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Identification of gtfB, gftI Sequence of Streptococci and its Prevalence in Cariogenic Patients Presented at University College Hospital of Medicine and Dentistry Lahore, Pakistan During 2016-2017

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Abstract

Streptococcus mutans and *Streptococcus sobrinus* have been considered to be the most important pathogens associated with dental caries. The purpose of current study is to detect and correlate the presence of *S. mutans* and *S. sobrinus* in the dental plaque of caries active patients using conventional methods, analytical profile index analysis and polymerase chain reaction (PCR) for identification of gtfI and gtfB of *streptococcus* species. Total of 130 carries and saliva mixed gingival fluid samples were collected from dental patients attending Outdoor Patient department (OPD) of University Dental Hospital, The University of Lahore during 2017. The samples were cultured on blood agar, subsequently shifted to mannitol salt agar (MSA). Each of the similar colony was than subjected for microbiological and molecular analysis using specific gentotype primers. It is revealed that 1mm pink colonies on MSA showed gram positive cocci bacteria arranged in chains under microscope and amplified 512bp and 712bp amplicon size PCR product for *S. mutans* and *S. sobrinus* respectively. The samples obtained from male dental patients ranging 8-10 years were 100% confirmed as *S. mutans* and *S. sobrinus* using either of the technique. Similarly, in female dental patients the highest prevalence of *S. mutans* (83.33%) and S. sobrinus (66.6%) were observed in same age group. Furthermore, the gender base frequency of *S. mutans* were found higher in male caries active patients (64.6%) as compare to female caries active patients (56.9%) while for *S. sobrinus*, the percentage positivity was observed comparatively lower in male dental patients (29.2%) than female (43%). The overall prevalence of *S. mutans* and *S. sobrinus* in all the samples were 67.52% and 40.17% respectively. Antibiotic susceptibility test results revealed that all screened isolates was highly sensitive to Chloramphenicol (C), Amoxicillin (AMX), Ciprofloxacin (CIP), Erythromycin (E) Tetracycline (TE) and Ampicillin (AM). Whereas, were resistant to Cefixime (CFM), Clindamycin (CD), Spectinomycin (SPT) and Kanamycin (K).

Keywords: *Streptococcus mutans*, *Streptococcus sobrinus*, Cariogenic Pathogens, Caries active patients, Dental caries

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Introduction

The oral cavity is a major gateway to the human body. Microorganisms from the oral cavity have been shown to cause a number of oral infectious diseases, including caries (tooth decay), periodontitis (gum disease), endodontic (root canal) infections, alveolar osteitis (dry socket), and tonsillitis.[1,2] Oral microbiota consists of variety of micro-organisms such as bacteria, protozoa, fungi and hardly viruses. Bacteria are the predominant population found as microbiota.[3,4] Bacteria play a major role in the initiation of disease facilitated by different factors like quality of dietary material, pH of saliva consumption of antibiotic whenever desired for treatment of other diseases.[5] Dental caries is one of the most common chronic infection of tooth enamel caused by the interaction of *Streptococcus mutans* and *Streptococcus sobrinus*. These bacteria break down sugars for energy, causing an acidic environment in the mouth and result in demineralization of the enamel of the teeth and develop dental caries.[6] Streptococci are the utmost group having number of species been reported as involved in damaging different components of oral cavity. However, *S. sangunis* and *S. mutans* formed colonization on the teeth in contrast to other

species are reported as restricted to the cheeks and gums. Staphylococcus and streptococcus constitutes the major portion of microflora of oral cavity.[7] Among the oral bacteria, Mutans streptococci have been implicated as a major cariogenic bacteria. The degree of colonization of these organisms correlates with the prevalence of dental caries in children and adults. Epidemiological studies have reported that S. mutansis more prevalent than *S. sobrinus* in the oral cavity, but have also shown that the prevalence of *S. sobrinus* is more closely associated with a high caries experience.[8] *Streptococcus mutans* has been in the focus of researchers and recognized as the primary contributing cause of human dental caries. The organism yields certain extrinsic factors like glucosyltransferases responsible for the production of glucans from sucrose.

