

"Vinegar" as Anti-bacterial Biofilm formed by *Streptococcus pyogenes* Isolated from Recurrent Tonsillitis Patients, *in vitro*

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Abstract

Failure of antibiotic treatment in eradication of bacterial tonsillitis induced us to postulate presence of "bacterial biofilms" attached to living tissues that can be considered as an etiologic factor, among others. The knowledge about biofilm existence supports a new concept to explain chronic infections. Tonsillectomy is often the required choice as a consequence of frequent tonsillitis during the previous years, subsequently a novel studies propose using of non-antibiotic and non-surgical modalities for eradication of biofilm-related tonsillitis. In our designed study we used "vinegar" as anti-biofilm agent due to its antimicrobial effect. Vinegar is a sour liquid composed mainly of acetic acid, which has an important role in disruption of biofilm aggregations. This study has been undertaken to determine biofilm production ability by *Streptococcus pyogenes* isolated from tonsillitis patients and to evaluate effectiveness of different types of vinegar for eradication of Streptococcal-tonsillar biofilm, *in vitro*. Twenty nine isolates of *S. pyogenes* belonged to 19 patients, were tested for biofilm production by using Microtiter-plate method. Also, Modified Microtiter-plate method was conducted to study the effect of different vinegar types (Date, Apple, and Grape) as anti-biofilm, *in vitro*. Out of 75.9% produced biofilm according to depending biofilm criteria, while 24.1% were non-biofilm producers. Biofilm producer isolates distributed into 12(54.5%) and 10(45.5%) which was detected on tonsil surface and crypts respectively. Concerning the anti-biofilm activity, our results demonstrated that types of vinegar eradicated biofilm by (100%), (95.5%), and (90.9%) for Date, Apple, and Grape vinegar respectively in compare with distilled water as negative control. It was concluded that there was no significant differences in biofilm production between *S. pyogenes* isolated from tonsil surface and crypts. Furthermore, our study concluded that the three types of vinegar eradicated streptococcal biofilm remarkably, but Date vinegar was the best for eradication of streptococcal biofilm *in vitro*.

Keywords: Tonsillitis, *Streptococcus pyogenes*, Biofilm eradication, Vinegar.

1. Introduction

Bacteria that attach to surfaces aggregate in an extracellular polymeric matrix of their own synthesis or host origin to form biofilms. These bacteria are resistant to host defense mechanisms and antibiotics (Fux *et al.*, 2005), 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 (Woo *et al.*, 2012). Biofilms are currently considered to be an important cause of chronic infection. Several studies have reported the presence of biofilms in patients with recurrent tonsillitis (Chole and Faddis, 2003; Al-Marzrou and Al-Khattaf, 2008). Several bacterial species are able to develop a biofilm, at the same time considered as important frequent pathogen for tonsillitis. For example

Streptococcus pyogenes, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Staphylococcus aureus* (Post *et al.*, 2004; Wang *et al.*, 2005; Galli *et al.*, 2007). They 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 (Woo *et al.*, 2012). Additionally, biofilms can directly colonize mucosal tissues, producing chronic and/or recurrent infections that are resistant to all types of antibiotic treatment (Galli *et al.*, 2007). The upper airways seem to be at high risk for this type of colonization since evidence has been documented in the nasal and sinus mucosa of subjects with chronic hyperplastic sinusitis (Perloff and Palmer, 2005), and in the tonsillar crypts (Chole and Faddis, 2003).

Over the years, tonsillectomy remains the golden step for treatment. In spite of postoperative complications, a novel studies suggest using of non-antibiotic and non-

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surgical modalities for eradication of biofilm-related tonsillitis (Smith *et al.*, 2011). Such as using of chemicals, surfactants (Suga and Igarashi, 2012) and other natural compounds (Hannig *et al.*, 2009). Among these material, is vinegar which is a sour liquid comprised mainly of acetic acid, typically 4-18% acetic acid by 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 (Nascimento *et al.*, 2003). Vinegar has also been shown to be effective in the prevention and control of microbial contamination in intra-canal treatment of apical periodontitis in teeth (Estrela *et al.*, 2004). Also, it has been used as removing agent for *Candida albicans* biofilm from Acrylic Resin Plates. The amount of acetic acid used as vinegar on a worldwide scale is not large, but is by far the oldest and best-known application (Jafari *et al.*, 2012).

The main goals of the present study were to determine biofilm production ability by *Streptococcus pyogenes* isolated from infected tonsil surface and crypts, and its relation with recurrent infection. In addition to evaluate the effectiveness of different types of vinegar (commercially available as table vinegar) for eradication of Streptococcal biofilm isolated from recurrent tonsillitis patients, *in vitro*.

