CHAPTER: V
DISCUSSION

Dental caries is a polymicrobial infectious disease that is prevailing among all the age groups (Kianoush et al., 2014). The prevalence and distribution of dental caries varies greatly in developed and developing countries and can reach over 90% (Shivakumar et al., 2009; Jain and Pundir, 2010). Dental caries prevalence in India is reported to be 50-60% (Shah, 2005). Most of the studies in India on dental caries have focused mainly on children and limited studies have been carried out among adults.

Streptococci are a group of oral bacteria, frequently associated with dental caries. There are about 19 distinct species of oral streptococci, and among these, mutans streptococci are implicated with dental caries (Colby and Russell, 1997). Mutans streptococci are a group of cariogenic species consisting of S. mutans (serotype c, e, f and k), S. sobrinus (d and g), S. downei (h), S. cricetus (a), S. rattus (b), S. ferus (c), and S. macacae (c) (Saravia et al., 2013; Nakano and Ooshima, 2009; Whiley and Beighton, 1998). Among mutans streptococci, the prime causative organisms which are involved in human dental caries are S. mutans and S. sobrinus and they are commonly isolated from dental plaques. Both S. mutans and S. sobrinus produce large amounts of acids and extra polysaccharides that cause demineralization of hydroxyapatite component of enamel and dentine thereby leading to dental caries (Kim et al., 2011; Matsuyama et al., 2005; Oho et al., 2000). The non mutans streptococci organisms constitute anginosus, bovis, mitis, pyogenic and salivarius groups.

The understanding of the genotypes of the mutans streptococci and their diversity in dental caries may aid in developing new treatment methods of caries and vaccine development strategies so as to promote health and to prevent the disease. The distribution of different genotypes in the caries subjects depends on many parameters including gender, food behavior, oral hygiene, etc (Moynihan and Petersen, 2004). Bengaluru, being a cosmopolitan city with multicultural people with
diverse food behavior, studying the dynamic or distribution of mutans streptococci in such population yield valuable data than studying the homogenous group of the subjects.

Four levels of strategies were followed in the present investigation to study the frequency or dynamics of mutans streptococci distribution in the dental caries. The lower two levels include morphological and biochemical identification of mutans streptococci which gives an idea about the mutans streptococci at species level and biotypes respectively. The third level of strategy including multiplex PCR and AP-PCR studies to corroborate the results of first two levels. Multiplex PCR strategies also used to identify the serotypes of mutans streptococci. Fourth level of strategy included 16S rDNA sequencing followed by phylogenetic analysis. The strategies were designed in such a way that the clinical isolates will respond to any one of them and get characterized.

In the present study, 35-44 years of age group was selected as standard monitoring group among the five age groups as per WHO guidelines. Based on earlier studies and to have a power of 90% using Z Test a sample size of 38 subjects along with reference strains was arrived.

The study was focused mainly on *S. mutans* and *S. sobrinus* as these species are strongly associated with human dental caries (Kim *et al.*, 2011). In the present study, it was observed that males had lower DMFT value (2.63 ± 0.90) than females (3.90 ± 1.56) which is in accordance with earlier reports (Petersen and Kaka, 1999; Luan *et al.*, 1989). Although the factors associated with the increased incidence of caries in females is unknown, it is believed that they may be due to difference in dietary habits (Luan *et al.*, 1989) and the type of mutans streptococci associated with caries.

The plaques samples plated on the MSB media showed three different colony morphologies (Table: 9) which were compared and analysed with standard reference strains (C1, C2, C3, C4, C5, C6, C7 and C8). Glistening bubble and extracellular
polysaccharide surrounded colonies were more prevalent among the variants of S. mutans and S. sobrinus. The S. mutans and S. sobrinus colonies identified in the study population are presented in Table 2. The present study is in agreement with Wu et al., 2003, who reported that S. mutan could not always be distinguished from S. sobrinus by colony morphology and is unreliable for species identification.