The detection and identification of oral streptococci in the dental biofilm is considered to be an important step for the understanding of dental caries. Nearly all investigations into the microbial pathogenesis have been done by cultivation of bacteria. This conventional method of culturing the bacteria is more time consuming and is sometimes inaccurate. It has also been reported that MS-bacitracin inhibits the growth of *S. sobrinus* to a greater extent than that of *S. mutans* on the agar medium, thereby providing inappropriate results.[9] However, the advanced molecular methods have revealed that the bacterial involvement in the development of dental caries is more complex than previously believed. In the present study, PCR method was used for amplification of the gene sequences of *S. mutans* and *S. sobrinus*. According to Igarashi PCR is a rapid, more sensitive and simpler method for the detection of micro-organisms. They also reported that the conventional cultural methods used for the detection of micro-organisms in dental plaque takes a long time nearly 1 week whereas, PCR method reduces the time to 6-7 hours.[10]

Biochemical analysis has proposed that the bacterium secretes two different types of gtfs. Both gtfI and gtfB act cooperatively and synthesize insoluble glucans involved in the colonization of bacteria on tooth surfaces.[11] So, the current study was undertaken to isolate the cariogenic Streptococcus and to relate the prevalence of its subtypes *S. mutans* and *S. sobrinus* through PCR amplification using type specific oilgos in the patients with the history of orthodental complication presented at University Hospital.

Materials and Methods

Collection Of Samples

Total of 130 samples were collected from dental patients attended outdoor patient department (OPD) of the University Dental Hospital, The University of Lahore, Punjab. Three ml of saliva mixed gingival fluid was collected from every patient with 2mm size of hard dental plaque and shifted to 20 ml sterile plastic container. Samples were stored at 4°C in a cool place and then transported to the laboratory. (Fig. 1)

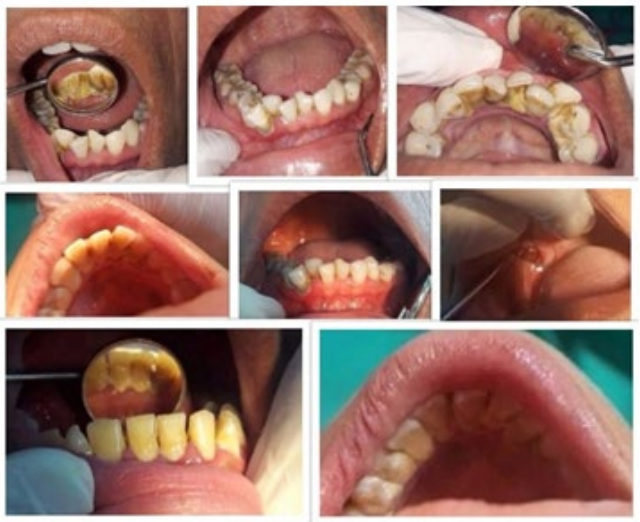

Fig 1: Collection of Saliva mixed gingival fluid from dental patients

Sample Size:

The sample size was calculated by using the formula of Kish & Lisle states that [12]

$n = z2\ p\ (1-p) / d2)$

Where, z = Score for 95% confidence interval = 1.96

p = Prevalence

d = Sampling error that could be tolerate= 5%.

1-p= Probability

Isolation of the Bacteria:

Specified grams of mannitol salt (MS) agar, nutrient agar, blood agar were weighed using digital balance (Shimadzu Scientific Instruments-Japan) and suspended in 1000 ml glass flask containing distilled water according to manufacturer instructions (Oxoid Ltd- England). The suspension was sterilized by autoclaving at 121°C for 15 minutes. 20 ml of each autoclaved nutritional media was poured on 9mm disposable petri plate and subjected for incubation at 37°C for 48 hours. Loopful of saliva mixed gingival fluid and 100 µl caries/ sulcus normal saline washing solution was streaked on the nutrient agar and MS agar. Cultures were incubated anaerobically for 48 hours at 37°C. (Fig. 2)

Fig. 2: Media Preparation

Identification of Isolates:

After the incubation period, the colonies were identified on the basis of colony morphology. The typical colonies from each sample plate were transferred to blood agar (Oxoid Ltd- England) for further purification and incubated anaerobically at 37°C for two days. The following steps were involved in identification and characterization of the isolates:

a. Colonial shape and form on MS-agar and Blood agar.
b. Gram-staining and microscopic examination.
c. Analytical profile index analysis
d. Antibiotic Susceptibility test
e. Molecular characterization through Polymerase chain reaction (PCR).

