Isolation and Characterization of *Streptococcus mutans* as Causative Agent of Dental Caries in Gaza Strip and their Antibacterial Susceptibility Pattern

Emad Abou Elkhair[a*], Abdallah Bashir[a], Mohammed Miqdad[b], Ibrahim Abu Ismail[c]

[a] Department of Biology, Faculty of Science, Al-Azhar University, Gaza, Palestine.
[b] Department of removable and fixed prosthodontics, Faculty of Dentistry, Al-Azhar University, Gaza, Palestine.
[c] Al-Amal medical laboratory

*Corresponding author: eabouelkhair@gmail.com, e.khair@alazhar.edu.ps

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Abstract:

Background: Dental caries is a well-known major oral health problem in most countries. The disease's multifactorial etiology includes multiple bacterial species; *S. mutans* is the main pathogen associated with the disease. *S. mutans* is a gram-positive facultative anaerobe commonly found in the oral cavity.

Objective: To isolate and characterize *S. mutans* as potential causative bacteria of dental carries and to study its sensitivity to antibacterial agents and antibacterial mouth rinses from patients visiting dental clinics in the Gaza strip.

Materials and methods: Open-label experimental study was performed on 300 patients and 300 control visiting dental

clinics in Gaza strip. All suspected *S. mutans* isolates were identified biochemically, tested against ten commonly used antibiotics by disc diffusion method, studied *in vitro* for efficacy of nine essential oils, five toothpaste and four mouth rinses.

Results: Among 300 patients tested in this study, 95 showed a positive result for the presence of *S. mutans* in their saliva and dental caries. The most positive cases for *S. mutans* in dental caries and saliva were detected in the age group 20-35 years and nonsmoker patients. Colgate showed the best antimicrobial activity among five different toothpastes**.** Antibiotic sensitivity test indicated that *S. mutans* was most susceptible to vancomycin (100%), doxycycline (84.2%), tetracycline (83.2%), ciprofloxacin (81.1%) and amoxyclav (75.8%). Gargarol was found to be the most effective mouth rinse, while clove bud, lemon grass and Tea tree were the most effective oils against *S. mutans***.**

Conclusion: *S. mutans* isolates were moderately resistant to antibiotics. Use of plant extracts (essential oils) may be recommended as a supportive or alternative option to conventional formulations.

Key words: *Streptococcus mutans*, Dental caries, Gaza strip, Essential oils, Tooth paste, Mouth rinses.

Background

Dental caries is a worldwide prevalent costly oral chronic disease, which is also known as dental decay or cavities and characterized as a destruction of the outer layer of teeth **(Bagramian et al., 2009)**. It affects humans throughout their life and is associated not only with pain in the oral cavity but can also cause endocarditis **(Banas 2004 ; Strużycka 2014)**.

Many factors can be associated with promoting the development of dental caries such as cariogenic bacteria**,** dietary sugar **(Touger-decker and Van Loveren 2003)**, and exposure to cariogenic effects **(Sayegh et al., 2002)** as well as diseases affecting the teeth leaving an individual at high risk to dental caries **(Ferraz et al., 2012).**

The most virulent of oral bacteria are *Streptococcus mutans*, which have been found to be the initiator of most dental caries (**Van et al., 2000**; **Tanzer et al., 2001**; **Li et al., 2014, and Ahrari et al., 2015**).

S. mutans are gram-positive facultative anaerobic cocci shaped bacteria, commonly found in the human oral cavity (**Loesche 1996**). The pathogenicity of *S. mutans* depends on their ability to form biofilms on solid surfaces such as teeth (**Ahn et al., 2008 and Krzyściak et al., 2014**). The pathogenic potential of *S. mutans* depend also on their ability to metabolize a wide range of sugars, to form biofilm and to create an acidic environment (**Argimón and Caufield 2011**). Also, the ability to survive this acidic milieu by production of branched amino acids like valines, leucines and isoleucines **(Santiago et al., 2012).**

Not only streptococci bacteria colonize the oral cavity, the mouth supports the growth of a wide variety of other microorganisms. These include diverse bacterial species, yeasts, viruses and, on occasions, protozoa **(Marsh 1999).**

In this work, we aimed to isolate and identify *S. mutans* from patients suffering of dental caries using conventional methods. We also studied the sensitivity of isolated *S. mutans* to different antibiotics, medicinal plants essential oils and mouth rinses.

