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Biofilm formation by Aggregatibacter actinomycetemcomitans

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ABSTRACT

Aims: The aims of study were to differentiate between biofilm former and biofiolm non former strains of *Aggregatibacter actinomycetemcomitance*, to reveal the effect of time and glucose concentration on formation and structure of *Aggregatibacter actinomycetemcomitance* biofilm using crystal violet assay(CVA), light microscope , scanning electron microscope (SEM).

Methods: Biofilm formation on glass surface was estimated by conventional crystal violet staining method *Aggregatibacter actinomycetemcomitans*, strains were cultivated overnight in in tryptic soy broth tube. The clinical isolates were classified according to the optical density in to positive and negative biofilm former, to study the effect of time, biofilm isolates were incubated for different times elapses (4,18, 24, 48)hrs after each incubation period optical density were measured and compared , biofilm structure examined and compared using light microscope and scanning electron microscope, to study the effect of glucose on biofilm, isolates were cultivated in TSB Supplemented with (no glucose ,0.5 % g glucose ,1% glucose),incubated for 18 hrs, absorbance were measured and compared, biofilm examined and compared using light microscope and scanning electron microscope**.**

Results: crystal violet method had been used to assess biofilm formation by measuring absorbance, to classify isolates in to biofilm former have (OD)more than 0.1nm while non biofilm former isolates have (OD) less than 0.1nm .Examination of biofilm by light and scanning electron microscopes showed larger microcolonies and increased amounts of exo-polysaccharides with increasing time and glucose concentration.

Conclusions: This study proved that some isolates of *A.actinomycetemcomitans* were biofilm former, time and glucose concentration effected on the structure of *A. actinomycetemcomitans* biofilm.

Key words: biofilm, micro colony, Exo-poly saccharide, *Aggrrgatibacter*

INTRODUCTION

Aggregatibacter actinomycetemcomitans is a gram negative coccobacillus, approximately 0.4x1.0 µm in size [1]. *Aggregatibacter actinomycetemcomitans* can be grouped into six serotypes (a-f) based on the polysaccharide antigen on the cell surface, Serotype b is commonly implicated in Localized Aggressive Periodontitis, while serotype c is related to periodontal health in adults[2][3]. Oral microbiologist pay a great attention for this species because it possesses many virulence factors like: Leukotoxin, Cytolethal distending toxin resistance to conventional antibiotic treatment Surface adhesion Lipopolysaccharides production and biofilm formation,[4]**.** Biofilms are a protected niches for microorganisms, complexity of biofilm structure make the microorganisms within the biofilm safe from antibiotic treatment which lead to chronic infection[5]**.** As biofilm cells undergo molecular alteration to accommodate with this complex and cooperated community, therefore several microbiologists have investigated the genetic and physiological nature of biofilm formation and structure for a huge number of bacteria [6].

Once the microorganisms adhered to the surface they began to produce an extracellular polymeric substances (EPS) consisting mainly of polysaccharides, proteins, nucleic acids, and lipids. The matrix not only provides mechanical stability to biofilms, but also mediates bacterial adhesion to surfaces [7]. The formation of three-dimensional biofilm structures is dependent on complex regulatory processes which involve the expression of genes important for biofilm formation in response to distinct environmental signals [8]. Mono species biofilm generated *in vitro* for a limited number of model bacteria have been a subject of intense research and have provided profound insights into dynamic structural and functional aspects of biofilm formation processes [9,10]**.**

MATERIALS AND METHODS

Samples collection and transport: sub-gingival plaque samples were collected by inserting sterile paper point size 50 into the deep pockets of patients, who attended the Dental hospital, Department of Periodontics, College of Dentistry at Mosul University asking for diagnosis and treatment and placed in sterile vials containing 5ml brain heart infusion broth.