Gram Staining:

Every pure culture grown on the surface of selective medium showing standard characteristics colony were selected for Gram Staining. It was performed according to standard procedure. Smear was established by placing a loopful of culture on microscope glass slide re-suspended with drop of distilled water in circular pattern. The smear was fixed by passing 3-4 times on flame followed by heat air drying. Straining was performed according to the method described by Bergey.[13]

Analytical Profile Index Analysis:

The analytical profile index of Gram positive cocci isolated from samples was determined through Remel RapIDTM STR System (Thermofisher scientific-USA), following instructions of the manufacturer. (Fig. 3)

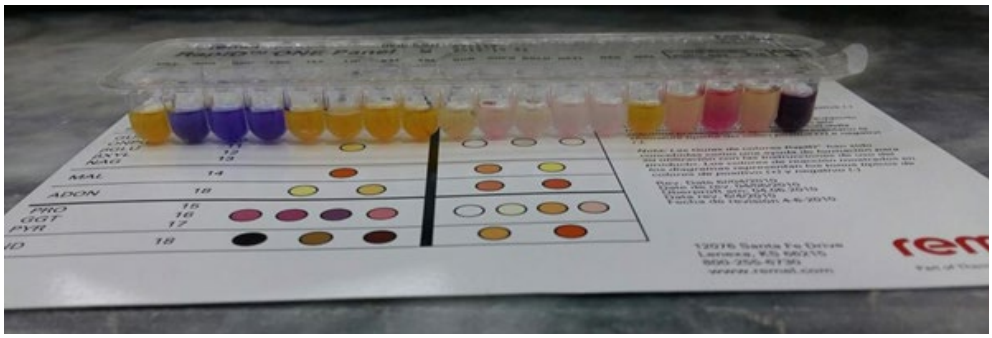

Fig. 3: Biochemical identification by using Analytical Profile Index (API)

Antibiotic Susceptibility Testing of Bacterial Isolates:

Antibiotic susceptibility test of the clinical isolates was done using modified Kirby-Bauer disc diffusion method in accordance with the guidelines of the Clinical and Laboratory Standards Institute.[14] The antibiotics used in this study include Amoxicillin (AMX), ciprofloxacin (CIP), erythromycin (E), chloramphenicol (C), tetracycline (TE), vancomycin (VA), cefixime (CFM), ampicillin (AM), clindamycin (CD), cefuroxime (CXM), bacitracin (B), spectinomycin (SPT), and kanamycin (K). The antimicrobial combinations used were AMC and AM/sulbactam (A/S). Bacterial inoculums were prepared by suspending the freshly grown bacteria in 2 ml of sterile brain heart infusion broth with yeast extract, and the turbidity of tube was matched with 0.5 McFarland turbidity standards and then swabbed onto Mueller-Hinton agar plate. To prevent condensation, antibiotic discs were allowed to warm to ambient temperature before application. Within 15 min of inoculation, the antibiotic discs were placed at side down on the seeded agar surfaces using sterile forceps, followed by incubating agar plates for 24 hours at 37°C. The relative susceptibility of the bacterial growth to each antibiotic shown by a clear zone of inhibition was measured in mm and zone diameter criterion was used to interpret the level of susceptibility to each antibiotic.

DNA Extraction:

All the samples including positive known bacteria were subjected for extraction of DNA by using QAIamp DNA Mini kit (QIAGEN-Germany) according to the manufacturer instructions. All the extracted DNA was separately stored in 1 ml labeled eppendorf tubes at -20°C. These samples were subjected to molecular identification.

PCR Idenfication:

The extracted DNA of all samples were subjected to PCR for amplification of the desired genes gtfB and gtfI of S. mutan and S. *sobrinus*. The sequence of these primers were:

S. mutans	Forward Primer	gtfB	ACTACACTTTCGGGTGGCTTGG
	Reverse Primer	gtfB	CAGTATAAGCGCCAGTTTCATC
S. sobrinus	Forward Primer	gtfI	GATAACTACCTGACAGCTGAC
	Reverse Primer	gtfI	AAGCTGCCTTAAGGTAATCACT