2. Materials and Methods

2.1. Specimens Collection and Bacterial Isolates

A total of 29 bacterial isolates of *Streptococcus pyogenes* were isolated from infected tonsils after tonsillectomy for 19 patients with recurrent tonsillitis were employed for this study. The ranges of patients' age group were from 4 to 14 years; 12 of them were female and 7 were male. Those were with a clinical history of at least five to six times of tonsillitis per year. Full informative history had been taken directly from patients and the information was arranged in detailed formula sheet with surgeon assistance. All patients subjected to tonsillectomy and the presented study extended from September, 2012 to January, 2013. Sixteen isolates of *S. pyogenes* were identified on the surface of tonsils, while 13 isolates were identified in the crypts of tonsils. Bacterial isolates of *S. pyogenes* were identified by standard methods. In brief, depending on the colonial morphology and β -hemolytic activity on 5% blood agar medium. In addition to some of biochemical tests specially bacitracin discs (0.04 units, Becton, Dickinson and company spark, MD (TAXO) BD, USA) were used for identification of *S. pyogenes* (Goldman and Green, 2009). Isolated bacteria were maintained for intermediate storage in brain heart infusion broth with 20% glycerol in -20°C (Baron *et al.*, 1994).

2.2. Microtiter Plate-Biofilm Production Assay

Quantitative determination of biofilm was made using microtiter plate adhesion assay in accordance with Stepanovic *et al.* (2000); Perez *et al.* (2011); Mulla and Revdiwala (2011) with some modifications. Briefly, three to five colonies were suspended with 5 ml of Trypticase

soy broth (TSB) (HiMedia, India) and incubated for 24 hrs. Then, diluted and adjusted to 0.5 McFarland turbidity standards to reach 10^5 CFU/ml. An aliquot of 200 μL of diluted bacterial suspension with 0.25% glucose (BDH, England), was added to each well of 96-well flat-bottomed polystyrene microtitre plates (Div. Becton, Dickinson & Co. Oxnard, California, USA) and incubated for 18-24 hrs. at 37°C . Media with suspended bacteria were then removed; the plates washed carefully 3-4 times with sterile distilled water and air-dried, then stained with 200 μL of 0.9% crystal violet (Fluka, Switzerland). After washing the dye, the attached bacteria solubilized with 95% ethanol and the optical density of the adherent biofilm was determined twice with a filter of 450/630nm in ELISA reader (STAT-FAX 3200, USA). In our experiment 200 μL of TSB broth with 0.25% glucose were used as a negative control to obtain a background absorbance, which was then deducted from absorbance values obtained from the wells containing study isolates. All isolates were tested in triplicate.

The interpretation of biofilm production depended on the criteria used by Perez *et al.* (2011) depending on standard calculations laid down by Christensen *et al.* (1985) by which the study isolates were classified as the following : Non-producer, weak, moderate and strong producer; if the $\text{OD}_{450/630} \leq \text{OD}_{\text{control}}$, $\text{OD}_{\text{control}} < \text{OD}_{450/630} \leq 2\text{OD}_{\text{control}}$, $2\text{OD}_{\text{control}} < \text{OD}_{450/630} \leq 4\text{OD}_{\text{control}}$, $\text{OD}_{450/630} > 4\text{OD}_{\text{control}}$, respectively. An optical density of 0.071 was chosen as guideline to distinguish biofilm producers from those that did not form biofilm.

2.3. Bactericidal Activity of Vinegar Against Streptococcal Biofilm, *in vitro*

In this study, 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 (Komiyama *et al.*, 2010), furthermore appeared to have antimicrobial potential (Motib, 2012). In addition to that, it is considered cheap and easy to obtain. Besides to vinegar types in our designed study, distilled water (D.W.) was used as a negative control to show inability of biofilm removal in compare to vinegar against study isolates. These types of vinegar include: Date vinegar (DV) (Al-Wafi co., Iraq) with 4.5-5 % acidity; Apple vinegar (AV) (Zer, Turkey) with 5 % acidity; and Grape vinegar (GV) (Al-Wadi Al-Akhdar, Lebanon) with 5 % acidity. All of them considered as table vinegar due to limitation of their acidity not more than 5% in order not to be corrosive for living tissues when used by human.

Determination of bactericidal activity of vinegar against biofilm was performed by using modified microtiter plate method (MMTP) as described previously by Srdjan *et al.* (2000); Tabak *et al.* (2007); Guinta (2010); Sendamangalam (2010) with some modifications. This assay was made by 10 μL (1:20) of the raw material of vinegar. Each type was added to prewashed biofilm of standardized bacterial suspension adjusted to 10^5

CFU/ml in microtiter plate for each study isolate, with gentle agitation or vortexing. Then, incubated for 18 hrs. at 37°C . The contents of each well was aspirated. The wells were washed three times with 250 μL of sterile distilled water. Plate was shaken well so that non-adherent

bacteria were removed. The bacteria attached to the walls were then fixed and washed. Furthermore, stained with crystal violet and re-solubilized by the same previous way in quantitative determination of biofilm. The optical density (OD) of each well was measured at the same previous wavelengths by ELISA reader. Similarly the optical density reading interpretations was depended on the previous biofilm criteria.

