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Study on biofilm-forming ability of environmental isolates of *Legionella, Sphingomonas* and *Elizabethkingia*

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Abstract : This study were assessed biofilm formation ability on coverslips and tube method by some isolates of *Legionella* species, *Sphingomonas paucimobilis* and *Elizabethkingia meningoseptica*, and investigate the effect of pipe material on biofilm formation by these isolates which isolated from cooling and drinking water in Baghdad City. The tested bacterial isolates were produced biofilm at a percentage of 81.25%; 88.24% and 75% from isolates of *S.paucimobilis*, *Legionella* spp and *E. meningoseptica* respectively by coverslips methods. While by tube method it were 76.47% of *Legionella* isolates, 57.14% of *S.paucimobilis* and 50% of *E. meningoseptica* were biofilm producer of tested isolates. All tested isolates are forming biofilm at different degree on all tested pipes materials and the bacterial count of biofilms was higher on galvanized iron pipe than other material pipes for S148, S8, Lp44 and Lm86 bacterial isolates, while cell count on the other material were varies according to the isolates. On the other hand stainless steel having the lowest bacterial count.
Key words : Biofilms, pipe material, *Legionella ,Sphingomonas, Elizabethkingia*.

Introduction

Biofilm is defined as complex microbial community distinguished by cells that are attached to a substratum and to each other by means of a matrix of self-produced extracellular polymeric substances (EPS)[1]. Interstitial water channels separate the microcolonies of bacteria that embedded in the EPS matrix, allowing transfer of oxygen, nutrients, genes and antimicrobial agents[2]. Biofilm production is consider as an indicator of clinically relevant infection. Previous studies have confirmed that biofilms forming organisms are resistant to antibiotics and variety of disinfectants[3] which suggest that their characterization is an essential aspect to control infection[4].

Generally , three discrete stages are involve in bacterial biofilm life cycle these include: First stage is attachment to substratum. The second stage is maturation by which attached bacteria start to grow and form microcolonies and an increase in number of the microcolonies that become embedded in EPS matrix. Third stage is detachment of cell and dispersal from biofilm to the environment by shedding of daughter cells during microbial growing, or shearing of biofilm mass as a results of water flow[1,2]. The goal of this study is to investigate the ability of three different water opportunistic pathogenic bacteria to form biofilm by two method and investigate effects of four pipe materials on biofilm formation by selected isolates.

Material and Methods

Bacterial isolates

Bacterial isolates were isolated from cooling and drinking water sample obtain from different place in Baghdad City these isolates were identified to species level by biochemical test, VITEK2 system device and by PCR .

Bacterial Biofilm Formation Assay

1- Coverslip Assay

This test was performed according to the procedure described early [4], for 64 *S.paucimobilis* isolates, *4 E.meningoseptica* isolates and 17*Legionella* species isolates.Briefly by preparing sterile test tubes containing 5 ml broth media according to tested isolates (TSB for *S.paucimobilis* and *E.meningoseptica* and BYEα broth for *Legionella* species) were inoculated by 1×10^8 cell/ml overnight culture. This culture was used to fill the bottom of sterile glass Petri-dish contain sterile glass coverslips which placed diagonally relative to plat bottom and incubated at 36±1°C for 48 hours. Then coverslips were removed, wash gently by sterile PBS to remove unattached cells and then one side of coverslips were clear by sterile cotton and fixed by ethanol, then stained by dipping in 0.1% safranin for 10 minutes and washed by submerging in two successive water baths, then allowed to air dry and visualized under compound microscope.

2- Quantitative Tube Assay

The biofilm assay described earlier [5]was applied on selected isolates as follows with some modifications:

Ten milliliter of bacterial suspension was prepared from each tested isolates as described above on glass tube. Negative control tube leaved without bacterial inoculums. All tubes were incubated at 36±1ºC for 48hr. After that, the tubes content was decanted and washed three times with phosphate buffer saline (PBS) to eliminate the unattached cells. 10 ml of 1% safranin stain solution was added to all tubes and rotated gently and leave for 10minutes at room temperature, then the contents was gently decanted and washed by sterile PBS to remove the stain which not adhered to the inner surface. The safranin bound to the bacterial biofilm was extracted later with 10ml of ethyl alcohol, and then absorbance was determined at 570nm.

