



Detection of biofilm formation and effect of vinegar on biofilm of *Streptococcus pyogenes* isolated from patients with tonsillitis

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Abstract : Background :Bacterial biofilms attached to living tissues that can be considered as an etiologic factor, among others. The biofilm existence supports a new concept to explain chronic infections.

Methods: *Streptococcus pyogenes* isolated from patients with tonsillitis admitted to Hilla Surgical Teaching Hospital in Babylon Governorate/Iraq was assessed for the biofilm formation by using Tissue culture plate.

Results: It was shown that biofilm formation was (76.9%) strong biofilm formation, (23%) moderately biofilm formation and no isolates that express non biofilm formation.

The effect of dates vinegar, apple vinegar and grape vinegar on bacterial isolates were studied and the results showed that date vinegar prevented biofilm formation in 13(100%) biofilm producer isolates of *S. pyogenes*. While Apple vinegar prevented biofilm formation in 12 (92.3%) biofilm producer isolates of *S. pyogenes*. Also, in the third type; Grape vinegar there were two isolates not eradicated, while 11 (84.6%) isolates were eradicated depending on biofilm criteria.

Keywords : *Streptococcus pyogenes* , Biofilm, tonsillitis, vinegar.

Introduction:-

Biofilm formation is an essential virulence factor in *Streptococcus pyogenes*, the ability to form biofilm is an innate property during the pathogenesis of *Streptococcus pyogenes* infection. Biofilm formation by *Streptococcus pyogenes* is important for maintaining an asymptomatic carriage state in the human tonsil [1]. The biofilms of *Streptococcus pyogenes* have been observed in the pharyngitis, necrotizing fasciitis, root canal infections, and skin [2].

On the other hand, biofilm play a main role in tonsillitis which is considered one of the best common pathologies in childhood [3]. However, bacteria in a biofilm can resist eradication causing chronic inflammation and permanent changes in the tonsillar lymphoid tissue [4]

The biofilm as a structure is too large to be engulfed by the host macrophages; therefore their presence in the tonsils will interfere with the normal functions of tonsillar lymphatic tissue which eventually leads to establish a recurrent or chronic infection [5]. This process explains the poor outcome of most therapeutic strategies to minimize the enlarged size of tonsils and avoid the choice of surgery. Failure to respond to antimicrobial therapy leaves the tonsillitis patients with no choice but surgery. However, despite the role of tonsillectomy in relieving the symptoms of tonsillar diseases, the more explanation for its effectiveness is the elimination of a possible biofilm infection [6].

Vinegar which is a liquid comprised mainly of acetic acid, typically 4-18% acetic acid of mass, which is prepared in households by the fermentation of many fruits. This solution is also commercially available, it is cheap and easily found in markets. There are various studies which support the antimicrobial effects of vinegar [7].

So, vinegar has been indicated as an antiseptic agent due to its medicinal properties and has been used for the treatment of infected wounds[8].The main content of vinegar represented by acids specially acetic acid [9].however, vinegar was used as anti-biofilm due to antibacterial effect of acetic acid that treats infections caused by fungiorbacteria [10]

The activity of acetic acid in prevention of biofilm formation may be attributed to the depression of the intracellular pH by ionization of the dissociated acid molecule, direct pH reduction of the substrate, or disruption of substrate transport by alteration of the biofilm bacterial permeability.

Materials and Methods

Patients:-

A total 100samples (throat swab), only thirteen isolates of *Streptococcus pyogenes* were obtained from patients suffering from tonsillitis by standard bacteriological methods. All samples were obtained from patients or individuals who admitted to Hilla Surgical Teaching Hospital in Babylon Governorate/Iraq.

Bacterial Identification:

The swab has been inoculated on culture media and incubated aerobically for 24hrs at 37°C. The gram-positive bacteria was performed by standard biochemical tests (catalase test, oxidase test, bacitracin sensitivity test, API 9 strepto system) [11].

Biofilm Production: Tissue culture plate method (TCP):

The tissue culture plate method assay described by [12]is most used and it was considered as a standard test for detection of biofilm formation. In the present study, all isolates were screened for their ability to form biofilm by tissue culture plate method as described by [12] with a modification in duration of incubation which was extended to (24) hours according to [13].