For the PCR reaction, 2µl of 10X PCR buffer, 1 µl of forward and reverse primer, 2 µl of 80 mM $MgCl_2$, 2 µl of DNTPs mixture and 1 µl of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) were added to the reaction mixture. Reaction volume was obtained by adding 9 µl of Nuclease-free Water and 2 µl of extracted DNA sample. The PCR tubes were incubated in thermal cycler (Applied Biosystems) with an initial denaturation of 94°C for 2 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 51°C, 56°C for 30 seconds and extension at 72°C for 45 seconds. Final extension was conducted at 72°C for 5 minutes. (Fig. 4)

Fig. 4: Polymerase chain reaction (PCR) of Extracted DNA

Fig 5: Gel Electrophoresis analysis of PCR product

Results

Caries is a polymicrobial infection and every bacterial species plays a role in determining the carcinogenicity of the biofilm or dental plaque. Out of 130 samples, 117(90%) pink colonies upto 1mm diameter on mannitol salt agar recovered form saliva mixed gingival fluid of caries active patients were declared as streptococcal species. Gram staining of such colonies showed 102(78.46%) non-sporeformer, round in shape forming long chains having purple color in compound microscope. The colonies showing alpha and gamma hemolysis on blood agar were selected and streaked on Mueller-Hinton agar media for antibiotic susceptibility pattern. Analytical profile index analysis revealed 73.50% colonies are positive for mannitol, sorbitose, oxidase and raffinose. While 26.50% showed negative reaction to hydrolysis of arginine production. Molecular characterization of streptococcus species isolated from dental patient showed 60% and 36% percentage positivity of *S. mutans* and *S. sobrinus* respectively. (Fig. 6, 7)

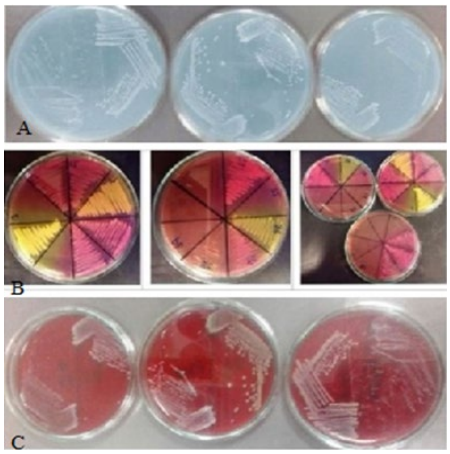

Fig. 6: Isolation and growth of bacteria on Selective Medium. A.Nutrient Agar. B. Mannitol Salt Agar. C. Blood Agar

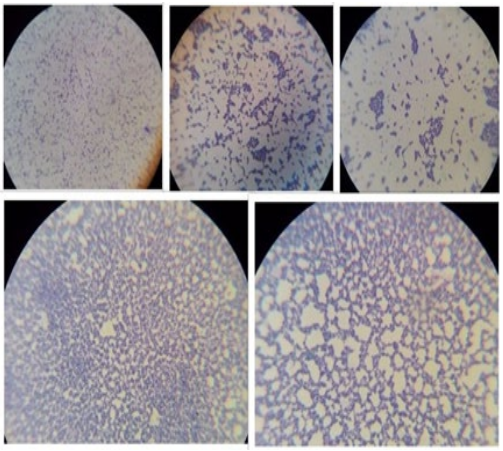

Fig 7: Gram staining and Microscopy of Isolated Bacteria

Kirby-Bauer disc diffusion analysis of clinical isolates against 15 commercially used antimicrobial drugs in dentistry. All isolates were sensitive against chloramphenicol (C), amoxicillin (AMX), ciprofloxacin (CIP), erythromycin (E), tetracycline (TE), ampicillin (AM), whereas least activity was shown by Bacitracin (B), while cefixime (CFM) and kanamycin (K), clindamycin (CD) and spectinomycin (SPT) showed no inhibition zones and isolates were reported as resistant against them. Combinations were used with an objective to broaden the spectrum of antimicrobial action. Among the antimicrobial combinations used, the greatest potency was shown by A/S followed by AMC. Among these antibacterial drugs tested, chloramphenicol showed maximum zone of inhibition followed by amoxicillin (AMX), erythromycin (E) and ciprofloxacin (CIP). Fig. 8)