The biofilm formation degree was calculated based on OD of biofilm -producing isolates were classified into the following categories: $OD \leq ODc$ (control) no biofilm producer, $ODc < OD \leq 2 \times ODc$ weak biofilm producer, $2 \times ODc < OD \leq 4 \times ODc$ moderate biofilm producer and $4 \times ODc < OD$ strong biofilm producer[6].

3- Biofilm Production on Solid Surface

One cubic centimeter (coupons) of each one of four types of solid surfaces which used in water pipes of distribution systems : polyvinyl chloride (PVC), galvanized iron(GI) , unplasticised polyvinyl chloride UPVC and stainless steel (SS) , were used for study biofilm productivity and adhesion on solid surfaces. These surfaces were soaked for one day in 95% ethanol and rinsed thoroughly by deionized distil water, after that, coupons were sterilized by autoclave for 15min at 121◦C[7], all assay was curry out with three replicate. This test was performs as follow:

Bacterial cells suspension of 1 $x10^8$cell/ml were prepared in 10 ml of sterilized BYEα broth for *Legionella* isolates and Tryptic soya broth for *S. paucimobilis* and *E. meningoseptica* isolates. One coupon from every type was put in a bacterial suspension. Control tube was leaved without bacterial inoculums, all tubes were incubated at 36±1°C for 48 hours. After incubation, all coupons were removed from bacterial suspension and rinsed by sterilized PBS to remove unattached cells. Each coupon was separately put in a tube with 10 ml sterilized tap water. The adhered bacteria were recovered from coupons by vortex vigorously. Then liquid was aseptically diluted and0.1ml of each dilute was cultured on BCYE plates and TSA and incubated at 36±1°C for 24-72 hours. CFUs were counted by viable plate count at 1cm² of solid surface.

Statistical Analysis

The Statistical Analysis System- SAS[8]program was used to found the effect of difference factors in study parameters . Least Significant Difference (LSD) test was used to significant compare between meansin this study.

Results and Discussion

1- Coverslip Method

A total of 85 isolates (64 *S.paucimobilis*; 17*Legionella* and 4 *E. meningoseptica* isolates) were subjected to biofilm formation by slide assay performance by assay described by Taj*et al.*[4], From which 70 isolates are biofilm producer by this methods distributed as fellow: 52 *S.paucimobilis*; 15 isolates of *Legionella*spp and 3 *E. meningoseptica* isolates are biofilm producer of tested isolates that comprise 81.25%; 88.24% and 75% from each species respectively (Figure1). Yim*et al.*[9] declared that biofilm forming property of *Sphingomonas* species may clarify their ability to live in chlorinated water and industrial water such as paper mill processing water, accordingly, both municipal and industrial water systems can be susceptible to contamination by this bacteria.

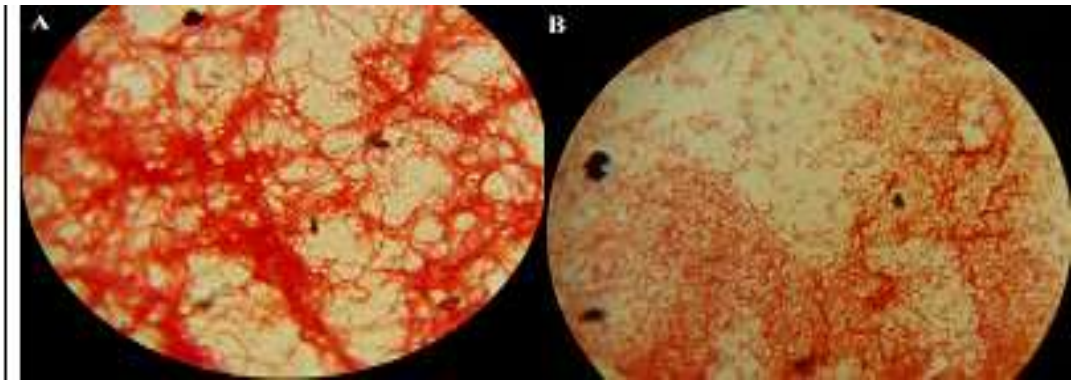


Figure (1): Biofilm formation assay by slid method of two isolates; A: *L.pneumophila* ; B: *S. paucimobilis* (×1000).

