Isolation and Identification of *Streptococcus mutans* from oral infection and testing their sensitivity to some plant extractions

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Abstract

Streptococcus mutans is the main etiological agents of dental caries. The aim of the study was to isolate, identify, characterize, and determine the minimum inhibitory concentration (MIC) and the inhibition zone of *S.mutans* from subjects to plant extractions.133 Swab samples were collected from dental caries, dental plaque and gingivitis subjects, processed and cultured on mitis salivarius agar (MSA). All the bacteria were subjected to morphotyping and the suspected colonies were identified by Biochemical tests and VITEK 2 compact and some isolates identified by 16S rRNA sequencing. The *S. mutans* strains were characterized by Sequence phylogenetic analysis. The MIC and zones of inhibition of plant extractions were determined by the double fold serial dilution and agar well diffusion methods. The study population, 56 isolates displayed typical colony morphologies of *Streptococcus spp*. The Biochemical tests and VITEK 2 compact results revealed that 14 isolates were *S. mutans* and 4 isolates were *S. sobrinus*. The 16S rRNA sequencing results revealed that the ratio of homogeneity between studied isolates and recorded strains ranged from 98%-100%. MIC of Alcoholic and Aqueous extracts to *Capparis spinosa* against *S. mutans* was 25mg/ml and 50mg/ml respectively. The average of zones of inhibition 11mm and 14mm to 50mg/ml and 16mm and 18mm to 100mg/ ml.

Introduction

The most common and important bacterial species in the oral cavity are attributed to the genus *Streptococcus* and in particular the members of the Viridans Streptococcus. the Viridans Streptococcus (VS) are Symbiosis in the oral cavity but has the potential to cause a variety of diseases[1], So they cause opportunistic pathogens[2]. The most common oral diseases in humans are Dental Caries and Gum diseases[3]. Plaque is the basis for these diseases[4]. Accumulative reports showed that among mutans streptococci (MS), *S. mutans* and *S. sobrinus* were the most isolated microorganisms from the majority of human dental caries. They are also the main factors of the disease, despite the many factors that cause it[5].The accurate identification of pathogens has been a key parameter as it directly involved in the treatment strategies. Over years, MS has been identified by various methods, including culturing, direct microscopy, biochemical tests enzyme-linked immunosorbent assays[6], polymerase chain reaction (PCR)-restriction fragment length polymorphism-PCR-RFLP[7], PCR-based species-specific primer[8] and PCR-based 16S rRNA gene[9]. Due to increased concern due to the emergence of antibiotic resistance, *S.mutans* has been assessed for some antimicrobial agents and some plant extracts because natural medicines are safer than chemical drugs.

Materials and Methods

Samples Collection: Swab samples were obtained from dental surfaces and gums of patients in the Specialized Dental Center in Amara and Some of these samples were collected from some schools in the city of Amara. The patients were from both sexes and from different ages. Swabs with transport medium are used to ensure the vitality of bacteria and not to contamination[10].

Sample of cultivation: The samples were immediately cultured in the laboratory on MSA medium for isolating the bacteria. The anaerobic incubation method was used for 48 hours followed by incubation for 24 hours[11].

Rapid differentiation of *S. mutans*: After the samples were cultured, the 10% mannitol sugar solution was spread over the growth colonies on the MSA medium, then the Triphenyl tetrazolium chloride 4% (TTC) solution is spread too. A change color of the colonies to the red indicates that the colonies belong to *S. mutans*[12].

Biochemical characterization: The ability of the bacterial isolates to utilize different carbohydrate sources was determined following the method described by. Brain heart infusion broth supplemented with a (0.02%) of phenol red and (10%) of each carbohydrate (mannitol, sorbitol, raffinose, sucrose, and Inulin). Suger solutions were sterilized by filtration and added aseptically to the autoclaved brain heart infusion broth medium with phenol red. A suspended media was inoculated with the tested isolates and incubated anaerobically at 37oC for 72 hrs. Change in the color of media from red to yellow as compared with the negative and positive control. Sucrose was used as positive control and brain heart infusion broth medium as a negative control [13].

