality along its passage by water pipes. This has been attributed in part to bacteria adhered (forming biofilm) inside of the water pipes. So the presence of bacteria forming biofilm inside water pipes in the municipality of Puebla, suggests contamination because to an inadequate sanitation. Bacteria forming biofilm inside the water pipes constitute a potential risk of infection for consumers.

It is important to search new water disinfectants to prevent biofilm formation inside of the water pipes.

Acknowledgements

Thanks to Rosa María Nava Nolazco by Biomedicina-BUAP for her invaluable technical assistance in the laboratory. Also thanks to PRODEP, VIEP-BUAP and Facultad de Medicina-BUAP for the facilities provied for the development of this work. Especially thank to the members of my family for their support and patience they have always given me.

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