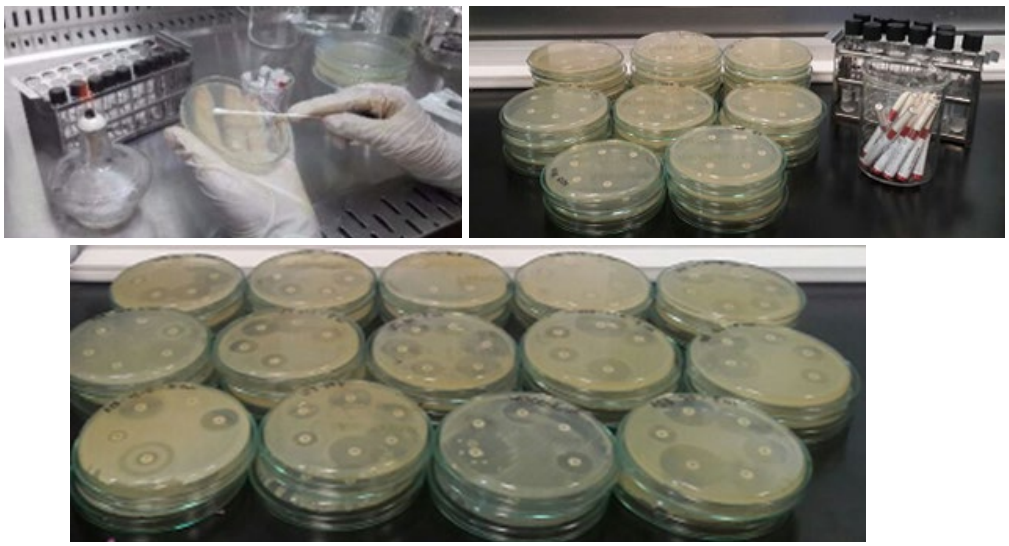

Fig. 8: Antimicrobial susceptibility pattern of isolated Streptococcus from Carries active patients

In male caries active patients, API based analysis revealed that 50(76.90%) samples were positive for Streptococci subsequently to high prevalence of S. mutans 42(64.6%) followed by S. *sobrinus* 19(29.2%) for gftB and gftI genotype sequences respectively. Moreover, 36(55.32%) samples of female dental patients with active caries were screened for significant bacterial count and identified as Streptococci in analytical profile index analysis. The frequency of gftB and gftI genotype sequences of S. mutans and S. *sobrinus* was 37(56.9%) and 28(43%) respectively.

Furthermore, Out of 5 samples collected from healthy caries individuals, 60% showed typical colonies. Whereas, only 2 samples were observed positive for microscopic morphology and API analysis. While none of the primer could amplify the extracted genome for a typical amplicon size.

The prevalence of S. mutans and S. *sobrinus* in male dental patients based on age group indicates that individuals of the 8-10 years age had high incidence (100%) than age group 11-30 years (60%) and 31-75 years (66.6% and 31.1%) respectively through PCR analysis. Streptococcus mutans and *Streptococcus sobrinus* specific amplicon were detected in (83.33%) and (66.6%) samples from the individuals of female caries active patients of 8-10 years age group. In contrast, the percentage positivity of both the Streptococcal isolates were slightly higher in age range of 11-30 years than age group 31-75 years. (Fig. 9, 10)

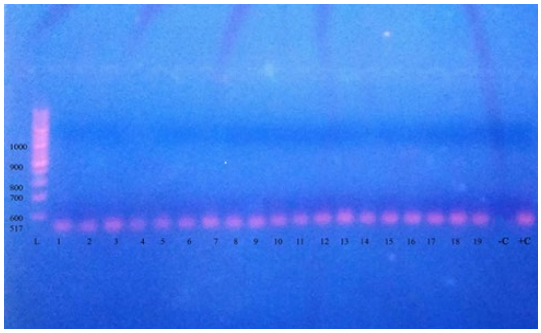

Fig 9: Gel Electrophoresis analysis of S. mutans. Lane L is 1000bp Ladder, Lane -C is negative control, Lane +C is positive control Lane 1-19 are isolates (517bp product).