Identification by using VITEK 2 compact: A wooden stick applicator was used to transfer bacterial colonies from a pure culture and was suspended in 3.0 ml of sterile saline in a 12 x 75 mm clear plastic test tube. The turbidity is adjusted to 0.8 and measured using a turbidity meter called the DensiChek, and then identification cards were inoculated with bacterial suspensions using an integrated vacuum apparatus. A test tube containing cells suspension was placed into a special rack (cassette) and the identification card was placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The filled cassette is placed either manually or transported automatically into a vacuum chamber station. After the vacuum is applied and the air is re-introduced into the station, the cells suspension was forced through the transfer tube into micro-channels that fill all the test wells. Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator. All card types were incubated on-line at 35.5, then Each card was removed from the carousel incubator once every 15 minutes, and

transported to the optical system for reaction readings, and then returned to the incubator until the next read time. Data were collected at 15-minute intervals during the entire incubation period[14].

Antibiotic Susceptibility Test: It was made by using the disk diffusion method according to Morello et al., 2006 on the Muller Hinton Agar medium with 5% human blood. A pure culture of previously identified bacteria was prepared by adding a growth from an isolated colony to 3 ml of sterile normal saline. The turbidity of the suspension was measured by a Densichek meter (0.50-0.63 mg). A sterile cotton swab was used to obtain inoculums to be streaked on the plate. The antibiotic discs were placed on the surface of the medium by sterile forceps. Incubate the plate for 24 hr. at 37°C. Zone diameter was compared to standard results being recommended by clinical laboratory standards institute documentations[15].

Identification by 16S rRNA sequencing: Genomic DNA extraction from bacterial isolates by using Genomic DNA Mini Bacteria Kit.PCR amplification of 16S rRNA region was done in 20 µl of reaction mixture containing 8 µl of nuclease-free water, 5 µl of master mix, 1 µl of 10 picomoles 16S forward primer (5'-GGTTACCTTGTTACGACTT -3'), 1 µl of 10 picomoles 16S reverse primer (5'-AGAGTTTGATCCTGGCTCAG -3'), and 5 µl of DNA template. The PCR temperature conditions for 32 cycles were as follows: Initial denaturation 94°C for 3 min, denaturation 94°C for 30 s, annealing 56°C for 30 s, extension 72°C for 1.5 min, and final extension 72°C for 6 min.

Phylogenetic analysis: The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura & Nei model[16].The phylogenetic analysis was carried out employing a program named MEGA7[17].

Collection of Plant Samples: In this study, the fruits of Capsicum annuum and seeds of Nigella sativa were obtained from the local market, while leaves of Alhagi maurorum and fruits of Capparis spinosa were collected from one of the villages of Amara city.

Samples Preparation and Extraction: Each of the plant material was ground and 20g of it was added to 200 ml of distilled water or 70%w/v ethanol in order to obtain water or ethanolic extract. This crude extraction was done in shaking incubator for 24 hours at 30◦C. Muslin cloth was then used to filter the plant residues. The crude Centrifuged at 250 x g for 10 minutes. The filtrate thus obtained was further purified by filtration through Whatman filter paper. Then The filtrate pours into glass dishes and enters into the oven at 40C for drying[18].

Determination MIC of the extracts on the test organisms: The initial concentration of the plant extract (100mg/ml) was diluted using double-fold serial dilution according to Ibekwe et al., 2001[19], by transferring 2ml of the sterile plant extract (stock solution) into 2ml of sterile Nutrient broth to obtain 50mg/ml concentration. The above process was repeated several times to obtain other dilutions: 25mg/ml, 12.5mg/ml and 6.25mg/ml. Each concentration was inoculated with 0.1ml of the bacterial suspension (0.50-0.63 mg) and incubation was done at 370c for 24 hours. The growth of the inoculum in the broth is indicated by turbidity or cloudiness of the broth and the lowest concentration of the extract which inhibited the growth of the test organism was taken as the MIC. Negative controls were set up as follows: Nutrient broth only; Nutrient broth and sterile plant extract; and finally positive control containing Nutrient broth, and a test organism.

Determination of Zones of Inhibition: It was made by using well diffusion method on the Muller Hinton Agar medium according to Prescott et al.1999[20]. The surface of this plate was streaked with the bacterial suspension(0.50-0.63 mg) from a pure culture. A sterile cork borer, (8mmin diameter) used to create a ditch at the center of the plate. The hole so created was then filled with the plant extract. The plates were allowed to

stand for one hour for pre-diffusion of the extracts and incubation was done at 37◦c for 24hours. At the end of the incubation period, the diameter of the zone of inhibition was measured in millimeter.