2.4. Statistical analysis

Data of our designed study were analyzed using the SPSS statistical program (Statistical Package for the Social Science) version 17.0, ANOVA test and LSD test for dependable samples and multiple comparisons. Statistical significance differences were taken with P-value <0.01. All the study graphics (bar chart, Pi-chart or other diagrams), were done by using Microsoft Excel (Dugard et al., 2012).

3. Results

Twenty nine bacterial isolates of *Streptococcus pyogenes* were isolated and identified from infected tonsils that belonged to 19 patients with recurrent tonsillitis. The mean of age groups was 8.21±3.19 years. Those were 12 female (63.2%) and 7 male (36.8%). Out of 29 isolates, 16 (55.2%) bacterial isolates were isolated from tonsil surface, while 13 (44.8%) were isolated from tonsil crypts. The means and Standard deviation for all readings of ODs: 450/630nm were: 0.1842 ± 0.1164, 0.2155 ± 0.1353 for all study isolates (biofilm and non-biofilm producers) which was detected on tonsil surface and crypts, respectively, as shown in table (1)

Regarding the tonsillar biofilm production *in vitro*; out of 29 isolates, 22(75.9%) of *S. pyogenes* were biofilm producers with Mean±StDev (0.2527±0.0866) for Optical densities (ODs) at 450/630nm; whereas 7(24.1%) isolates were negative for biofilm production. Twenty-two biofilm producer isolates were distributed in to 10 (34.5%), 9 (31.0%), and 3 (10.4%) as: Strong producer (SP), Moderate producer (MP), and Weak producer (WP) respectively, as shown in figure (1). Mean±StDev of optical density readings (ODs) at 450/630nm were: 0.3241±0.0580, 0.2197± 0.0304, and 0.1140± 0.0246 for SP, MP, and WP, respectively.

Table 1. Optical density readings at 450/630nm with their means and standard deviations for all *S. pyogenes* isolates (biofilm & non-biofilm producers) isolated from tonsil surface and crypts

Tonsil surface			Tonsil crypts		
no.	Isolate code	ODs: 450/630nm	no.	Isolate code	ODs: 450/630nm
1	1s	0.235	1	1c	0.191
2	2s	0.019	2	2c	0.304
3	3s	0.104	3	3c	0.227
4	4s	0.217	4	5c	0.208
5	5s	0.176	5	6c	0.473
6	6s	0.236	6	9c	0.013
7	8s	0.030	7	11c	0.051
8	9s	0.024	8	12c	0.005
9	10s	0.096	9	20c	0.142
10	11s	0.364	10	21c	0.294
11	12s	0.206	11	22c	0.316
12	20s	0.293	12	24c	0.291
13	21s	0.281	13	25c	0.286
14	23s	0.046			
15	24s	0.285			
16	25s	0.335			
Mean±		0.1842	Mean±		0.2155
StDev		0.1164	StDev		0.1353

The Mean ± StDev of optical density readings (ODs) at 450/630nm for biofilm producer *S. pyogenes* isolated from tonsil surface and crypts were: 2357 ± 0.0830, 0.2732 ± 0.0907, respectively (P-value= 0.323).

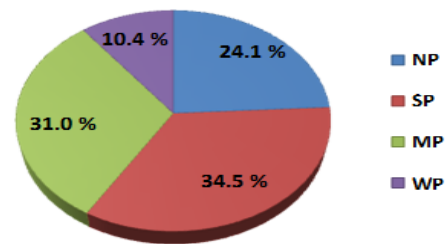


Figure 1. The distribution of *S. pyogenes* isolates according to biofilm production

According to biofilm production criteria, the twenty-two biofilm producer bacteria isolated from surface (54.5%) and crypts (45.5%) were distributed as in figure 2 below:

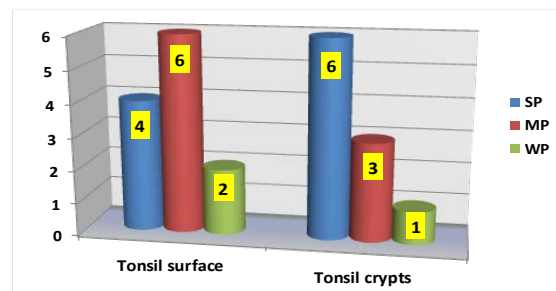


Figure 2. The distribution of biofilm producer *S. pyogenes* numbers isolated from tonsil surface and crypts

The concerned part of the study included examining the impact of three types of vinegar as anti-biofilm *in vitro*, showed that the three types of vinegar, eradicated streptococcal biofilm with excellent degrees. Table 2 demonstrated the readings of optical densities at 450/630nm with their means and standard deviations for

study isolates before and after using of three types of vinegar and distilled water