Isolates from fresh agar plates were inoculated in tryptic soy broth and incubated for 18 hrs. at 37°C in stationary condition and diluted (1) in (100) with fresh medium. Individual wells of sterile polystyrene (96) well-flat bottom tissue culture plates were filled with (0.2ml) aliquots of the diluted cultures and only broth served as control to check sterility and non-specific binding of media. The TCP were incubated for (18 hours) and (24 hours) at (37°C). After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with (0.2 mL) of phosphate buffer saline (pH 7.2) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent bacterial cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density of stained adherent bacteria was determined with a micro ELISA auto reader at wavelength of 450/630nm.

Experiment was performed in triplicate and repeated three times, the data was then averaged, and the results were interpreted according to [13] as:

Strong=>0.29

Moderate=0.15-0.29

Weak=<0.15

Effect of vinegars(Date, Apple, Grape) on biofilm formation:

The identical procedure described in tissue culture plate method for detection biofilm formation were done with some modification, vinegar(date, apple, grape)5% were added to samples in wells of sterile

polystyrene 96 well-flat bottom tissue culture plate, after fixed with sodium acetate for half an hour and all steps done as the same steps that described previously.

Ethical approval:

The necessary ethical approval from ethical committee in Hilla Surgical Teaching Hospital was obtained. Moreover, all subjects involved in this work were informed and the agreement required for doing the experiments and publication of this work was obtained from each one prior the collection of samples.

Results:-

Biofilm formation by *S. pyogenes*:

The results revealed that 10/13(76.9%) of *S. pyogenes* isolates were high biofilm former, the moderate biofilm formation were accounted for 3/13 (23%) of *S. pyogenes* isolates, while there is no isolated that express non biofilm formation as shown in table (1).

Table 1: Formation of biofilm in *S. pyogenes* by Tissue culture plate.

Bacterial Isolates(no.)	Biofilm formation		
	Strong*	Moderate*	Weak*
<i>S. pyogenes</i> (13)	10(76.9%)	3 (23%)	0 (0%)

Table (2) Effect of Date vinegar in biofilm formation.

Bacterial Isolates(no.)	Biofilm after adding Date vinegar		
	Strong	Moderate	Weak
<i>S. pyogenes</i> (13)	0%	0%	13 (100%)

Table (3) Effect of Apple vinegar in biofilm formation

Bacterial Isolates(no.)	Biofilm after adding Apple vinegar		
	Strong	Moderate	Weak
<i>S. Pyogenes</i> (13)	0%	1(7.7%)	12(92.3%)

Table (4) Effect of Grape vinegar in biofilm formation

Bacterial Isolates(no.)	Biofilm after adding Grape vinegar		
	Strong	Moderate	Weak
<i>S. Pyogenes</i> (13)	0%	2(15.4%)	11(84.6%)

Effect of vinegar (Date, Apple, Grape) in biofilm formation:

The three types of vinegar (date vinegar, apple vinegar, grape vinegar) used as anti-biofilm formation *in vitro* by growing bacterial isolates on polystyrene plates in tryptic soy broth supplemented with date vinegar, apple vinegar, grape vinegar at (5% concentration). After an 18-hour incubation time, the biofilms were stained with CV and OD value 450/630nm was measured. The results is shown in table (2),Table (3) and Table (4).The date vinegar prevented biofilm formation in 13(100%) biofilm producer isolates of *S. pyogenes* throughout readings of optical densities and comparison with depending criteria. In apple vinegar 12 (92.3%) isolates were

eradicated, except one. Also, in the third type; grape vinegar there was two isolates not eradicated, while 11 (84.6%) isolates were eradicated depending on biofilm criteria.

Discussions:-

Tonsillitis is one of a major public health concern both in developed and developing countries. *Streptococcus pyogenes* is considered one of the most prevalent human pathogens, which causes a wide of infections and the most frequent of them is tonsillitis, so it's responsible for a variety of infectious diseases and immunological complications [14]

Biofilm formation on polymeric surface was tested in the semi quantitative microtiter plate test (biofilm assay) using tryptic soy broth. This assay was repeated as triplicate to increase the accuracy of assay. The results revealed that 10/13(76.9%) of *S. pyogenes* isolates were high biofilm former, the moderate biofilm formation were accounted for 3/13 (23%) of *S. pyogenes* isolates, while there is no isolated that express non biofilm formation.

This result is agreement with the result obtained by [15] who were reported the percentage about 75.9% also this result is correlated with result obtained by [16] who reported that the percentage about (70.8%). Classification of bacteria as weak, moderate, or strong biofilm producers regulated by diverse factors, including the biotypes and the growth medium [17]. According to data obtained in this study, the presence of strong or moderate production of biofilm will confer bacteria to adhere strongly to the tonsil. However, weak positive may express the bacteria may be under stress condition or the growth is weak that made the biofilm weak or cannot be produced.

