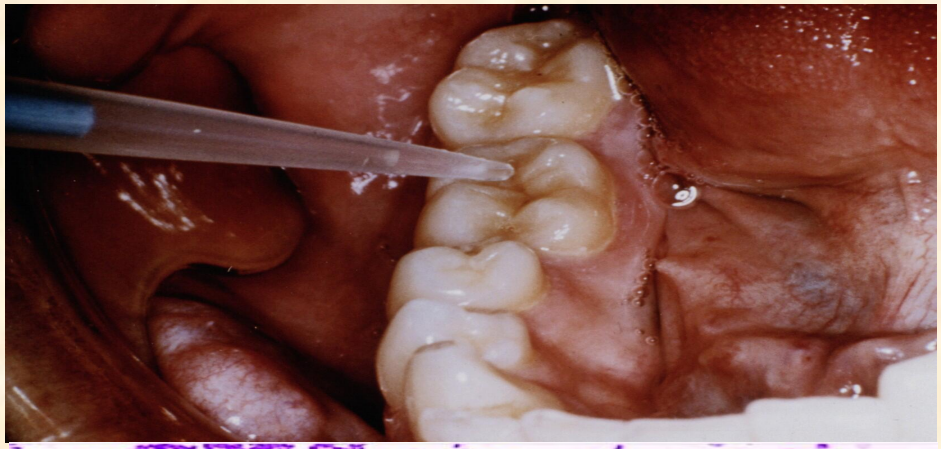

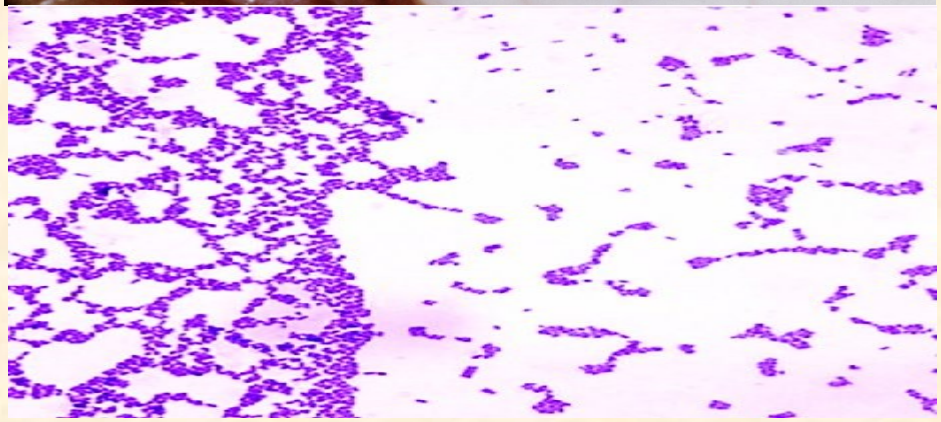

ISBN: 978-93-94638-03-7

Publishers:
Thanuj International Publishers,
8/173-B, Vengayapalayam, Rasipuram,
Namakkal, Tamil Nadu, India – 637406.
E-mail: thanujinternationalpublishers@gmail.com

Printers:
Dhazh Computers (Graphic Designer)
No: 442- A, 10th East Cross Street,
Munthirithoppu, Annanagar,
Madurai – 20, Tamil Nadu, India.
E-mail: narennarayanasamy@gmail.com

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First Edition

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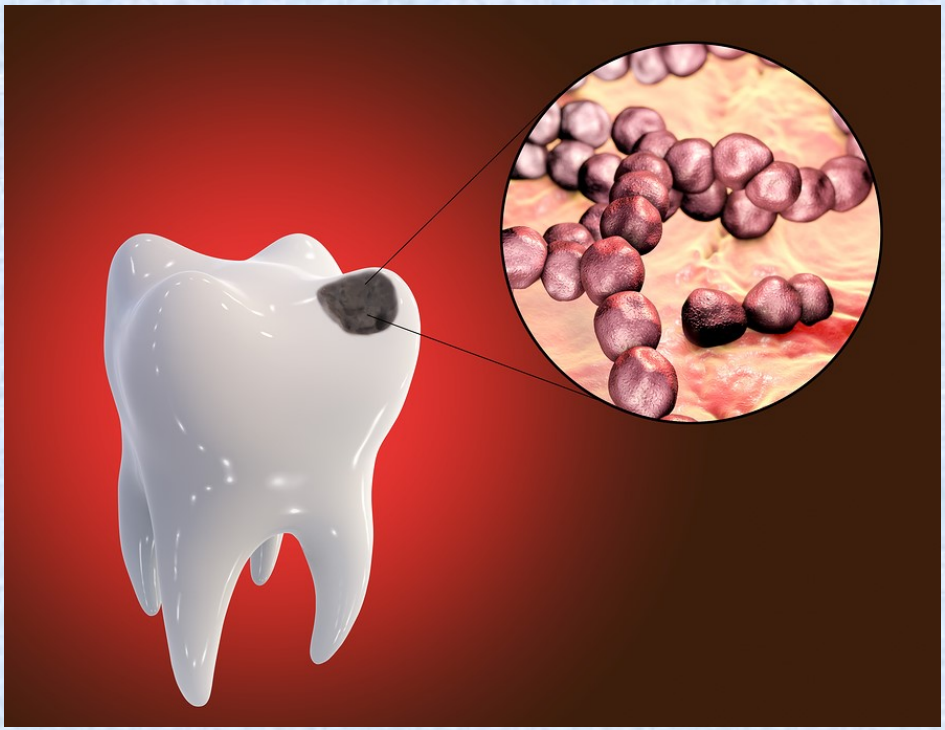