Bacterial cultivation: Dentaid-1 was prepared using brain heart infusion agar to which the following compounds were added: 5 g yeast extract, 1.5 g sodium fumarate Difco Comp., and 1 g sodium formate per liter. The medium was autoclaved for 15 min at 121°C. The final pH was 7.2 ± 0.2. Once the medium was cooled to 50°C, vancomyc in was added to a final concentration of 9 μg/ml[11], incubation at 37°C for 48-72 hours under anaerobic condition using anaerobic candle jar. Identification of *A.actinomycetemcomitans* based on colony morphology, gram stain (gram negative *coccobacilli*), catalase test(rapid catalase positive)and molecular (PCR) identification kit (Genekam Company. Germany).

Biofilm formation assessment: A qualitative assessment of biofilm formation was determined as previously described by Christensen et al.[12]. Biofilm formed on glass surface was estimated by conventional crystal violet staining method *A. actinomycetemcomitans* strains were cultivated overnight in tryptic soy broth prepared by dissolving 3g tryptic soy broth Difco Comp. in 100ml distilled water and supplemented with 0.5g yeast extract, 0.8g glucose, 0.4g sodium bicarbonate (20mL) The tubes were decanted and washed with phosphate buffer saline (pH 7.3). The cells adsorbed on the culture vessel were scraped off with sterile spatula and the aggregates briefly dispersed by vortex then a culture containing approximately 6×10^8 colony-forming units(tube 2 macCferland was inoculated in sterile 20ml of TSBY broth tubes containing 6 glass slides measured (1cm × 1cm) ,after cultivation anaerobically for 48 hrs at 37°C the glass slides were gently washed in (PBS pH 7.2) and the cells adsorbed on the culture vessel were stained with crystal violet (0.1%)for 10 minute.

Excess stain was removed, the dye-eluted solution were 20 fold diluted with 95% ethanol, then the absorbance was measured at 570 with spectrophotometer [13]. The clinical isolates were classified into positive and negative (planktonic) biofilm former according to the optical density (OD mean strong biofilm formation while very little OD mean weak or non-biofilm former, positive biofilm former have(OD) more than 0.1 and negative biofilm former (planktonic) have (OD) less than 0.1 [14]. Ring formation at the liquid interface was not indicative of biofilm formation. Experiments were performed in triplicate for each isolates of *A.actinomycetemcomitans* [12].

Examination of biofilm by light microscope

For examination under light microscope the cells adsorbed on the slides stained with 1 ml of 0.1% crystal violet for 10 minute gently washed in phosphate buffer saline (PBS, pH 7.3). Excess stain was washed with PBS.

Preparation of biofilm samples for examination by scanning electron microscope

Two slides in each experiment was washed with PBS and fixed in 2% glutteraldehyde in PBS for 1hr at 37°C, washed again with PBS two times and left to dry and send for examination under scanning electron microscope in University of Tehran School of metallurgy and material engineering [15] .

The effect of time on biofilm formation assay by *A.actinomycetemcomitans*

Biofilm formation on glass surface was estimated as described by Christensen *et al*[12], to determine the effect of time on biofilm formation ,the biofilm former isolates have been used in biofilm formation assay described by Christensen *et al.* [12]. incubated for different times elapses (4,18,24,48)hrs after each incubation period optical density were measured and compared , biofilms structure were examined and compared using light microscope and scanning electron microscope after each incubation time .

The effect of glucose concentration on biofilm formation assay by *A.actinomycetemcomitans*

Biofilm formation on glass surface was estimated as described by Christensen *et al*[12], to determine the effect of glucose concentration on the ability of biofilm former isolates of *A.actinomycetemcomitans* using (20mL) vial tryptic soy broth tube (3% tryptic soy broth Difco Comp. Supplemented with (0%,0.5%,1%)glucose without addition of yeast extract and sodium bicarbonate ,incubated for 18 hrs, absorbance were measured and compared, biofilms structure were examined and compared using (100x) power light microscope (Olympus Optical Company, LTD, Japan) and scanning electron microscope (University of Tehran School of mettulorgy and materials engineering), for each concentration of glucose[16].

RESULTS AND DISSCUSION:

Biofilm formation assay: Absorbance was measured for 21 isolate using crystal violet method were 16 isolates gave absorbance more than 0.1nm classified as biofilm former while 5 isolates gave absorbance less than 0.1 nm classified as planktonic isolates.