Gram staining and catalase test results demonstrated that all the clinical isolates belong to the genus Streptococcus. The biotyping data of thirty eight isolates showed that biotypes I, IV and V were present in the subjects (Table 7). The discrimination of mutans streptococci biotypes among male and female subjects of the study population revealed that the S. mutans (biotype 1) is predominant in both the sexes (Fig. 1), whereas there is a significant difference in the proportion of other biotypes. The proportion of biotype IV is high in female subjects when compared to male (Fig. 1).

The relationship between caries active females and high incidence of S. sobrinus is explaining the involvement of biotype IV in severe dental caries in female. This was earlier demonstrated by Wu et al., 2003.

Multiplex PCR experiment detected and differentiated S. mutans and S. sobrinus among mutans streptococci at their species level. Among thirty eight isolates, five were remained unidentified, whereas twenty five isolates were identified as S. mutans and eight as S. sobrinus. Among the five unidentified isolates, four strains (P3, P4, P8 and P33) could neither be identified by biotyping (Shklair and Keene, 1974; 1976) nor by multiplex PCR (Wu et al., 2003) targeting gtfB and gtfI genes while one isolate (P9) identified as S. sobrinus by biotyping remained unidentified by multiplex PCR. The possible explanation for not identifying four strains by biotyping and PCR methods is that they may belong to non-mutans streptococci in accordance to the results of Yoo et al., 2005 and Russell, 2009. Since it is expected that all S. sobrinus strains should respond for a GTFI primers (Wu et al., 2003), the above strain couldn’t be confirmed as S. sobrinus. Further molecular
technique like 16S rDNA sequencing was carried out, to identify the unidentified strains.

Kappa statistics was performed to compare the agreement between the three methods namely morphology, biochemical tests and multiplex PCR experiments. Biochemical tests showed better agreement with PCR than morphology (Table 10). The present study results of Kappa statistics confirmed the findings of Wu et al., 2003.

Multiplex PCR experiment for serotyping was conducted with primers specific for four serotypes namely c, e, f, and k (Table 2). Previous epidemiological studies revealed that serotype c is the most common serotype isolated from dental plaque, being found in nearly 80% of S. mutans positive samples. Serotypes e and f are found in about 20% and 2% of patients, respectively (Abranches, 2011). Strains belonging to serotype k is most infrequent and only reported from the subjects of Japan, Thailand and Finland (Nakano et al., 2007; Nakano and Ooshima, 2009; Lapirattanakul et al., 2009). Our studies also confirm that serotype c was the predominant one in the subjects, though all four were reported. Serotype k which is most infrequent serotype was also reported in the present study. As per our limited knowledge this may be the first time that serotype k is reported in Indian population. In the present study, the serotyping of S. mutans was carried out by designing a multiplex PCR for all the four serotypes (Plate 14 and 15). This strategy can be developed into a strong diagnostic tool for detection of S. mutans serotypes.

AP-PCR fingerprinting technique was employed to identify and differentiate S. mutans and S. sobrinus species. Among the eight working primers, except 970-11 primer, other primers could not able to identify and differentiate S. mutans and S. sobrinus species. Among the eight working primers, 970-11 primer alone was able to identify and differentiate S. mutans and S. sobrinus species. The 970-11 primer generated characteristic and unique AP-PCR fingerprints for S. mutans and S. sobrinus in agreement to the study of Truong et al., 2000.
It can be seen from the AP-PCR fingerprints with primer 970-11 (Plate 23 and 31) two amplicons of around 1500 bp and 700 bp were common in all the 25 isolates of *S. mutans* and reference strains C1 (MTCC 497), C2 (ATCC 25175), C3 (MTCC 890) whereas around 600 bp and 500 bp were common in all the eight isolates of *S. sobrinus* including reference strains C4 (ATCC 33478) and C5 (KCOM 1221). The amplicons on the gel, indicated that these could be used as a species specific marker. This is in consistent with the results of Troung et al., 2000. From the results of AP-PCR fingerprinting, it can be concluded that the common amplicons present in *S. mutans* and *S. sobrinus* isolates can be considered for the development of Sequence Characterized Amplified Region (SCAR) which is a valuable technique in disease diagnosis.