Table 2. Optical density readings at 450/630nm with their means and standard deviations before and after using the three types of vinegar and distilled water

no.	Isolate code	ODs: 450/630nm	Result of Biofilm production	ODs:450/630nm With different Types of vinegar			Negative control Distilled water
				Date vinegar	Apple vinegar	Grape vinegar	
1	1s	0.235	MP	0.044	0.038	0.046	0.233
2	1c	0.191	MP	0.027	0.052	0.029	0.191
3	2c	0.304	SP	0.061	0.070	0.058	0.303
4	3c	0.227	MP	0.016	0.069	0.040	0.227
5	3s	0.104	WP	0.002	0.004	0.009	0.101
6	4s	0.217	MP	0.018	0.038	0.017	0.215
7	5s	0.176	MP	0.019	0.003	0.011	0.175
8	5c	0.208	MP	0.021	0.001	0.019	0.205
9	6s	0.236	MP	0.023	0.014	0.039	0.233
10	6c	0.473	SP	0.069	0.159	0.183	0.472
11	10s	0.096	WP	0.001	0.013	0.028	0.092
12	11s	0.364	SP	0.033	0.051	0.162	0.364
13	12s	0.206	MP	0.015	0.029	0.029	0.205
14	20c	0.142	WP	0.009	0.002	0.011	0.141
15	20s	0.293	SP	0.051	0.069	0.053	0.292
16	21c	0.294	SP	0.009	0.065	0.068	0.294
17	21s	0.281	MP	0.036	0.070	0.044	0.280
18	22c	0.316	SP	0.011	0.070	0.063	0.315
19	24s	0.285	SP	0.030	0.065	0.037	0.283
20	24c	0.291	SP	0.043	0.065	0.046	0.288
21	25s	0.335	SP	0.021	0.053	0.066	0.333
22	25c	0.286	SP	0.031	0.036	0.027	0.283
Mean±		0.2527		0.02682	0.04709	0.04932	0.2511
StDev		0.0866		0.01811	0.03581	0.04374	0.0870

Our results showed that Date vinegar (DV) prevented biofilm formation in 22(100%) biofilm producer isolates of *S. pyogenes* throughout readings of optical densities and comparison with depending criteria. In Apple vinegar (AV), 21(95.5%) isolates were eradicated, except one. Also, in the third type; Grape vinegar (GV), there was two isolates not eradicated, while 20 (90.9%) isolates were eradicated depending on biofilm criteria. There was a high statistically significant difference before and after using of these three types of vinegar (P -value=0.000<0.01). Otherwise, the negative control (Distilled water) showed no biofilm eradication for all study isolates. There was no significant difference (P -value was high 0.931>0.01). On the other hand, there was no statistically significant difference among the three types of vinegar according to P -values, as shown in the table 3.

Regarding biofilm categories, the biofilm eradication ability by the three types of vinegar and distilled water was different among biofilm categories (Strong, moderate, and weak) and distributed as shown in figure 3.

vinegar and distilled water.

Table 3. Multiple comparisons by LSD test, among original readings and the three types of vinegar with control, with Standard errors and P -values

		Mean difference	Std. Error	P -value
Original	Grape	0.2034091	0.0183829	0.000
	Apple	0.2056364	0.0183829	0.000
	Date	0.2259091	0.0183829	0.000
	Control	0.0015909	0.0183829	0.931
Grape	Original	-0.2034091	0.0183829	0.000
	Apple	0.0022273	0.0183829	0.904
	Date	0.0225000	0.0183829	0.224
	Control	-0.2018182	0.0183829	0.000
Apple	Original	-0.2056364	0.0183829	0.000
	Grape	-0.0022273	0.0183829	0.904
	Date	0.0202727	0.0183829	0.273
	Control	-0.2040455	0.0183829	0.000
Date	Original	-0.2259091	0.0183829	0.000
	Grape	-0.0225000	0.0183829	0.224
	Apple	-0.0202727	0.0183829	0.273
	Control	-0.2243182	0.0183829	0.000
Control	Original	-0.0015909	0.0183829	0.931
	Grape	0.2018182	0.0183829	0.000
	Apple	0.2040455	0.0183829	0.000
	Date	0.2243182	0.0183829	0.000

Regarding biofilm categories, the biofilm eradication ability by the three types of vinegar and distilled water was different among biofilm categories (Strong, moderate, and weak) and distributed as shown in figure 3.

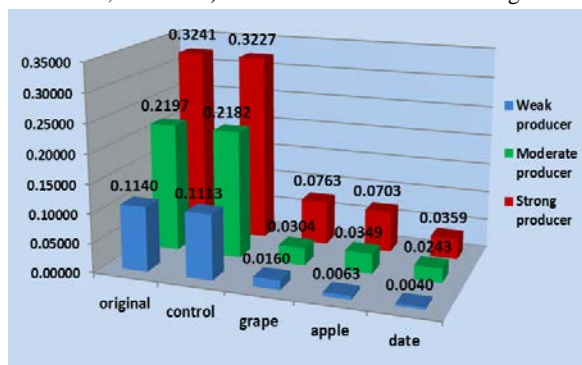


Figure 3. Distribution the biofilm eradication ability means by the three types of vinegar in compared with control and original readings according to biofilm categories