The effect of time on biofilm formation of biofilm former isolates of *A.actinomycetemcomitans:*

[Table 1] a and b, [Figures 1] a, b, c, d, [Figures 2] a, b, c, d [Figure 3] showed the effect of time on the biofilm formation of biofilm former isolates of *A.actinomycetemcomitans*. This work demonstrated the effect of time on the structure of biofilm of *A.actinomycetemcomitans* using crystal violet assay, in which a-ANOVA b- Duncan's test showed significant effect of time [Table 1] a and b [Figure 3], biofilm also examined by electron microscopes [Figure 1] a, b, c, d: revealed a biofilm after 4 hrs in which the micro colonies consist of small number of cell and encased by little amount of exo-polysaccharide, b)after 18 hrs there was greater microcolonies and greater amount of exo-polysaccharide, (c) after 24hrs the microcolonies disappeared because they were fully covered with more and more exopolysaccharide, (d) after 48hrs maturation of biofilm was obvious as biofilm possess geometrical three dimensional structure pores appeared through the biofilm. Examination of biofilm by light microscope (100 X) [Figure 2] a, b, c and d confirm those of electron microscope showed that the amount of crystal violet (according Christensen 1982) absorbed by the biofilm (cells and exo-polysaccharide) were increased with time (a) after 4hrs showed small size microcolonies attached to the exo-polysaccharide which appeared like lines or rays, (b) after 18 hrs larger size microcolonies, (c) after 24hrs the microcolonies covered with stain and disappeared, (d) after 48hrs stain appeared darker and pores appeared in the biofilm. In similar manner time used to evaluate *Vibrio cholerae* biofilm development employing time-lapse confocal laser scanning microscopy with very-high-resolution and bounded extracellular components consist of adherent cells finally being embedded and enrolled inside an extracellular membrane in relation to time revealed temporal maturation of matrix constituents through the early time of biofilm development [17]. Another study, utilize a semi-quantitative procedure to evaluate biofilm development employ *V. fischeri* as a an example bacteria, use a dissecting microscope with camera connected to monitor recording biofilm development as wrinkled colony formation with time on a solid agar surface, using time course assay three dimensional development structure can be recorded at which a strain start to form three dimensional structure in comparison to that of control strains [18].

The effect of glucose concentration on biofilm formation of *A.actinomycetemcomitans:*

[Table 2]a and b ANOVA and Duncan's, [Figure 6] [Figures 4] a, b and c [Figures 5] a , b and c Showed significant effect of glucose on the biofilm formation of *A.actinomycetemcomitans* biofilm isolates. To demonstrate the effect of glucose concentration during biofilm formation of *A.actinomycetemcomitans* , crystal violet assay were used, a-ANOVA b- Duncan's test showed significant effect of glucose concentration at :(0% , 1 %, 0.5%), [Table 2] a and b, [Figure 6]. Imaging biofilm by scanning electron microscope (SEM) in University of Tehran School of metallurgy and material engineering showed different 3D structure of biofilm at different glucose concentration which can be explained by increase the amount of extracellular polysaccharide surrounding and incorporated between microcolonies, [figures 4] a, b and c .Biofilm examination by and light microscope also show different structure and density at different glucose concentration which ensure examination by (SEM), which can be explained by increase extracellular polysaccharide that absorbed more stain in crystal violet assay[figure 5] a, b and c. Some investigators have shown that, among *E. faecalis*, glucose supplementation enhances biofilm formation [19]. Glucose facilitate &increase the multicellular accumulation step during biofilm development [20]. The effect of glucose concentration (0 to 2.7%) on biofilm induction was investigated through the *rbf* gene, which was important in biofilm development formed in laboratory on polystyrene and glass, biofilm assessment method employed the wild type, (isogenic *rbf* mutant), and the genetically engineered *rbf*-complemented strain with the availability of different concentration of glucose the mutation in *rbf* resulted in a loss of glucose-induced biofilm formation at all glucose concentrations tested, indicating that the *rbf* gene mediates biofilm formation on polystyrene in response to glucose[21] .