Materials and Methods

Open label experimental study was performed on 300 patients visiting dental clinics in Gaza strip. The bacterial isolates (*S. mutans*) were conventionally identified and tested for antimicrobial susceptibility using disk diffusion method. Screening for the antibacterial activity of different medicinal plants essential oils, toothpaste and mouthwashes were also performed.

Inclusion criteria for selection:

- Age more than 20 years.

The Exclusion criteria for selection:

- Marked intra oral soft tissue pathology.
- Patients with history of taking antibiotics three months prior or during the course of study.
- Medically compromised patients.

Chemicals and Reagents

All chemicals and reagents in this study were of analytical grade. Bacteriological media were purchased from Hi Media Company (India) and Sigma Aldrich Company (Germany). Other disposable materials like Petri dishes, cotton swabs and plastic loops were purchased from local distributors in Gaza city.

Commercial Kits, Chemicals, media and Reagents

Chemicals and kits			
Absolute ethanol		Anaerobic gas bags	
Brain heart- infusion agar		Bile Esculin Agar	
Gram stain kit		Brain heart infusion broth	
Mitis salivarius agar		Mannitol salt agar	
Antibiotics	**Abbreviation and potency**	**Antibiotics**	**Abbreviation and potency**
Amoxicillin	AMX (30 µg)	Amoxicillin-clavulanic acid	AMC (30 µg)
Ampicillin	AMP (2 µg)	Clindamycin	CD (2 µg)
Ciprofloxacin	CIP (5 µg)	Doxycycline	DO (30 µg)
Erythromycin	E (15 µg)	Oxacillin	OX (1 µg)
Tetracycline	TE (30 µg)	Vancomycin	VA (5 µg)
Mouth rinses			
Gargarol		Garosept	
Iodocare		Septoral	
Tooth paste			
B-white		Colgate	
Paradontax		Sensodyne	
Signal			

List of essential oils used in the antimicrobial assay

- **List of essential oils used in the antimicrobial assay**

Local Arabic name	Commercial name	Scientific name	Family	Sources
النعناع الفلفلي	Peppermint oil	*Mentha piperta* Eugenia	Labiateae	Germany
القرنفل	Clove bud oil	Caryophyllata part bud	Myrtaceae	Germany
حشيشة الليمون	Lemon grass oil	*Cymbopogon citratus*	Poaceae	Germany
الزعتر	Thyme oil	*Thymus sature* iodes coss	Labiateae	Germany
الزنجبيل	Ginger oil	*Zingiber officinale*	Zingiberaceae	Germany
مريمية	Sage	*Salvia officinalis*	Lamiaceae	Germany
ملالوكا	Tea tree	*Melaleuca alternifolia*	Myrtaceae	Germany
قرفة	Cinnamon leaf	*Cinnamomum zeylanicum*	Lauraceae	Germany
إكليل الجبل	Rosemary	*Rosmarinus officinalis*	Lamiaceae	Germany

Enumeration of *S. mutans* in saliva

Materials:

Four sterile test tubes that contain 0.9ml of sterile normal saline

0.1ml saliva sample from patients and controls

Procedure:

100µl of saliva sample were mixed in 0.9ml sterile saline. Serial dilutions were prepared as followed: 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. 0.1 ml of each dilution was spread on 2 different agar media: Brain Heart Infusion (BHI) agar and Mannitol Salt Agar (MSA) and incubated at 37 °C in anaerobic jar containing carbon dioxide bag for 48 hours. Developed colony forming units were counted on each agar media **(Gallez et al., 2000 and Ogawa et al., 2012).**

Determination of MIC and MBC for essential oils, toothpaste and mouthwashes:

The MIC (Minimal Inhibitory Concentration) of a bacterium to a certain antimicrobial agent gives a quantitative estimate of the bacterial susceptibility to this antimicrobial agent.