Bereschenko *et al.*[10]demonstrated that *Sphingomonas* spp. secrete exopolysaccharides which are the main components of biofilm and they were responsible for initial biofilm production, consequently, any *Sphingomonas* cell that survives from the disinfection process can speed up biofilm formation in distribution system and further deteriorate drinking water quality. In natural environment *Legionella* seem to be a secondary colonizer of biofilms already exists.Declerck*et al.*[11]found that *L. pneumophila* rapidly colonizes biofilm communities within less than two hours, and Abdel-Nour*et al.*[12]revealed that thepresent of *P. aeruginosa* enhance *fla* A expression of *L. pneumophila* by 40%. *Fla*A is one of the proteins concerned in the assembly of *L. pneumophila* flagellum, which enables the bacteria to move toward the biofilm. Mahapatra *et al.*[13]found that in coverslips method strong producers could be simply detected whereas difficult to distinguish between moderate and weak producers while quantitative tube method distinguished between these three biofilm degree easily, also discrimination non-biofilm producers was very clear by these two methods.

2- Quantitative Tube Method

Twenty eight isolates were subject to biofilm forming trait as describe by Pfaller*et al.*[5]with some modification. All *Legionella and E. meningoseptica* isolates and seven isolates of *S. paucimobilis* which positive for biofilm production by slide methods and which conceder of the most antibiotic resistant isolates of this species were subjected to this test. Based on intensity of color by OD measured for each test tube at 570nm using spectrophotometer, and OD values were compared with the OD of negative control, the results could be expressed for all isolates to as strongly adherent, moderately, weakly, and non-adherent. Results show that 28.57 % were strong biofilm producer, 21.43 % were mild producer, 14.28%were weak biofilm producer and the other 35.71% were non biofilm producer. As seen in figure (2) 76.47% of *Legionella* isolates were biofilm producer distributed as strong29.4%, mild23.52% and weak producer 23.52% while 23.52% were non producer.

One isolates of *E. meningoseptica* was strong biofilm producer and one mild producer while the other two isolates were non producer. 42.86% of *S. paucimobilis* isolates were biofilm non producer and 57.14% are biofilm producer distributed as fellow: 28.57% of isolates are strong biofilm producer, 14.28% mild and 14.28% weak producer, that is close to Gusman*et al*.[14]whom noticed in their studies which was done in Serbia that all *S. paucimobilis* tested isolates are strong biofilm-producer which resembling to what found in the current study.Similar findings have been observed by other researcher in different countries, including in Finland, Hungarian and Canada[15,16]which is close to this study finding. Gusman*et al*.[14]emphasized that the *S. paucimobilis* strains tested have a strong biofilm-producing ability which may consider a potential pathogenic features of this bacterium, so the presence of these isolates in drinking water and distribution systems is not desirable. Consequently, adequate management of drinking water and biofilm degradation in water distribution networks which will guarantee safety drinking water is recommended.

Liu *et al*.[17]found that *E. meningoseptica* established biofilms in the lens cases, with significant numbers of CFU recovered and the biofilm of *E. meningoseptica* were metabolically active. Soman*et al*.[18]emphasized that biofilm formation is an important property of this bacterium, which decrease antibiotic susceptibility.