4. Discussion

Recurrent tonsillitis is a common upper aerodigestive tract infection with local and systemic symptoms, such as sore throat, odynophagia, fever, and myalgia. These symptoms can be improved by antibiotic therapy but they tend to relapse (Woo *et al.*, 2012). The failure of antibiotic treatment in the eradication of susceptible organisms has recently induced microbiologists to hypothesize the presence of bacteria ordered in communities, attached to surfaces, identified as "biofilms" (Bjarnsholt *et al.*, 2011). It has been investigated that *S. pyogenes* was able to form biofilm as an alternative method to escape antibiotic treatment and host defenses leading to recurrent infections (Baldassari *et al.*, 2006). A biofilm is a colony of single or multiple bacterial species embedded in a self-producing polymeric matrix, this matrix guarantees better survival and protection from macrophage action, antibiotics, temperature and pH fluctuations (Galli *et al.*, 2007; Bjarnsholt *et al.*, 2011; Post *et al.*, 2004). One of the best known biofilm-specific properties is antibiotic resistance, which can be up to 1000-fold greater than that seen with planktonic cells (Ogawa *et al.*, 2011). So biofilm-associated infections are difficult to eradicate by routine antibiotic doses in compare with planktonic form of bacteria. They need thousands times of doses used for non-biofilm infections (Al-Ani, 2008). As in biofilm formed by *Streptococcus pyogenes* in pharyngitis patients, which evading high antibiotic concentrations greater than 10-folds minimum inhibitory concentration (MIC) for planktonic *S. pyogenes* (Ogawa *et al.*, 2011).

Focus on treating established biofilms may need to shift from antibiotic to non-antibiotic therapy to effectively eradicate established biofilms. Among these modalities, were physical (laser-produced pressure waves, pulsed ultrasound) and chemical methods such as using of surfactants, which may soon replace traditional surgical techniques (Smith *et al.*, 2011). Researchers have also demonstrated ENT biofilm (ear, nose, and throat) prevention using many various techniques that have been shown to disrupt established biofilms, including the use of probiotics and surfactants (Free *et al.*, 2001; Free *et al.*, 2003; Rodriguez *et al.*, 2004). To the best of our knowledge, this is the first study that has evaluated effect of vinegar on biofilm formed by *S. pyogenes* isolated from recurrent tonsillitis patients.

In this study, with regard to tonsillar biofilm production; 22(75.9%) study isolates of *S. pyogenes* produced biofilm on tonsils, whereas 7 (24.1%) isolates were non-biofilm producer. Ability and inability of biofilm formation by *S. pyogenes* may be due to variance in their strains within group A-streptococci. Different data indicated that there is a necessary protein component for initial adherence and/or aggregation leading to biofilm maturation, made them hypothesized that high levels of streptococcal cysteine protease (SpeB) may be responsible for the biofilm-deficient phenotype, suggesting that SpeB degrades GAS proteins needed for establishment of the biofilm. It is worth nothing that the activation and inactivation of streptococcal regulator of virulence gene (Srv) controlled SpeB production in the

strain (Doern *et al.*, 2009). Also this result may explain biofilm-related recurrence in tonsillitis patients and presumed the association between frequent tonsillitis and biofilm presence. This result was agreed with Woo *et al.* (2012) that showed presence rate and grade of biofilms were significantly higher in recurrent tonsillitis patients than the control group. Furthermore, other studies reported similar results as mentioned by Chole and Faddis (2003) who identified biofilms in 73.3% of their tonsillitis specimens using light and transmission electron microscopy, whereas Kania *et al.* (2007) reported that biofilms were present in most 70.8% of patients with tonsillitis.

Tonsillar biofilm producing isolates of *S. pyogenes* were distributed into (54.5%) on the surface, and (45.5%) in the crypts; with *P*-value 0.323 as in figure (2), and the means of ODs were 0.1842 and 0.2155 for all *S. pyogenes* (biofilm and non-biofilm producers) isolated from tonsil surface and crypts, table (1). There was no statistically significant difference between biofilm producer *S. pyogenes* isolated from tonsil surface and crypts but there is a slight preference to the tonsil crypts according to the mean of optical density readings for biofilm production. The tonsil moves dynamically during swallowing and comes into direct contact with food, which makes tonsillar surface vulnerable to biofilm development. In particular, the tonsillar crypts were a preferred site for bacterial attachment and biofilm formation because it is formed by invagination of the surface epithelium and tends to trap foreign material (Balogh and Pantanowitz, 2007; Woo *et al.*, 2012). As in Diaz *et al.* (2011) who reported presence of bacterial biofilms in the crypts of 77.28% of the studied patients, he strongly suggested occurrence of a chronic inflammatory underlying pattern with poor immune response.