Table (1) a-ANOVA b- Duncan's Multiple Ranges Tests .The effect of time on the biofilm formation of biofilm former isolates of *A.actinomycetemcomitans*

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	6.427	3	2.142	54.275	**.000**

Within Groups	2.368	60	.039		
Total	8.796	63			

Time	No. of isolates	Mean±S.D.	Dungan's Gp.
4 h	16	0.5344 ± .032697	**A**
18h	16	0.10575±.065815	**A**
24h	16	.62875±.223931	**B**
48h	16	.77813±.319921	**C**

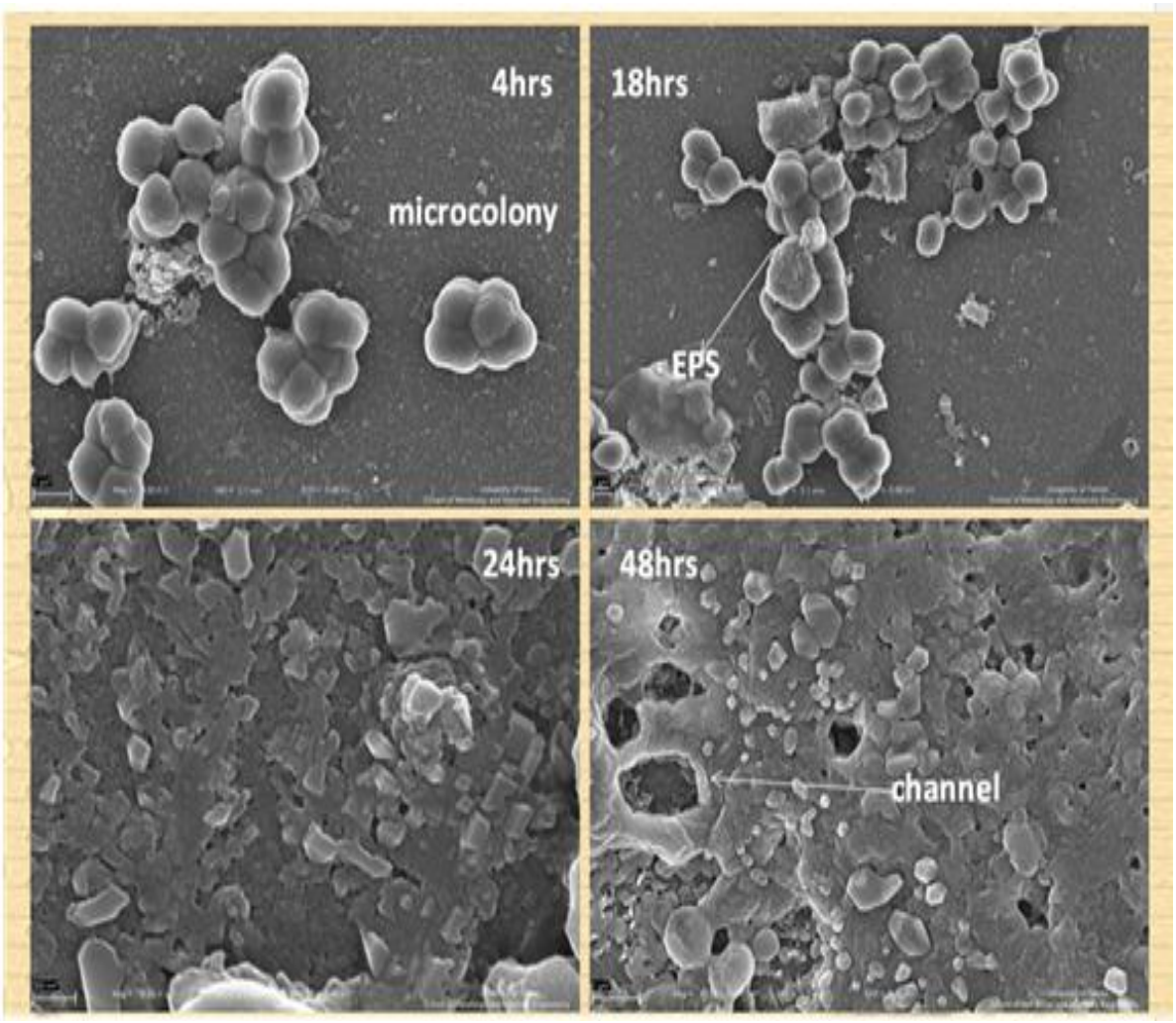

Figure 1: a, b, c, d Examination by Scanning electron microscope of biofilm formed on glass slides by A actinomycetemcomitans after different times