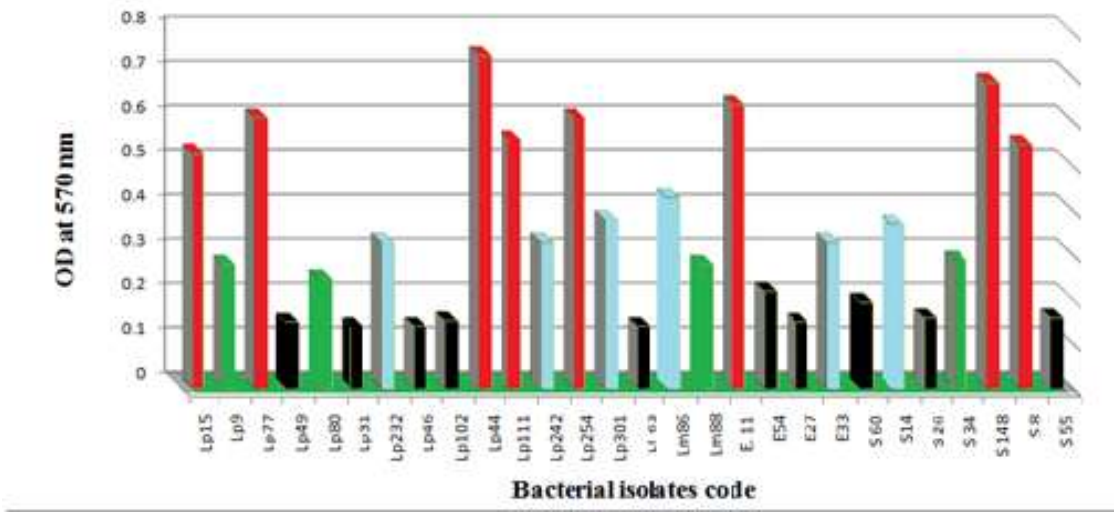


Figure (2): Biofilm forming ability represented by OD values of selected isolates isolated from drinking water and cooling water. Each datum is a mean of triplicateandsubtracted from (control OD value). Green: weak producer; Blue: mild producer;Red: strong producer.

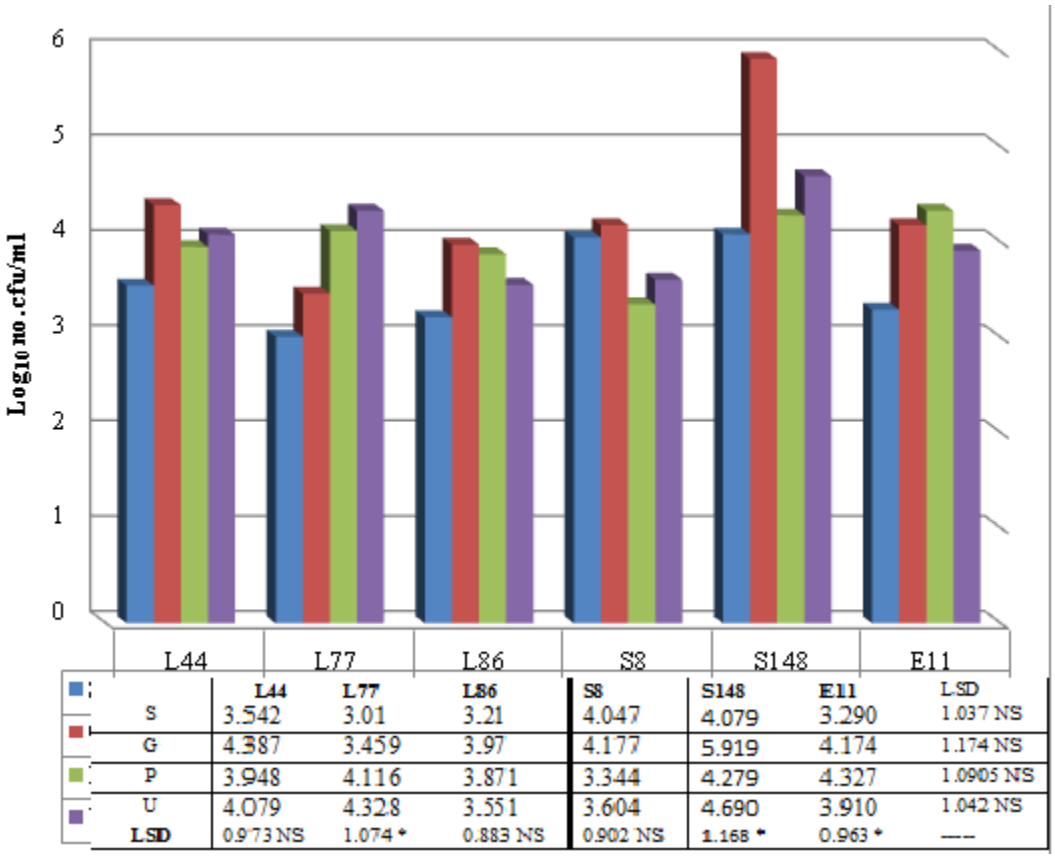
Where Lp: is *L. pneumophila*; Ll: is *L. longbeachae*; Lm: is *L.micdadei*; E: is *E. meningoseptica* and S: is *S. paucimobilis*.