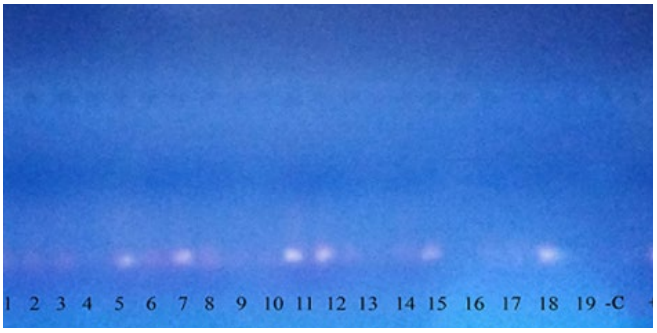

Fig. 10: Gel Electrophoresis analysis of *S. sobrinus*. Lane L is 1000bp Ladder, Lane -C is negative control, Lane +C is positive control Lane 1-19 are isolates (712bp product).

	Test Code	Reactive Ingredients	Colours	Results
Before Reagent Addition				
1	ARG	L-arginine	Red or Dark Orange	Negative
2	ESC	Esculin	Black	Positive
3	MNL	Mannitol	Yellow or Yellow Orange	Positive
4	SBL	Sorbitol		Positive
5	RAF	Raffinose		Positive
6	INU	Inulin	Yellow, Yellow Orange or Orange	Positive
7	GAL	ρ-Nitrophenyl-α,D-galactoside	Yellow	Positive
8	GLU	ρ-Nitrophenyl-α,D-glucoside		Positive
9	NAG	ρ-Nitrophenyl-n-acetyl-β,D-glucosaminide		Negative
10	PO4	ρ-Nitrophenyl phosphate		Negative
After Reagent Addition				
12	TYR	Tyrosine β-naphthylamide	Light Purple or Purple	Negative
13	HPR	Hydroxyproline β-naphthylamide		Negative
14	LYS	Lysine β-naphthylamide	Very Dark Purple	Positive
15	PYR	Pyrrolidine β-naphthylamide		Negative

Table I: Identification of Isolates by Using RapID RemelTM STR System

Source of isolates (ne=135)	Age	Sample #	Sample showing typical features		Biochemical reactions***	PCR results (Prevalence %)	
			Typical* colonies	Microscopic** characteristic		S. mutans	S. sobrinus
Caries active male-	8-10	1-5	5(100%)	5 (100%)	5 (100%)	5 (100%)	5 (100%)
	11-30	6-20	15 (100%)	15 (100%)	10 (66.66%)	7 (47%)	0 (0%)
	31-75	21-65	40 (100%)	25 (62.5%)	14 (35%)	13 (32.5%)	5 (12.5%)
Caries active female	8-10	66-105	30 (100%)	28 (93.3%)	24 (80%)	25 (83.33%)	22 (73.33%)
	10-30	106-120	25 (100%)	22 (88%)	20 (80%)	16 (64%)	12 (48%)
	31-75	121-130	5 (100%)	5 (100%)	5 (100%)	4 (80%)	2 (40%)
Control		130-135	3	2	2	0 (0%)	0 (0%)

Table II: Distribution of Percentage Positivity of S. mutans and *S. sobrinus* Regarding the Age groups in Gender

*Facultative Anaerobic, visible growth in 24 hours at 37° C, Pink colonies up to 1mm in diameter colonies on MS agar, Gamma hemolysis on blood agar

** Gram positive, cocci in chains, non-spore former

*** The isolate of each of the typical colony showed positive reaction to mannitol, sorbitose, oxidase, Raffinose. Moreover each of the isolate showed negative reaction to hydrolysis of arginine production

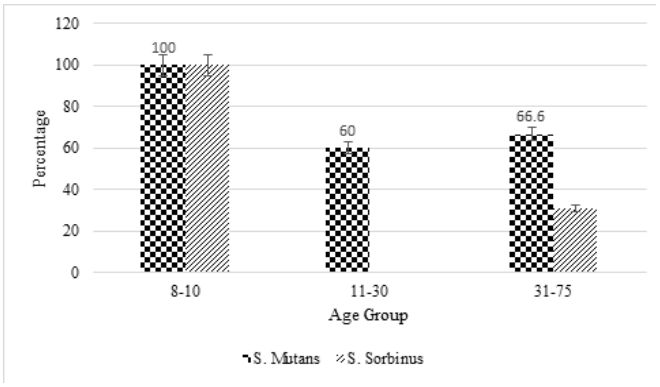

Fig 11: Prevalence of *S. mutans* and *S. sobrinus* on the basis of age distribution in Males caries active patients.