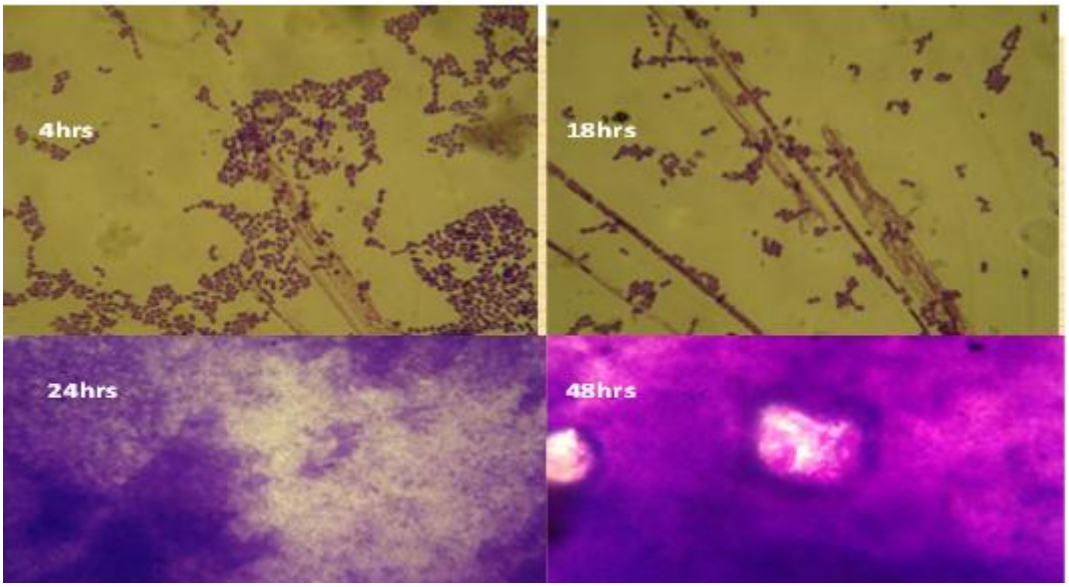

Figure 2: a, b, c, d Examination by light microscope(100X) of biofilm formed on glass slides by *A actinomycetemcomitans* after different times

a-ANOVA b- Duncan's Multiple Ranges Tests. The effect of glucose Conc. on the optical densities of biofilm formation of A. a

A- Anova

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	.176	2	.088	42.408	**.000**
Within Groups	.093	45	.002		
Total	.269	47			