Statistical analysis: Statistical analysis was performed using the software platform offers advanced statistical analysis (SPSS) The statistical analysis result with $P \leq 0.05$ was considered statistically significant.

Results

Identification of the isolates at the genus level has been depending on catalase test, microscopic examination, and gram staining. The streptococci are spherical or ovoid occurring pairs or chains, stain gram-positive and catalase negative bacteria[21]. Identification isolates to species level depending on colonies shape on the surface of MSA. Further specific identification down to distinguished *S. mutans* from other oral streptococci down by using the staining the cells with 2,3,5- triphenyl tetrazolium chloride (TCC) and changed color to dark pink. Ten isolates were stained with dark pink color and considered to be a positive result, Also were dispersed *S. mutans* from other oral streptococci down by sugar fermentation test [Table 1].

Table 1: Sugar fermentation for isolates of oral Streptococci

Sugar fermentation				Catalase	Species
Inulin	Raffinose	Sorbitol	Mannitol		
+	+	+	+	-	*S.mutans*
+	V	V	+	-	*S.sobrins*

V –	V –	V –	– –	– –	*S.sanguins* *S.orilius*
– +	– +	– –	– –	– –	*S.mitis* *S.salivirius*
+ = Positive, – = Negative, V= Variable					

Among the study population, 14 (25%) and 4 (7%) identified as *S. mutans* and *S. sobrinus*, respectively, based on VITEK 2. However, 38 (68%) isolates were other species non-MS[Table 2].

Table 2: Identification of oral Streptococci by VITEK 2

Number	confidence	Number	Species	No
14	**Excellent**	**14**	*S.mutans*	**1**
8	**Excellent**	**16**	*S.sanguins*	**2**
8	**very good**			
10	**Excellent**	**16**	*S.orilus*	**3**
4	**very good**			
2	**good**			
4	**Acceptable**	**4**	*S.sobrins*	**4**
4	**Excellent**	**2**	*S.salivirius*	**5**
4	**good**	**2**	*S.ovis*	**6**
2	**Excellent**	**2**	*S.mitis*	**7**

56		**56**	**Total**	

Antibiotic sensitivity test may be used as a criterion for separating oral Streptococcus from that belongs to *Streptococcus pneumoniae*, Also resistant viridans streptococcus plays a role as a reservoir for other bacteria of the oral microbiota especially *S. pneumoniae*[22]. The results appeared that p-vague = 0.00 and all isolates of *S. mutans* were sensitive 100% to Clindamycin, Penicillin, Tetracycline, Vancomycin, and 63% to Chloramphenicol. It is also 100% resistant to the Optochin, Bacitracin and 38% to Streptomycin (Figure1).

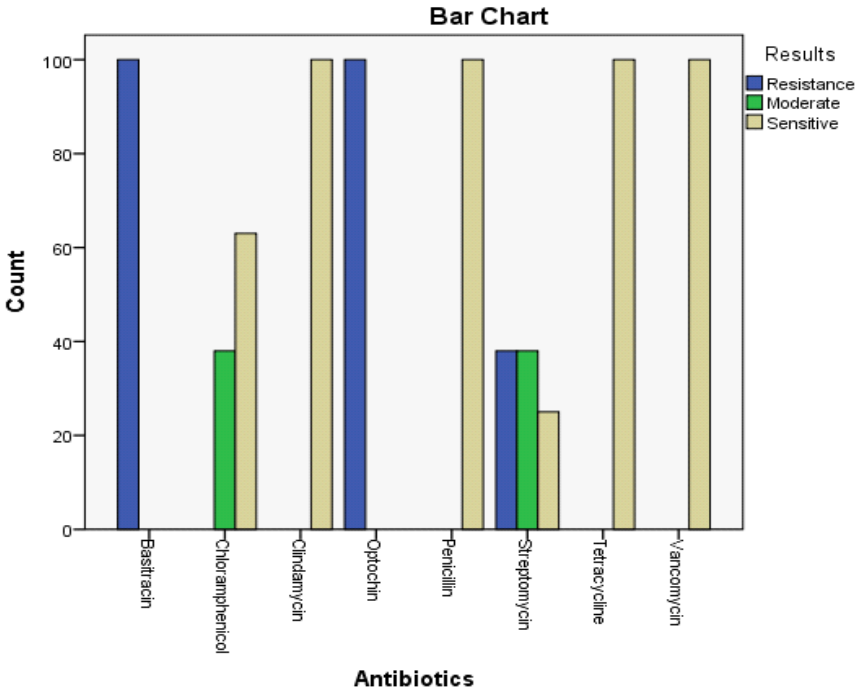

Figure 1 Antibiotic sensitivity of *S. mutans* isolated from oral diseases against eight antibiotics