MIC is defined as the lowest concentration of antimicrobial agent required to inhibit growth of the organism. In principle, agar plates, tubes or microtitre trays with two-fold dilutions of antibiotics are inoculated with a standardized inoculum of the bacteria and incubated under standardized conditions following CLSI guidelines. The next day, the MIC is recorded as the lowest concentration of antimicrobial agent with no visible growth.

MIC-determination performed as agar dilution is regarded as the gold standard for susceptibility testing **(CLSI 2011).**

Tube Dilution Method:

It's used to obtain Minimum Inhibitory Concentration (MIC). The microbial inoculum was standardized at 0.5McFarland, 100µl of bacteria were aseptically introduced of *S. mutans* were added to each tubes that contains 5ml of sterile Brain heart infusion broth with different concentration of 9 essential oils, 5 tooth pastes and 4 mouth washes. After the tube of suspensions were done, all tubes were incubated at 37^{o}C for 24-48 hours. Assess the result of incubation by looking at the tube blurriness then MIC can be determined. Three randomly selected isolates of *S. mutans* were chosen for the purpose of determining the MIC **(Nwaokorie et al., 2010).**

To obtain Minimum Bactericidal Concentration (MBC), the suspension of each tubes is streaked on the Blood agar plate as many as 0.1 ml. Then it has incubated in anaerobe environment at 37°C for 24-48 hours. Colony forming unit can be seen after the incubation **(CLSI 2011).**

Statistical analysis

Data generated from this work were tabulated, entered into Microsoft excel sheets and uploaded to SPSS (Statistical Package for

Social Sciences version (22) software. Frequencies, cross tabulation and appropriate statistical tests as Chi-square test, fisher exact test and others were performed.

RESULTS

Antimicrobial susceptibility

As shown in Figure 1, vancomycin, doxycycline, tetracycline, ciprofloxacin and amoxyclav were the most effective antibiotic respectively.

Among 300 patients tested in this study, 95 showed a positive result for the presence of *S. mutans* in their saliva and dental caries. The most positive cases for *S. mutans* in dental caries and saliva were detected in the age group 20-35 years and nonsmoker patients.

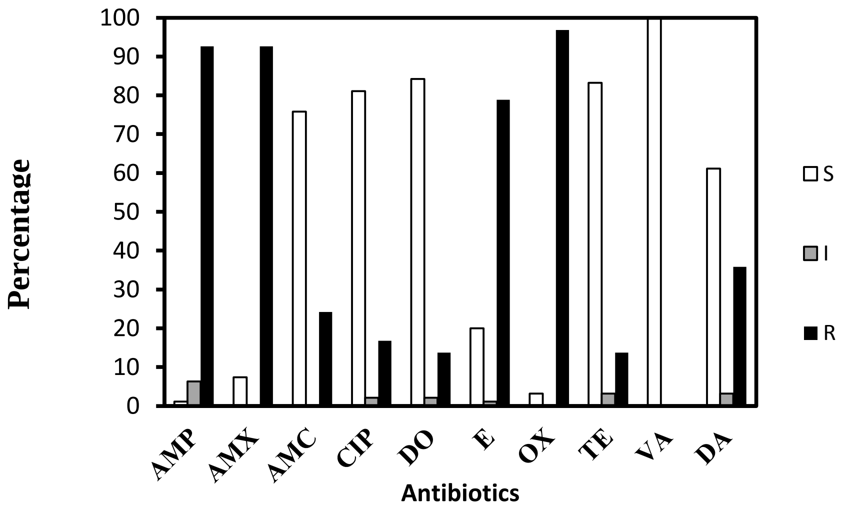


Figure (1): Antibiotics susceptibility for *S. mutans*

Antimicrobial activity of essential oils against three isolates of *S. mutans*

Nine essential oils were evaluated for their antimicrobial activity against 3 selected isolates of *S .mutans* (X, Y and Z). Clove bud oil has the highest antibacterial effect against the three isolates while ginger oil had the lowest antibacterial effect. **Figure (2)** illustrate an example of the inhibitory effect of clove bud oil against *S. mutans*.