B-Duncan's

N.	mean±SD	Duncan's Gp.
16	06388±.035556	A
16	09313±.034394	A
16	20438±.20438	B

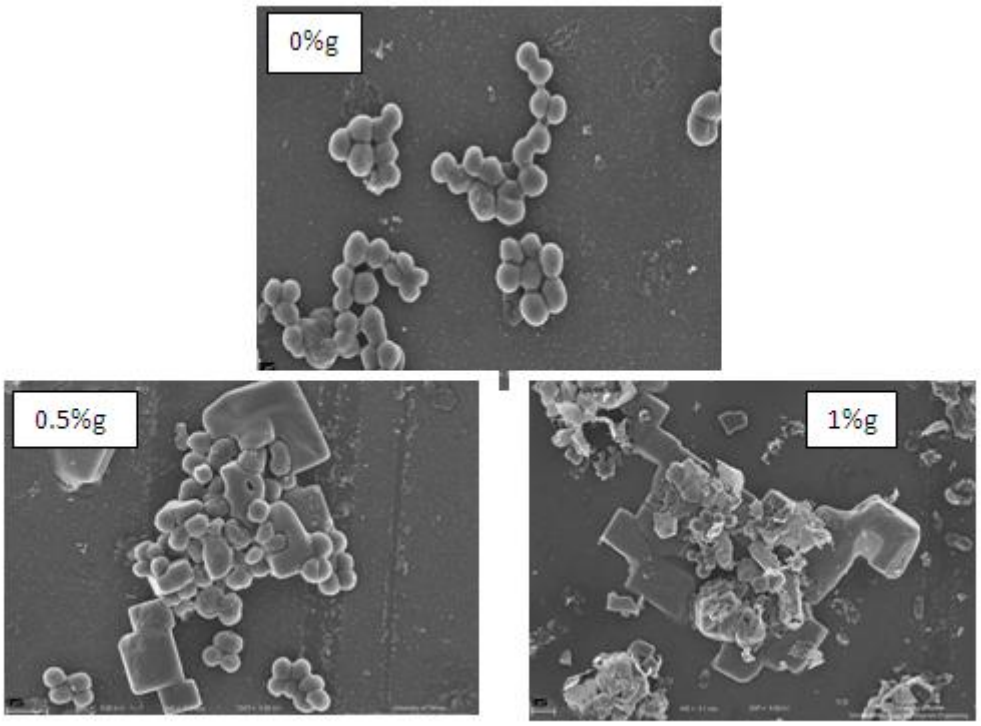

Figure 3: Examination by Scanning electron microscope of biofilm formed on glass slides by A actinomycetemcomitans using different conc. of glucose

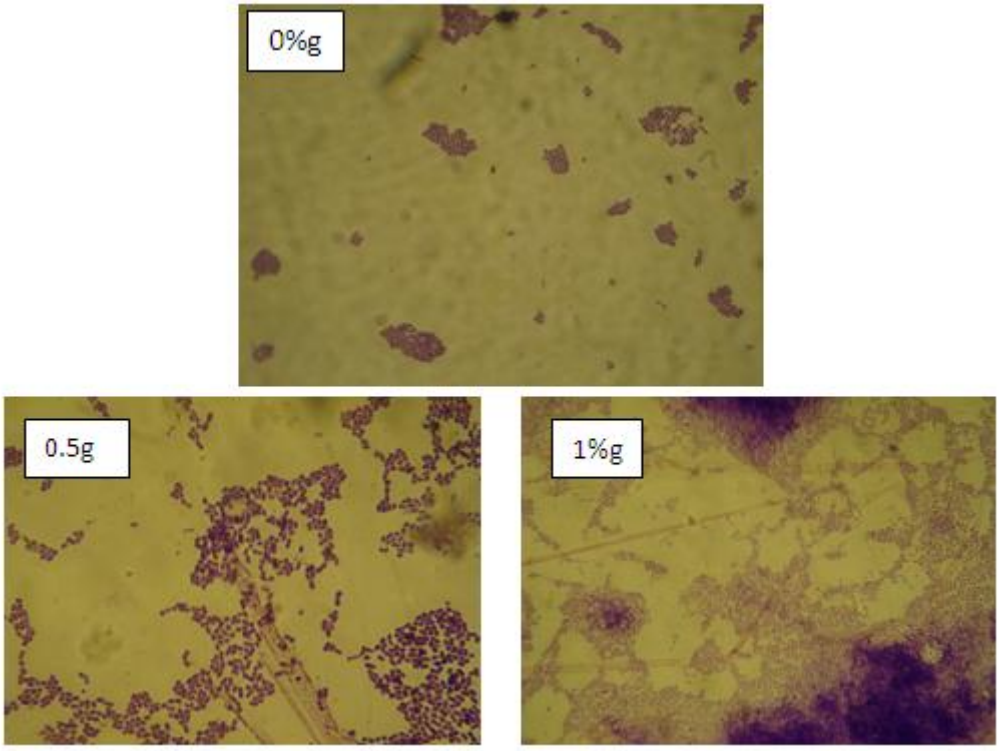

Figure 4: Examination by light microscope of biofilm formed on glass slides by A actinomycetemcomitans using different conc. of glucose

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