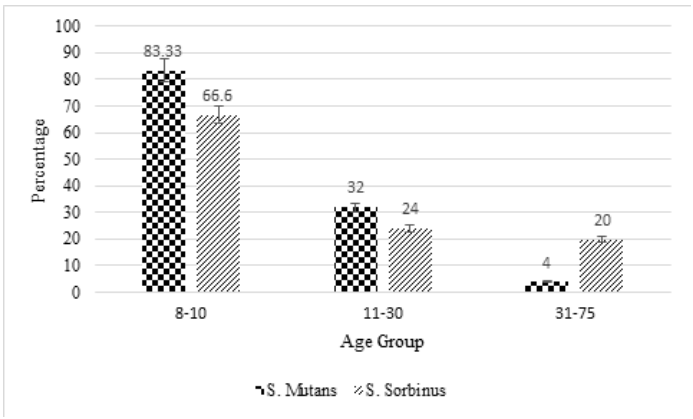

Fig 12: Prevalence of *S. mutans* and *S. sobrinus* on the basis of age distribution in Females caries active patients

Source of isolates n=135	Caries active Sample #	Sample showing typical features		Biochemical reactions***	PCR results (Prevalence %)	
		Typical* colonies	Microscopic** characteristics		S. mutans	*S. sobrinus*
Male	1-65	65 (100%)	58 (89.2%)	50 (76.9%)	42 (64.6%)	19 (29.2%)
Female	66-130	52 (80%)	44 (67.6%)	36 (55.3%)	37 (56.9%)	28 (43.0%)
Control	130-135	3	2	2	0 (0%)	0(0%)

Table III: Distribution of Percentage Positivity of *S. mutans* and *S. sorbinius* Isolates Regarding the Gender

*Facultative Anaerobic, visible growth in 24 hours at 37° C, Pink colonies up to 1mm in diameter colonies on MS agar, Gamma hemolysis on blood agar
** Gram positive, cocci in chains, non-spore former
*** The isolate of each of the typical colony showed positive reaction to mannitol, sorbitose, oxidase, raffinose. Moreover each of the isolate showed negative reaction to hydrolysis of arginine production

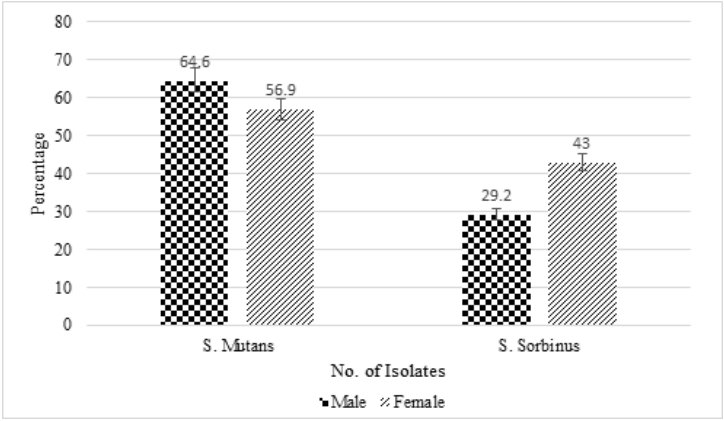

Fig 13: Comparison between *S. mutans* and *S. sobrinus* on the basis of Gender

Discussion

Dental caries is an infectious disease in which members of the Mutans streptococci have been implicated as the etiological agents. In this study we analyzed samples of saliva mixed gingival to determine the presence of *S. mutans* and *S. sobrinus* and to relate it to the presence caries on the basis of gender and age. The saliva mixed gingival fluid was used as a source for detection of cariogenic bacterial spieces. The intention is to relate the presence of cariogenic bacteria and dental caries by establishing an effective association. Although the presence of *S. mutans* is high in saliva, it is lower on the surface of enamel, where the bacteria actually manifests its capacity to produce acids leading to subsequent demineralization.[14]

The colonization by mutans streptococci in the oral cavity are important factors for dental caries.[15] Bacteria can be isolated by colony morphology, biochemical assay, DNA sequence analysis, species specific PCR, antibiotic susceptibility assay indicates some of the strains are resistant to one or more antibiotics.[16] In the present study, oral cariogenic pathogens were isolated and identified on MS and Blood agar, showed typical colonies which were further stained and microscopic observations revealed purple colored interconnected cocci forming long chains. The pure cultures grown on the selective medium were identified through commercially available API kit showed high percentage prevalence of *S. mutans* 67.52% followed by S. sobrinus 40.17%. Antimicrobial susceptibility was performed for all the clinical isolates using Kirby-Bauer's disk diffusion method. Nucleic acid of the isolates was amplified using type specific primers displayed particular bands through PCR.