In our study, biofilm formation ability was evaluated among *S. pyogenes* isolates using standard Microtiter plate assay. These isolates were classified in our study into three groups which are: strong, moderate and weak producer; 10(34.5%), 9(31.0%), and 3(10.4%), respectively, according to previous depending biofilm criteria. Most of isolates were strong and moderate depending on adhesion ability to microtiter plate wells, figure (1). This result is different from biofilm production grading that was reported by Torretta *et al.* (2012) who investigated that (82.4%) were weak producers, and (17.6%) were moderate, but did not report any strong producer isolate among nasopharyngeal biofilm-producing pathogens. Classification of bacteria as moderate, strong or weak biofilm producers regulated by diverse factors, including the growth medium, but still poorly understood (Stepanovic *et al.*, 2004). Therefore, they speculate about the reasons for different influence of nutritional content of the growth medium on biofilm formation. One possible explanation for the different response of bacteria to environmental conditions could be the results of mutations in genes that control biofilm formation (Romling *et al.*, 1998). While, other results of biofilm assay (tissue culture plate) showed that moderate producer isolates constituted high percentage compared with strong or weak producers. These observations

suggested a strong reliance between growth condition and biofilm formation and using of various sugar supplementations is essential for biofilm formation (Mathur *et al.*, 2006).

In the field of studying the effect of vinegar as anti-biofilm, our study presented eradication of Streptococcal biofilm using 4.5-5% date vinegar, 5% apple vinegar and 5% grape vinegar solutions and compared for their removing abilities. Our results explained that all types of used vinegar presented biofilm eradication ability remarkably, *in vitro*. There was no obvious difference among their prevention ability according to *P*-values as in table (3), also through means of optical density readings, figure (3). The main constituents of vinegar represented by acids specially acetic acid (Jafari *et al.*, 2012), so vinegar used as anti-biofilm in our designed study, due to antibacterial effect of acetic acid that treats infections caused by bacteria or fungus (Jaber *et al.*, 2011). Furthermore, vinegar used as a surfactant against *S. pyogenes* (Motib, 2012). Acetic acid-based solutions (vinegar) used for the disinfection and treatment of oral inflammatory processes (as a mouthwash) and as an anti-septic for sores which has been reported in previous studies (Utyama, 2003).

Although there was no obvious 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. Al-daihan and Bhat (2012) showed that Date extracts were effective inhibitors for bacterial growth. This may be due to presences of carbohydrates, alkaloids, steroids, saponins, flavonoids and tannins in this fruit according to the chemical analysis. They recorded mean of inhibition zones as 18 mm \pm 0.08 for these extracts against *S. pyogenes*. Also, apple vinegar showed amazing biofilm eradication ability however less than date vinegar. This antimicrobial effect may be due to high content of phenolic compounds in apple as proved by Alberto *et al.* (2006) who demonstrated direct relationship between phenolic content of apple extract and the antimicrobial effect on human pathogens. Sendamangalam, (2012) explained that polyphenols reduced streptococcal biofilm formation through inhibition of exopolymers-producing enzymes, exopolymers are a major component in biofilm formation. He observed that biofilm reduction ability was ranged between 62.8 - 75.7% for used polyphenols. Grape vinegar had the least effect in compared with other two types, however it prevented biofilm remarkably. This may be due to difference in concentrations of grape components, as explained by Smullen *et al.* (2006) who concluded that grape extract containing high levels of polyphenols inhibited streptococcal biofilm. It has been found that grape extracts include polyphenols, flavanol, catechins and anthocyanins, which exhibited different antimicrobial activity according to their concentrations.

The study concluded that there was obvious correlation between biofilm production by *S. pyogenes* and recurrent tonsillitis, while there was no significant difference in biofilm production between *S. pyogenes* isolated from tonsil surface and crypts. Furthermore, our study concluded that the three types of vinegar eradicated streptococcal biofilm remarkably, *in vitro*, although date

vinegar was the best for eradication of streptococcal biofilm.

On the other hand, our results showed a huge difference in the biofilm eradication ability before and after using of vinegar types, but there was no effect for distilled water (negative control), figure (3). These results correspond with Jafari *et al.* (2011) who found that 99-100% of biofilm formed on the denture surface was removed by using two concentrations of white vinegar (5,10)% and 1% sodium hypochlorite.