Figure (3) shows the different types of oils and their measured inhibitory effect (in mm) on *S.*

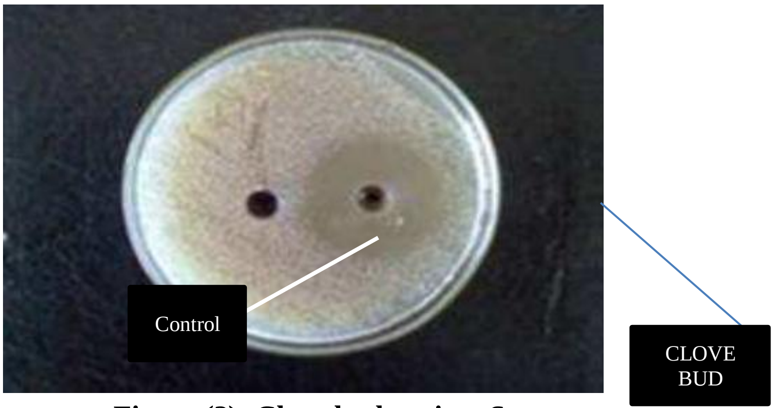


Figure (2): Clove bud against *S. mutans*

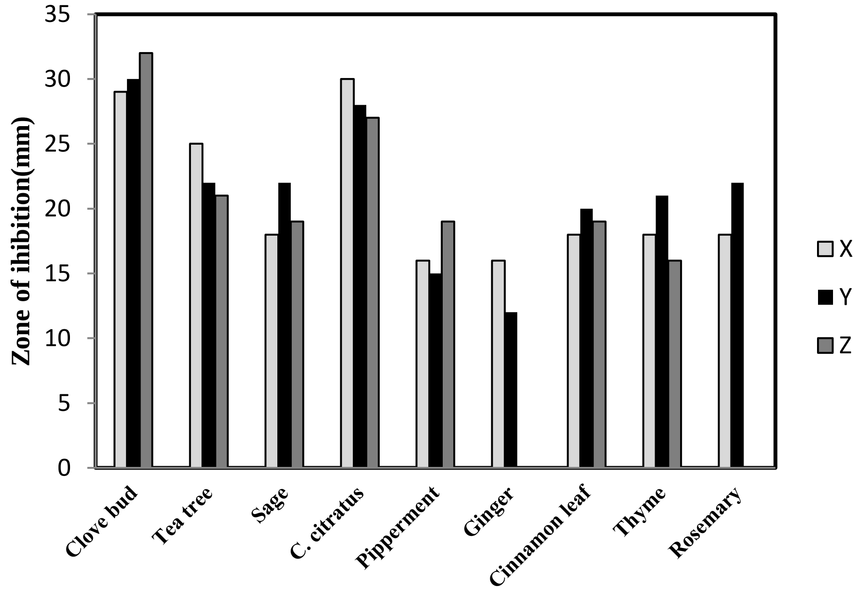
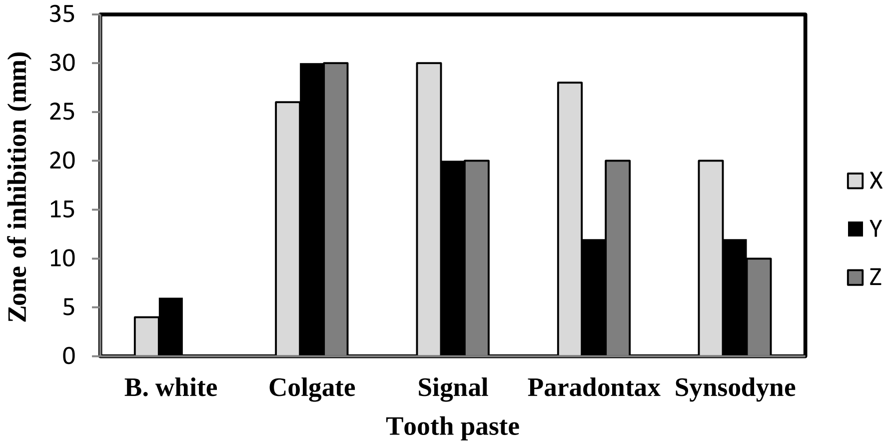


Figure (3): Antimicrobial activity of essential oils against *S. mutans*

Efficacy of toothpaste against three isolates of *S .mutans*

The antimicrobial activity of different toothpaste against *S. mutans* was examined. As shown in Figure (4), Colgate was the most effective against the three isolates of *S. mutans* while B. white was the least effective.