It was observed that conventional techniques for detection of microorganisms are economic, easier to execute and still applicable however, results could be obtained in limited period of time by using advanced technique of PCR. Observation of the current study regarding dominance of PCR over conventional methods of microbiology for the identification of *S. mutans* is reported in comparative order. PCR was done as the previous tests did not provide the genotypic data of the samples so it was concluded from PCR results *S. mutans* (60.70%) and S. sobrinous (36.10%) that PCR is more sensitive and specific genotypically as compared to previous conventional methods.

Selection of appropriate antibiotic and effective dosage for treatment after detection of dental caries is vital to limit the infection. The results are similar to the observations reported by Hirose et al., 1993 showed that all isolates were highly sensitive to Chloramphenicol (C), followed by Amoxicillin (AMX), Ciprofloxacin (CIP) and Erythromycin (E) whereas, Cefixime (CFM) and Kanamycin (K) were reported as resistant against them.[17]

Genotypic prevalence of gtfB and gtfI sequence of *S. mutans* and *S. sobrinus* was significantly higher (100%) in male caries active patients of age group ranging 8-10 years than 31-75 years age (66.6%, 31.1%) followed by 11-30 years age group (60%). Similarly, the sample of female dental patients also showed high frequency of both isolates in 8-10 years age group (83.33%, 66.6%). The high prevalence revealed its virulence nature in the form of dental caries. The results of the current study are well supported by the observations of Carlsson et al., 1968, who reported the high frequency of *S. mutans* in children aged 3 to 14 years.[18] Fujiwara et al., 1991 and Roters et al., 1995 were recorded the similar observations which revealed that *S. mutans* was isolated more frequently from children aged 6 years or slightly older.[19,20]

The mechanism behind all the damage caused by bacteria is cariogenic species in the biofilm is composed of *streptococcus mutans*. When sucrose, disaccharide sugar from food is broken down by bacteria in mouth, glucose and fructose are produced. The glucose is used to make dextran which is part of the extracellular matrix of the biofilm. Fructose is fermented producing organic acid such as lactic acid. The acids dissolve the minerals of the tooth including enamel. The acid work even more quickly on exposed dentine. Over time, the plaque biofilm can become thick and eventually calcify and become hardened in this way it is called as the dental calculus. If the pulp is affected than the root canal may be needed to completely remove the infection.[21]

According to the current study, the overall percentage prevalence of *S. mutans* and *S. sobrinus* in caries active male patients (64.6%, 29.2%) were significant higher as compare to female caries active patients. The results collaborates with Levine et al., 2014 who reported the prevalence of *S. mutans* (73%) and *S. sobrinus* (48%) higher in male dental patients than female dental patients.[22] Mattos-Graner et al., 2000 suggested that the ability to synthesise water-insoluble glucan is an important virulence factor in initial caries development, in that it increases mutans streptococcal adherence and accumulation in the plaque of young children.[23] Gronroos et al., 1998 found that the mutacin activity of clinical isolates is reasonably stable, and this virulence factor seems to be of clinical importance in early colonisation by S. mutans.[24] An understanding of the route of transmission of mutans streptococci may help clinicians to develop measures to prevent, delay, or reduce colonization, thereby decreasing caries incidence.[25,26]

The microbes present in oral cavity can cause damage to the teeth and can also lead to the infection i.e. cariogenic bacteria causes damage to enamel, dentine and eventually inner most structures the pulp resulting in infection leading to abscess. Tooth decay results from metabolic activities of microbes that are present on tooth.[27] A layer of proteins and carbohydrates form when clean teeth come in contact with saliva. Microbes are attached to food and forms a thin biofilm on all the surfaces of the oral cavity. This thin biofilm is also known as plaque which is composed of food debris, bacteria and saliva *Streptococcus mutans* and *Streptococcus sobrinus* are the prominent member of caries causing bacterial family.[28]

It was observed that bacteriological methods are consistent in microbial diagnosis in limited resources but it requires considerable time however, PCR is highly sensitive and more reliable technique could be used for the further genotyping.

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