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References

- Al-Ani NFI. 2008. Microbiological Aspects in Biofilm Produced by Some Uropathogens Isolated from Patients with Indwelling Bladder Catheters. MSc dissertation, University of Anbar, Ramadi, Iraq.
- Alberto MR, Canavosio MAR and de Nadra MC. 2006. Antimicrobial effect of polyphenols from apple skins on human bacterial pathogens. *Electron J Biotechnol*, special issue, **9**: 205-209.
- Al-Daihan S and Bhat RS. 2012. Antibacterial activities of extracts of leaf, fruit, seed and bark of *Phoenix dactylifera*. *Afr. J. Biotechnol.*, **11**: 10021-10025.
- Al-Marzrou KA and Al-Khattaf AS. 2008. Adherent biofilms in adenotonsillar diseases in children. *Arch Otolaryngol Head Neck Surg*, **134**:20-23.
- Baldassarri L, Creti R, Recchia S, Imperi M, Facinelli B, Giovanetti E, Pataracchia M, Alfarone G and Orefici G. 2006. Therapeutic failures of antibiotics used to treat macrolide-susceptible *Streptococcus pyogenes* infections may be due to biofilm formation. *J Clin Microbiol*, **44**: 2721-2727.
- Balogh K and Pantanowitz L. 2007. Mouth, nose, and paranasal sinuses. In Mills SE (Eds.), **Histology for Pathologists**. Philadelphia, PA: Lippincott Williams & Wilkins, pp: 403-430.
- Baron EJ, Peterson LR and Finegold SM. 1994. Bailey and Scott's. Diagnostic Microbiology. 9th ed. Toronto: C.V. Mosby Company.
- Bjarnsholt T, Moser C, Jensen PO and Hoiby N. 2011. **Biofilm Infections**. Springer, New York; London.
- Christensen GD, Simpson WA, Younger JA, Baddour LM, Barrett FF, and Melton DM. 1985. Adherence of coagulase negative *Staphylococci* to plastic tissue cultures: a quantitative model for the adherence of *Staphylococci* to medical devices. *J. Clin. Microbiol*, **22**:996-1006.
- Chole RA and Faddis BT. 2003. Anatomical evidence of microbial biofilms in tonsillar tissues: a possible mechanism to explain chronicity. *Arch Otolaryngol Head Neck Surg*. **129**: 634-636.
- Diaz RR, Picciafuoco S, Paraje MG, Villegas NA, Miranda JA, Albesa I, Cremonezzi D, Comisso R and Paglini-Oliva P. 2011. Relevance of biofilms in pediatric tonsillar disease. *Eur J Clin Microbiol Infect Dis*, **30**:1503-1509.
- Doern CD, Roberts AL, Hong W, Nelson J, Lukomski S, Swords WE and Reid SD. 2009. Biofilm formation by group A *Streptococcus*: a role for the streptococcal regulator of virulence (Srv) and streptococcal cysteine protease (SpeB). *Microbiology*, **155**: 46-52.