All the thirty eight isolates were subjected to 16S rDNA sequencing for studying the lineage among the mutans streptococci isolates. From the 16S rDNA sequencing results, it can be concluded that multiplex PCR gave reliable results for identification and differentiation of *S. mutans* and *S. sobrinus*. Among the five unidentified isolates, it was found that four isolates were non-MSO (*S. anginosus*- P3, P4, and P8 and *S. sanguinis*- P33) and one was found to be mutans streptococci (*S. downei*).

*S. downei* was first isolated from dental plaque of monkeys and were initially designated as *S. mutans* serotype *h* by Beighton et al., 1981 and later in 2005, Yoo et al., reported in Korean human subjects. To the best of our knowledge, this is the second report of isolation of *S. downei* from humans in India. Further investigations are recommended to determine the role of *S. downei* in caries by studying their virulence factors.

Among thirty eight clinical isolates from caries active subjects, four (10.52%) were detected to be non-MSO and the findings are in accordance to Russell, 2009. The non-mutans streptococci organisms were unable to be differentiated from mutans streptococci based on colony morphology. The results from the present study indicate that the MSB agar could not comprehensively inhibit non-MSO and inadequate to
differentiate the oral streptococci species. Our study is in agreement with Russell, 2009 in making Type II error, failing to detect the true bacteria, based on morphology.

After the identifying of the non-MSO from 16S rDNA sequencing, it was found that there were different biotypes of *S. anginosus* (Table 7) in study population. The possible explanation for detecting *S. sanguinis* is that they usually compete with the mutans streptococci group for colonizing on the surface of the tooth (Yamaguchi, *et al.*, 2006) and Takahashi, 2005 has reported that mutans streptococci are inhibited by hypothiocyanite which is produced by the metabolic activity of *S. sanguinis*. Yamaguchi *et al.*, 2006 has reported that *S. sanguinis*, has low cariogenicity and aids in aggregation of oral bacteria and maturation of dental plaque.

In one of the earlier study, it was found that *S. anginosus* were able to grow on MS-SOB medium which contains multiple antibiotics namely Bacitracin, Aztreonam and Fosfomycin (Sasaki *et al.*, 1995) and in an another study, Yoo *et al.*, 2005 reported that eight strains of *S. anginosus* and one strain of *S. sanguinis* were able to grow on MSB agar. These investigations clearly indicate that both species have gained resistance over time. There are sufficient studies on *S. anginosus* involvement in carcinogenesis (Sasaki *et al.*, 1995; Sasaki *et al.*, 1998) but there is a scarcity of information on *S. anginosus* involvement in caries. The present study indicates that both *S. sanguinis* and *S. anginosus* have acquired resistance to both 20% sucrose and 0.2 units/ml bacitracin, and was able to grow on MSB agar in accordance with previous studies (Yoo *et al.*, 2005).

16S rDNA sequences obtained from thirty eight isolates were deposited in NCBI and accession numbers were received (Table 13). The phylogenetic tree (Fig. 7) distinctly highlighted five clusters among the clinical isolates. These clusters clearly show that each species have evolved distinctly. Among mutans streptococci group, *S. mutans, S. sobrinus* and *S. downei* were closely clustered when compared with non mutans streptococci organisms that are *S. anginosus* and *S. sanguinis*. *S. downei* showed that it has branched from *S. sobrinus* isolate, which is in agreement with Whiley *et al.*, 1988 and Yoo *et al.*, 2005. From the phylogenetic tree, it was observed
that the multiple serotypes of clinical isolates (P17, P20 and P29) clustered together between serotype e and c. The possible explanation for detecting multiple serotypes is that, these isolates may have evolved from serotype e and c (Fig. 7).