3 - Biofilm Production on Solid Surface

A single distribution system, usually includes different materials such as metal pipes like iron and stainless steel, and synthetic polymers such as PVC, on which divers bacterial community at different growth rates and bacterial densities were occur[19], also pipe material composition influences biofilm development on pipe surfaces[20].The effects of four pipe materials on biofilm formation by selected isolates were investigated , and these pipe materials wereStainless steel (SS) , galvanized iron(GI) , polyvinyl chloride (PVC) and unplasticised polyvinyl chloride(UPVC). In this study the tested isolates were biofilm producer selected from previous experiments results. Five isolates were tested, three of them were *Legionella* isolates, two of them belong to *L. pneumophila* and the other isolate was belong to*L.micdadei*. Other tested isolates were two *S. paucimobilis* and one belong to *E. meningoseptica.* Results show that all isolates are forming biofilm at different degree on all tested pipes materials (Figure 3) that the results revealed that obviously the bacterial count of biofilms was higher on galvanized iron pipe than other material pipes for S148, S8, Lp44 and Lm86 bacterial isolates, while cell count on the other material were varies according the isolates. On the other hand stainless steel having the lowest bacterial count during the experiment at the end of the operation. This finding was very close to Jang *et al*.[21]whom found that biofilm bacterial count was about one hundred times higher on steel pipe than other material pipes during the experiment and that the SS pipe havingthe lowest bacterial count

as it was confirmed by present study results. Also similar to Prest *et al*.[20]observation, that iron pipe permit the highest bacterial densities with up to 45 times bacterial biomass on iron coupons more than on plastic materials. And they emphasized that iron pipe corrosion give rise to deposit formation and release of particles on which inorganic and organic compounds adsorb, and which form an attachment sites, on which bacteria are protected from disinfectant residuals[20].

Bucheli-Witschel *et al*.[22]found that polymeric pipe materials such as PVC release biodegradable organic substances which can modify the available nutrient sources for bacteria.



	L44	L77	L86	S8	S148	E11	LSD
S	3.542	3.01	3.21	4.047	4.079	3.290	1.037 NS
G	4.387	3.459	3.97	4.177	5.919	4.174	1.174 NS
P	3.948	4.116	3.871	3.344	4.279	4.327	1.0905 NS
U	4.079	4.328	3.551	3.604	4.690	3.910	1.042 NS
LSD	0.973 NS	1.074 *	0.883 NS	0.902 NS	1.168 *	0.963 *	——

Figure (3) : Number of bacterial cells attached on four solid surface after 48hs incubation at 36°C and pH7. Wherestainless steel (S) ,galvanized iron(G) , polyvinyl chloride (P) and unplasticised polyvinyl chloride(U) . And Lp77& Lp44= *L. pneumophila*; Lm86 = *L. micdadei* ; S148 &S8= *S. paucimobilis* ; E11: *E. meningoseptica*

Regarding the bacterial species, results in current study show that *S. paucimobilis* have the highest ability to form biofilm on the all four pipe material compare to other tested isolates and *L. micdadei* have the lowest bacterial count on biofilm forming on the four pipe materials. Other three isolates were nearly close to each others.

Interestingly, previous study was conducted the effects of pipe materials (polyvinyl chloride, steel, copper, polystyrene and stainless steel) in tested isolates ability to biofilm accumulation, have demonstrated that apart from thepipe materials, the predominant species in all biofilms was *Sphingomonas*[14,21]. Also Chao*et al*.[23]observed that pipe material have considerable effect on microbial community constitution of the drinking water biofilms as it was confirmed in the present study (Figure 3), revealing that the type of pipe material could be important feature that governing the structure of drinking water biofilm communities.

Young *et al*.[24]revealed thatthe ability of *E. meningoseptica* to form biofilms and survive for long periods in water sources as well as tap water or other moist environment, indicating its importance in intensive care environments biofilms. And they observed that *E. meningoseptica* biofilm is difficult to eradicate after organism has established on indwelling device like endotracheal tube or a vascular line, since transmission of this organisms from environmental reservoirs to patients is a known infection outbreak scenario.

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