VITEK 2 were identified by 16S rRNA sequencing to confirm the diagnosis. Identification of *S.mutans* isolates by using 16S rRNA is more accurate than bacteriological and biochemical assays. Flayyih et al. 2016[23] have stated that 22 (100%) isolates are identified as *S. mutans* by 16S rRNA gene, Also useful for identification of bacterial pathogens in patients pretreated with antibiotics. In this study, one of the isolates was diagnosed using VITEK 2 as *S.sanguinis* characterized by the 16S rRNA sequencing as *S. mutans*. The NCBI GenBank three accession numbers of *S. mutans* sequences are MH613069, MH613221, MH613224 and two accession numbers of other streptococci. The phylogenetic analysis of the 16S rRNA sequences constructed by[Figure2].

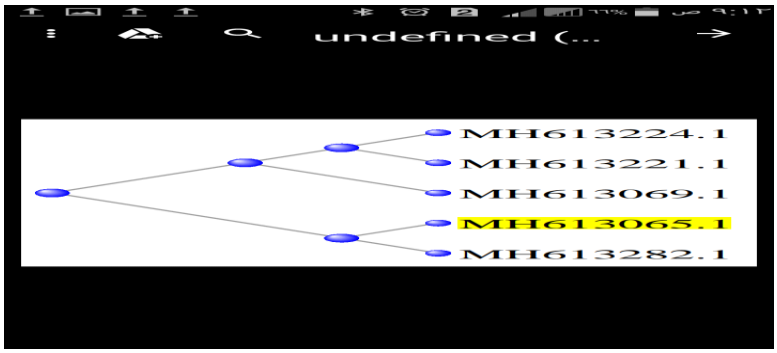

Figure 2: Phylogenetic analysis of the three *S.mutans* and two other streptococci strains based on 16S rRNA sequences. The accession numbers are shown.

The results of MIC showed that *S. mutans* were not inhibited by the extracts at the test concentration used. However, the extracts of *Capparis spinosa* had MIC of 50.0mg/ml to water extract and 25.0 mg/ml to alcoholic extract. The results obtained in the Agar diffusion plates followed the same trend with what was obtained in the Minimum Inhibitory tests respectively. The mean average of the water extract of *Capparis spinosa* to 100mg/ml in the present study was16.00 ± 0.00 while the mean average of the alcoholic extract was18.00 ± 0.00 and L.S.D was 0.86(Figure 3).