- Dugard P, Todman J and Staines H. 2012. **Approaching Multivariate Analysis, A Practical Introduction**. 2nd Ed. Routledge, Taylor and Francis group.
- Estrela C, Holland R, Bernabe PF, de Souza V and Estrela CR. 2004. Antimicrobial potential of medicaments used in healing process in dogs' teeth with apical periodontitis. *Braz Dent J*, **15**: 181-185.
- Free RH, Busscher HJ, Elving GJ, van der Mei HC, van Weissenbruch R and Albers FW. 2001. Biofilm formation on voice prostheses: *in vitro* influence of probiotics. *Ann Otol Rhinol Laryngol*, **110**: 946-951.
- Free RH, van der Mei HC, Elving GJ, Van Weissenbruch R, Albers FW and Busscher HJ. 2003. Influence of the Provox flush, blowing, and imitated coughing on voice prosthetic biofilms *in vitro*. *Acta Otolaryngol*, **123**: 547-551.
- Fux CA, Costerton JW, Stewart PS, and Stoodley P. 2005. Survival strategies of infectious biofilms. *Trends Microbiol*, **13**: 34-40.
- Galli J, Calo L, Ardito F, Imperiali M, Bassotti E, Fadda G and Paludetti G. 2007. Biofilm formation by *Haemophilus influenzae* isolated from adeno-tonsil tissue samples, and its role in recurrent adenotonsillitis. *ACTA oto rhi nol aryngologica italic*, **27**: 134-138.
- Goldman E and Green LH. 2009. **Practical Handbook of Microbiology**. 2nd Ed. Taylor & Francis Group, NW.
- Guinta AR. 2010. New approaches for controlling biofilm formation. MSc dissertation, University of New Jersey, New Brunswick, New Jersey.
- Hannig C, Sorg J, Spitzmuller B, Hannig M and Al-Ahmad A. 2009. Polyphenolic beverages reduce initial bacterial adherence to enamel *in situ*. *J of Dent*, **37**: 560-566.
- Jabir HB, Abbas FN and Khalaf RM. 2011. *In vitro* assessment of antifungal potential of apple cider vinegar and acetic acid versus fluconazole in clinical isolates of Oromycosis. *Thi-Qar Med J*, **5**: 126-133.
- Jafari AA, Tafti AF, Lotfi-Kamran MH, Zahraei A and Kazemi A. 2012. Vinegar as a removing agent of *Candida albicans* from acrylic resin plates. *Jundishapur J Microbiol*, **5**: 388-392.
- Kania RE, Lamers GE, Vonk MJ, Huy PT, Hiemstra PS, Bloembergen GV and Grote JJ. 2007. Demonstration of bacterial cells and glycocalyx in biofilms on human tonsils. *Arch Otolaryngol Head Neck Surg*, **133**: 115-121.
- Komiyama EY, Back-Brito GN, Balducci I and Koga-Ito CY. 2010. Evaluation of alternative methods for the disinfection of toothbrushes. *Braz Oral Res*, **24**: 28-33.
- Mathur T, Singhal S, Khan S, Upadhyay D J, Fatma T and Rattan A. 2006. Detection of biofilm formation among the clinical isolates of *Staphylococcus*: An evaluation of three different screening methods. *Ind J Med Microbiol*, **24**: 25-29.
- Motib AS. 2012. The effect of vinegar solution on the bacteria that cause impetigo. *Diyala J pure sci*, **8**: 60-67.
- Mulla SA and Revdiwala S. 2011. Assessment of biofilm formation in device-associated clinical bacterial isolates in a tertiary level hospital. *Indian J Pathol Microbiol*, **54**: 561-564.
- Nascimento MS, Silva N, Catanzi MP and Silva KC. 2003. Effects of different disinfection treatments on the natural microbiota of lettuce. *J Food Prot*, **66**: 1697-1700.
- Ogawa T, Terao Y, Okuni H, Ninomiya K, Sakata H, Ikebe K, Maeda Y and Kawabata S. 2011. Biofilm formation or internalization into epithelial cells enable *Streptococcus pyogenes* to evade antibiotic eradication in patients with pharyngitis. *Microbiol Pathogenesis*, **51**: 58-68.
- Perez LRR, Costa MCN, Freitas ALP and Barth AL. 2011. Evaluation of biofilm production by *Pseudomonas aeruginosa* isolates recovered from cystic fibrosis and non-cystic fibrosis patients. *Braz J microbiol*, **42**: 476-479.
- Perloff JR and Palmer JN. 2005. Evidence of bacterial biofilms in a rabbit model of sinusitis. *Am J Rhinol*, **19**: 1-6.
- Post JC, Stoodley P, Hall-Stoodley L and Ehrlich GD. 2004. The role of biofilms in otolaryngologic infections. *Curr Opin Otolaryngol Head Neck Surg*, **12**: 185-190.
- Rodriguez L, van der Mei HC, Teixeira J and Oliveira R. 2004. Influence of biosurfactants from probiotic bacteria on formation of biofilms on voice prostheses. *Appl Environ Microbiol*, **70**: 4408-4410.
- Romling U, Sierralta WD, Eriksson K and Normark S. 1998. Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the agfD promoter. *Molecular Microbiology*, **28**: 249-264.
- Sendamangalam V. 2010. Antibiofouling Effect of Polyphenols on *Streptococcus* Biofilms. MSc dissertation, The University of Toledo, Ohio, United States.
- Smith A, Buchinsky FJ and Post JC. 2011. Eradicating chronic ear, nose, and throat infections: a systematically conducted literature review of advances in biofilm treatment. *Otolaryngol Head Neck Surg*, **144**: 338-347.
- Smullen J, Koutsou GA, Foster HA, Zumbe A and Storey DM. 2007. The antibacterial activity of plant extracts containing polyphenols against *Streptococcus mutans*. *Caries Res*, **41**: 342-349.
- Srdjan S, Dragana V, Ivana D, Brainslava S and Milena SV. 2000. A modified microtiter plate test for quantification of staphylococcal biofilm formation. *J Microbiol Meth*, **40**: 175-179.
- Stepanovic S, Cirkovic I, Ranin L and Svabic-Vlahovic M. 2004. Biofilm formation by *Salmonella spp.* and *Listeria monocytogenes* on plastic surface. *Lett in Appl Microbiol*, **38**: 428-432.
- Stepanovic S, Vukovic D, Dakic I, Savic B and Svabic-Vlahovic, M. 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods*, **40**: 175-179.
- Suga H. and Igarashi J. 2012. Amide compound or salt thereof, and biofilm inhibitor, biofilm remover and disinfectant containing the same. University of Tokyo, Otsuka Chemical Co. Ltd. (Osaka-shi, Osaka, JP). *Online Patent US*. Publication numbers: US20120296094A1- US20090068120- US8258307B2.
- Tabak, M, Scher K, Hartog E, Romling U, Matthews KR, Chikindas ML and Yaron S. 2007. Effect of triclosan on *Salmonella typhimurium* at different growth stages and in biofilms. *FEMS microbiol letters*, **267**: 200-206.
- Torretta S, Marchisio P, Drago L, Baggi E, De Vecchi E, Garavello W, Nazzari E, Pignataro L and Esposito S. 2012. Nasopharyngeal biofilm-producing otopathogens in children with nonsevere recurrent acute otitis media. *Otolaryngol Head Neck Surg*, **146**: 1-6.
- Utyama IKA. 2003. Evaluation of antimicrobial activity and cytotoxicity *in vitro* of vinegar, acetic: perspective in the treatment of wounds. MSc Dissertation, University of Sao Paulo, Ribeirao Preto.
- Wang EW, Jung JY, Pashia ME, Nason R, Scholnick S and Chole RA. 2005. Otopathogenic *Pseudomonas aeruginosa* strains as competent biofilm formers. *Arch Otol Head Neck Surg*, **131**: 983-989.
- Woo JH, Kim ST, Kang ILG, Lee JH, Cha HE, and Kim DY. 2012. Comparison of tonsillar biofilms between patients with recurrent tonsillitis and a control group. *Acta Oto-Laryngologica*, **132**: 1115-1120.