Figure(4) :Inhibitory effect of some tooth paste against *S. mutans*

Antimicrobial activity of mouth wash against *S. mutans*

This section deals with the results of inhibitory growth effect of some mouthwashes against *S. mutans*. As shown in Figure (5), Gargarol was the most effective against the three isolates of *S. mutans* while Garosept was the least effective**.**

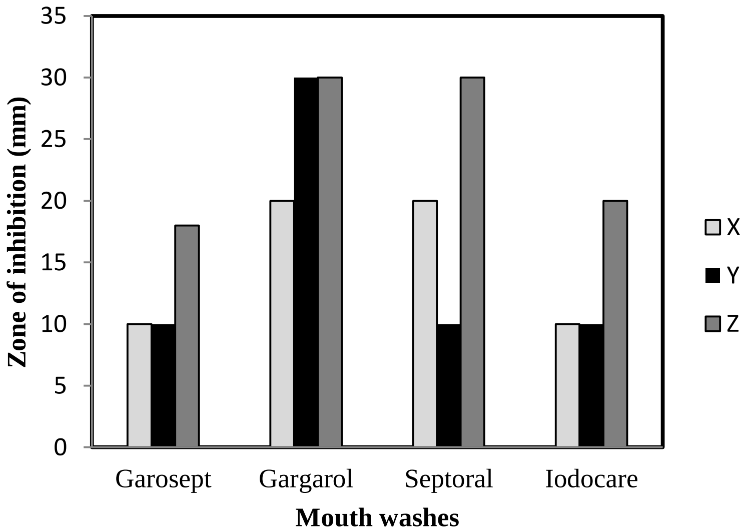


Figure (5): Mouthwashes and their effects on *S. mutans*

MIC and MBC determination

MIC and MBC of nine essential oils (µl/ml)

Figure 6 shows the essential oils which exhibited antibacterial activity, were further determined for MIC and MBC values. The essential oils demonstrated MIC values ranging from 100 to 1500 µl/ml, and their MBC values was recorded less than 100 to 500 µl/ml. For Rosemary oil the result showed that MIC was 1500 µl/ml which mean MBC will be higher than1500 µl/ml so it was non effective for using as antibacterial agent. *C. citratus*, Pipperment, Cinamon leaf and Thyme showed bacteriocidal effect against *S. mutans* where they have almost same concentration of MIC and MBC as showed in Fig. 6

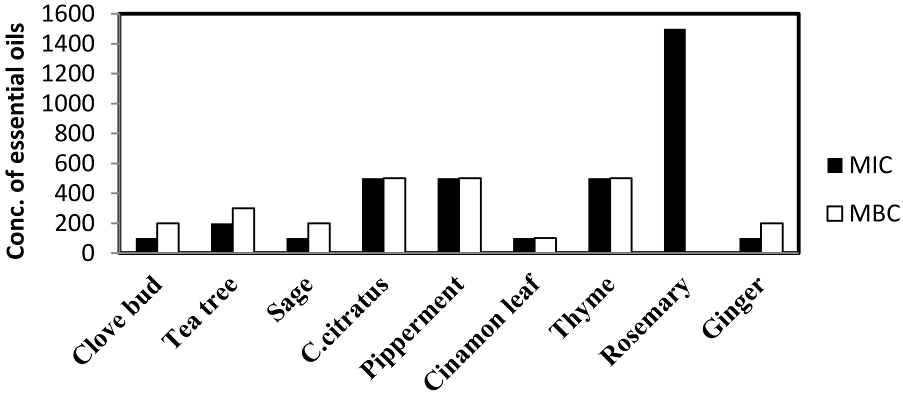


Figure (6): MIC and MBC of essential oils against *S. muta*

Discussion

Antibacterial agents and *S. mutans*

Our results indicate that *S. mutans* have been found to be most susceptible to vancomycin (100%), doxycycline (84.2%), tetracycline (83.2%), ciprofloxacin (81.1%) and amoxyclav (75.8%). Dentists commonly prescribe most of the antibiotics employed in this study. A study conducted by Jubair 2015 indicated that most *S. mutans* isolates were resistance to amoxicillin and erythromycin with 90% and 78% from all isolates **(Jubair 2015)**. In addition, this result was agreed with **(Muna, 2011)** in which found rate resistance for each amoxicillin and erythromycin was 87.5%. **(El Sherbiny 2014)** found that rate resistance to tetracycline was 35% from all *S. mutans* isolates. This difference may be due to the influence of many factors like the age of the patient, the season of sample collection and the frequently and uncontrolled use of antibiotics in Gaza strip.