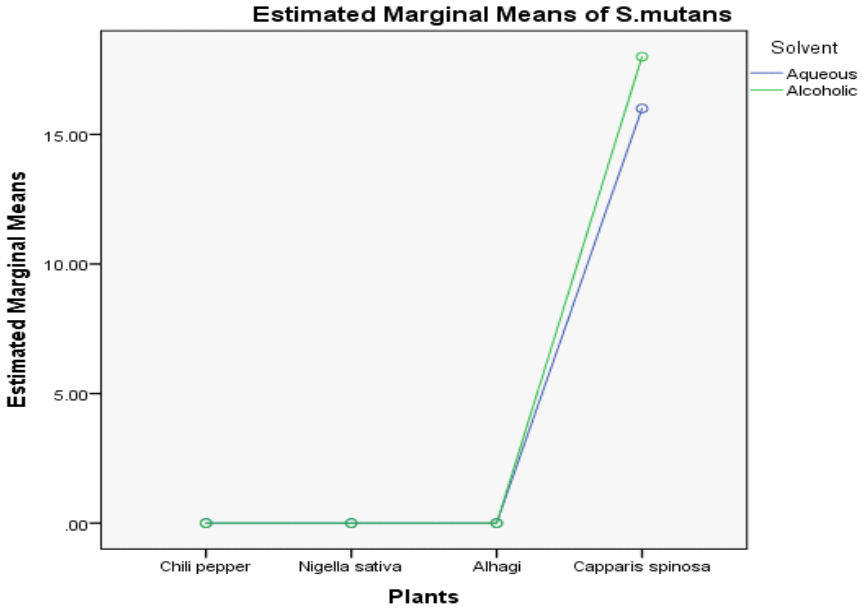

Figure 3: *S.mutans* sensitivity for plant extracts 100mg/ml

The mean average of the water extract of Capparis spinosa to 50mg/ml was 11.00 ± 0.00 while the mean average of the alcoholic extract was14.00 ± 0.00 and L.S.D was 0.863 (Figure4)

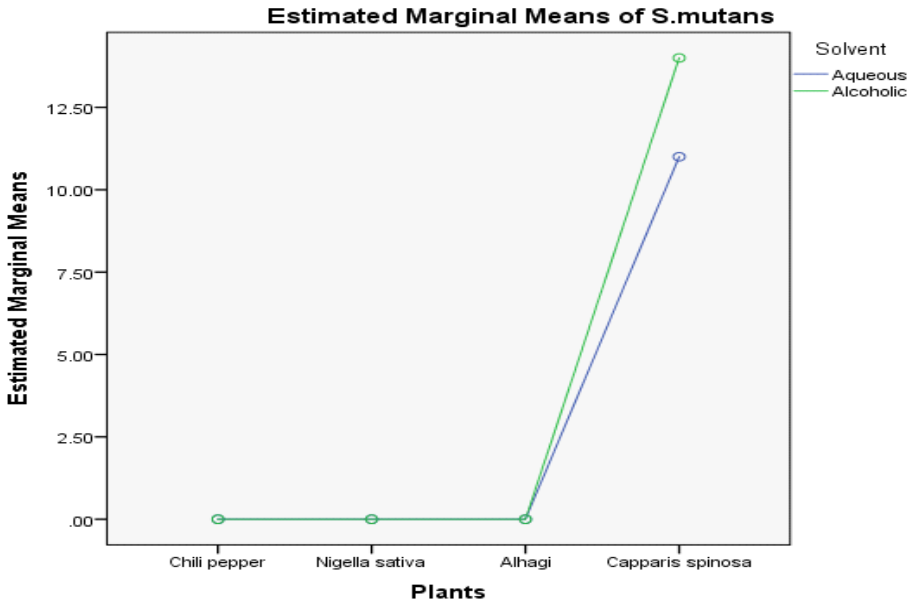

Figure 4*: S. mutans* sensitivity for plant extracts 50mg/ml

Discussion

Dental caries remains the most prevalent disease worldwide, burdening billions of people, especially children, with pain and subsequently poorer quality of life and general health [24]. *S mutans* a primary etiologic agent of human dental caries. Morphotyping was noted to be unreliable for species identification which was in agreement with the earlier report. Especially, that both *S. mutans* and *S. sobrinus* exhibited similar colony morphologies. Staining the cells of *S. mutans* with 2,3,5-triphenyl tetrazolium chloride (TCC) and changed color to dark pink due to hydrolysis of mannitol to the acid by mannitol-1-phosphate dehydrogenase and a reduction of (TTC). Accurate results were obtained when the S. mutans were identified with VITEK 2. However, one isolate that had been identified as *S. sanguins* was found to belong to *S. mutans* after being identified with 16S rRNA sequence. The results of *S. mutans*

sensitivity for antibiotics were consistent with most previous reports, except for penicillin which in turn agreed with the researchers[25,26]. This may be due to the lack of excessive treatment of patients. The sensitivity for plant extractions statistical analysis showed that there were significant differences between plants and solvents used in the study. Identification results of *S. mutans* with 16S rRNA sequence were high resolution and consistent with previous reports[27,28].

Conclusion

The 16S rRNA sequencing was an impeccable method for *S. mutans* identification when compared with morphotyping, Biochemical tests, and VITEK 2 compact methods. The study also showed that the *Capparis spinosa* extracts of alcohol and Aqueous are effective against *S. mutans* without other extracts used in the study. The alternative of antibiotic like plant extract is possibly preferred for the approaching years to avoid the incoming microorganism resistance to the antibiotic.

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