[18] were demonstrated the crystal violet microtiter plate test as a simple and rapid method to quantify biofilm formation of different bacterial strains. It is a basic dye known to bind to negatively charged molecules on the cell surface as well as polysaccharides and nucleic acid and therefore gives an overall measure of the whole biofilm. Crystal violet was used as a standard technique for rapidly accessing cell attachment and biofilm formation in a range of gram positive and gram-negative bacteria as well as yeast

They speculate about the reasons for different influence of nutritional content of the growth medium for biofilm formation. The possible explanation for the different response of bacteria to environmental conditions could be the results of mutations in genes that control biofilm formation [19], lead to propose a strong dependence between using of various sugar supplementations is essential for biofilm formation and growth condition biofilm formation and [13].

Streptococcus pyogenes is resistant to antibiotics and host defense mechanisms [20], but they can be released and cause acute infections. Antibiotic therapy can improve symptoms caused by bacteria that are released from biofilms, but cannot eliminate biofilms. For this reason, bacterial biofilms cause recurrent infections, until they are removed completely using surgical methods [15].

Biofilms are considered to be an important cause of chronic infection. Some studies have been reported the presence of biofilms in patients with recurrent tonsillitis [21].

Some bacterial species are able to evolve a biofilm, at the same time considered as important frequent pathogen for tonsillitis. For example, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* [22],[23], [24].

[25] which they have demonstrated the symptoms like apnea, tonsillar and adenoids hypertrophy, harsh raucous sound, and cervical adenopathies to be related to the presence of biofilm in the tonsils.

Streptococcus pyogenes has ability to invade tonsil crypts and proliferate, causing an acute inflammatory reaction and pus formed in the crypts. The infection occasionally extends to adjacent tissues. So, tonsillectomy is advised after at least five repeated attacks of tonsillitis per year [15].

The presence of *Streptococcus pyogenes* biofilm in tonsillar tissue demonstrate one mechanism for the increased survival of this microorganism during antimicrobial therapies[26]. Biofilms of *Streptococcus*

pyogenes play a role in the severity and progression of life-threatening human disease [27]. It also play an important role in most of the human infections caused by bacteria.

On the other hand, the effect of vinegar (date, apple and grape) on biofilm formation was also studied. So, vinegars has a clear inhibitory effect on the formation of the biofilms; this result is in agreement with results obtained by [28]. Three different types of vinegar were used on considering that the main components of them were acids specially acetic acid, which has been used as disinfectant against various contaminants [29].furthermore it appeared to have antimicrobial potential .In addition to that, it is considered easy to obtainand cheap. These types of vinegar include: date vinegar with 5 % acidity; apple vinegar with 5 % acidity; and grape vinegar with 5 % acidity. All of them considered as table vinegar due to the limitation of their acidity not more than 5% in order not to be harmful for living tissues when used by human.

Vinegar was used as anti-biofilm, because of antibacterial effect of acetic acid that treats infections caused by fungusorbacteria [10]. Furthermore, vinegar used as a surfactant against *Streptococcus pyogenes* [30]. It also used for the disinfection ,as anti-septic for sores and treatment of oral inflammatory processes [31].

There were no observable difference among types of vinegar, date vinegar was the best for biofilm prevention, which is possibly due to one of its components that have antibacterial activity besides acetic acid also may be due to presences of flavonoids, alkaloids, tannins, saponins, carbohydrates and steroids in this fruit according to the chemical analysis [32].

In addition, apple vinegar is essential role in biofilm eradication, however it less than date vinegar. This antimicrobial effect may be due to high content of phenolic compounds in apple as illustrate by [33] who was explained the direct relationship between phenolic content of apple extract and the antimicrobial effect on human pathogens.

Also, grape vinegar had the least effect in biofilm compared with the other two types, however it prevented biofilm remarkably. This may be due to difference in concentrations of grape components, as demonstrate by [34] who explained that grape extract containing high levels of polyphenols inhibited streptococcal biofilm. It has been found that grape extracts include flavanol, polyphenols, anthocyanins and catechins, which exhibited different antimicrobial activity according to their concentrations, also it is strongly bactericidal and is used in traditional medicine to cure infections. Moreover, grape vinegar is low in calories and consumption is associated with a number of health benefits.

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