Essential oils and its effect on *S. mutans*

Agar diffusion tests are often used as qualitative methods to determine whether a bacterium is resistant, intermediately resistant or susceptible. However, the agar diffusion method can be used for determination of MIC values provided the necessary reference curves for conversion of inhibition zones into MIC values are available. Diffusion tests are cheap compared to most MIC-determination methods, but has been developed to give an approximate MIC-value.

Well standardized methods are essential for all kinds of susceptibility testing, since the methods are highly sensitive to variations in several factors, such as size of inoculum, contents and acidity of the growth medium, time and temperature of incubation. The agar diffusion methods are also strongly influenced by factors, such as agar depth, diffusion rate of the antimicrobial agent and growth rate of the specific bacteria.

The MIC-determination and disk diffusion methods described in this protocol are in accordance with the international recommendations given by the Clinical and Laboratory standard Institute (CLSI). In our study 9 essential oils have been tested for *in-vitro* antimicrobial activity and some have demonstrated to be possessing potential antimicrobial activity.

The effect of essential oils against *S. mutans* in our study showed that Clove bud, lemon grass and Tea tree were the most effective oils against *S. mutans* respectively. A study was conducted by Chaudhari et al., showed that Cinnamon oil was the most effective against *S. mutans* followed by lemongrass oil, cedar wood oil and clove bud oil **(Chaudhari et al., 2012)**.

Efficacy of tooth paste and mouth rinses on *S. mutans*

The results of our study showed that different toothpaste brands exhibited wide range of inhibitory activity against the three tested isolates of *S. mutans*. Among all the investigated toothpastes, Colgate toothpaste has emerged as the most effective formulation compared to all other toothpaste formulations (with inhibition zones ranged from 25 to 30 mm) followed by Signal, Paradontax and Sensodyne. The lowest inhibitory effect was expressed by B white toothpaste with inhibition zones ranging from 0 to 5 mm. The mean inhibition zone diameters of the five toothpaste brands at full strength ranged between 5 and 30 mm. Colgate toothpaste emerged as the most effective against all the three isolates tested, this may be due to presence of sodium mono fluorophosphate and sodium fluoride. Signal toothpaste has significant antimicrobial activity against the tested organisms. It contains sodium mono fluorophosphate and calcium glycerophosphate as active ingredients. **Prasanth 2011,** demonstrate the fact that toothpastes containing mono fluorophosphates have inhibitory effect not only on *S. mutans* but also on oral pathogens like *Escherichia coli* and *Candida albicans*. Fluorides are popularly used in many oral health products as reported to help in caries prevention **(Marinho 2009)**. Sensodyne toothpaste had low inhibitory effect on tested pathogens that may be due to the presence of a single ingredient in its formulation (strontium chloride hexahydrate).

In case of mouth rinses, Gargarol was found to be the most effective mouth rinse, which showed maximum antimicrobial efficacy against the tested pathogens. This may be due to the presence of Chlorhexidine gluconate as major ingredient in its formulation; this observation fit with the results achieved by **Prasanth 2011**, which demonstrate that mouth rinse containing Chlorhexidine formulation has significant antimicrobial activity compared to those do not have this formulation.

Conclusion

The results of this study showed that S. mutans have been found to be most susceptible to vancomycin, tetracycline, doxycycline, ciprofloxacin and amoxyclav. Clove bud, lemon grass and Tea tree were the most effective oils against S. mutans respectively. Colgate toothpaste has emerged as the most effective formulation compared to all other toothpaste formulations, and Gargarol was found to be the most effective mouth rinse.

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