From our comprehensive investigation, we conclude that a multiplex PCR has to be designed and validated for identifying S. mutans, S. sobrinus, S. downei, S. anginosus and S. sanguinis to overcome type II error in identifying S. anginosus and S. sanguinis (Yoo et al., 2005).

Bacteria utilize a cell-cell communication process termed “Quorum Sensing” (QS) to monitor cell population density and to regulate gene expression in response to fluctuations in cell numbers (Miller and Bassler, 2001). Many streptococci use QS systems to regulate several physiological properties, including ability to incorporate foreign DNA, tolerate acid, form biofilms and become virulent. These QS systems are primarily made of small soluble signal peptides that are detected by neighboring cells via a histidine kinase/response regulator pair (Cvitkovitch et al., 2003).

In vitro QS studies using reference strain (MTCC 497) confirmed the biofilm formation which was supported by light microscopic visuals (Plate 33). These biofilms simulated in the study formed the source of QS signals in S. mutans. Peptide purification and characterization revealed that a spectrum of peptides were present in the culture broth. Five peptides of different molecular weights were reported in the MS spectrum (Fig. 8) with the molecular weights of 1589.008, 1831.161, 2066.292, 2365.608 and 3136.265 Da. When the above mentioned peptides were searched against database quorumpeps (http://quorumpeps.ugent.be), one peptide with a molecular mass of 2365.608 Da matched with the signal peptides quoted in the reference. Based on the molecular weight and quorumpeps reference database search, the peptide was identified as CSP. This is in line with finding of Petersen et al., 2006. This simulated study once again confirmed the role of CSP in biofilms formation and QS in S. mutans.
The spread of antibiotic resistance among pathogens has emerged into most challenging phenomena of reversing progress in therapeutic success since it causes increase in rate of mortality. The scientific approaches of the past are not adequate to address these challenges and consequently require innovative strategies to minimize these threats. At present, biofilms mediated antibiotic resistance has become a major cause of anxiety for many clinical and device associated infections. In our study *S. mutans* shows strong tendency of biofilms formation. The role of QS in biofilms mediated virulence is well reported. QS inhibition also called quorum quenching is considered an attractive modality of therapeutic intervention to overcome the antibiotic resistances and revolutionary pressure on the targeted bacterial population.

Modern approaches for the development and validation of quorum sensing inhibition involves strategies targeting quorum sensing signal production, strategies targeting signal themselves, and strategies targeting receptor interactions. Quorum quenching using second strategy is more promising and the agents for such activities have been reported in many natural resources including plants. In this study, pomegranate dried peeled methanolic extract powder was used as quorum quenching agent. The MIC of PME against reference strain and clinical isolates was found to be 230 µg/ml. It was noticed that PME does not possess minimum bactericidal effect up to 500 µg/ml concentration. The sub-MIC concentration of PME was found to be 110 µg/ml and the highest level of quorum inhibition was expected at this level. In growth curve study assay, the PME did not alter the growth of *S. mutans* but it significantly interfered in biofilm formation. The light microscopic analysis and confocal microscopy (Plate: 37 and 39) with COMSAT analysis showed a significant negative influence of PME over the biomass and maximum thickness of the biofilm formation.

The HPLC chromatogram of PME exhibited the presence of various compounds. Among them, ellagic acid presence was noticed which can be suspected for antibiofilm activity as reported by Bakkiyaraj *et al.*, 2013. Ellagic acid prevents the biofilm formation by precipitating the vital proteins involved and alters the charge of the surface thereby interfering with cell-substratum interactions and biofilm development (Liu *et al.*, 2008). The lower sub-MIC concentration of PME may be a
promising quorum quenching candidate which holds the potential for use as anticaries drug which can be commercially exploited.

The diversity and dynamics of mutans streptococci observed in the present study may aid in designing comprehensive treatment strategy and possible cure for candidate vaccine development based on serotypes, whereas QS and quorum quenching studies may help us to formulate commercial personal hygiene products against dental caries.