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ISBN: 978-81-955196-4-4

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First published in India in 2022

This edition published by Thanuj International Publishers

ISBN: 978-81-955196-4-4

Price: Rs: 800.00

Published by:

Thanuj International Publishers,
8/173-B, Vengayapalayam, Kakkaveri, Rasipuram,
Namakkal, Tamil Nadu,
India – 637406.
www.darshanpublishers.com
E-mail: thanujinternationalpublishers@gmail.com

Printed by:

Dhazh Computers (Graphic Designer)
No: 442- A, 10th East Cross Street,
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Contents

1. INTRODUCTION

Oral diseases related to dental biofilms, grief the majority of the world's population and dental caries is still the single most prevalent and costly oral infectious disease (Costerton *et al.,* 1987; Aas *et al.,* 2005). Dental caries is one of the most common diseases of childhood and transmissible infectious human disease that still represent a significant public health problem. Even it is common in the children but experience of caries among adults and old age is also as high as 80% in developing countries (Andreasen and Andreasen, 2002). The distribution and severity of dental caries indicates significant geographical variations and further shows marked differences with the same region or country, (Brown *et al.,* 2000; Carvalho *et al.,* 2001; Bonecker *et al.,* 2003). The WHO reports and observation of several epidemiological surveys has indicated reduced experience of dental caries in children as well as adults. Decline in incidence of caries observed in children and adolescents from developed countries while doing it in the developing countries show a tendency to increase. Reduction in the caries in developed countries is a result of a number of public measures coupled with changing living conditions, life styles and improved self-care practices (Paterson *et al.,* 2005). According to the WHO, dental caries is still a major public health problem in most industrialized countries affecting 60-90% of children and the vast majority of adults and is a most prevalent oral disease in several Asian countries while it appears to be less common and less severe in African countries.

Incidence of caries in all age groups is expected to be high where consumption of sugar and inadequate expose to fluorides and less awareness of oral

hygiene (Peterson, 2003). Since 1940, fluoride used as anti-caries agent in toothpaste and mouthwash solutions to minimize the incidence of dental caries especially in the school children (Koch and Poulsen, 2006). However, Excessive and continuous use of fluoride leads to many side effects like teeth fluoresces and joint pains. Therefore, oral hygiene is one of the main factors in caries control strategies. Many studies reports that incidence of caries is more in rural areas than in urban so it clearly indicates that lack of oral hygiene and health care awareness (Lenander-Lumikari and Loimaranta, 2000).

Individuals consuming tobacco in either form of smoking or chewing experienced more oral diseases. In tobacco smokers the saliva secretion is not affected however it affects the physiology of the saliva by changing the pH, buffering capacity and immunity. This change enhances the growth of few bacterial species especially the lactobacilli and streptococci become opportunistic pathogens and increases the dental caries in susceptible individuals (Sajith *et al.,* 2006). Association between the cigarette smoking and dental caries has well established in the old age peoples (Locker, 1992; Jette *et al.,* 1993). Among middle age group (Axelsson *et al.,* 1998) and young adults (Sgan-Cohen *et al.,* 2000) results are inconstant. Over all predisposal factors of dental caries are genetic factors, oral hygiene, diet habits and the general health of individuals.

The oral ecosystem comprises several distinct habitats each supporting the growth of a characteristics microbial community adapted to the local environment (Theilade and Theilade, 1985). The oral microflora has the ability to adjust their physiology to the changing environments, although the nature and extent of the ability to adapt varies among different strains and species. These changes in the balance between the host and the oral microbial flora may lead to mucosal infections and increase the

prevalence of both dental caries and periodontal diseases. The oral bacteria are also involved in many systemic diseases such as bacterial endocarditis, arthritis, aspiration pneumonia, coronary heart disease and strokes (Gudachun *et al.,* 2001; Dom *et al.,* 1999; Genco *et al.,* 2002). Thus, the above reported facts create an impetus to study systematically the organisms of the oral cavity and their role in oral and systemic diseases. Further, these studies provide an insight for diagnosing and treating diseases caused by these organisms.

Primarily the initiation of caries takes place by the demineralization of enamel of the tooth by the action of acids produced by the cariogenic bacteria present in the dental plaque. Plaque formation starts with deposition of salivary components on the surface of the tooth, which act as substrate for the fixation of cariogenic and other bacteria to form biofilm called dental plaque. This biofilm matrix derived from these bacteria (Flamming and Wingender, 2010) becomes niche for as many as 200-300 bacterial species. Many reports have been emphasizing that presence of *Streptococcus mutans* consistently linked with the formation of dental caries in humans (Loesche, 1986). May be *S. mutans* survives in an extremely diverse, high cell biofilm on the tooth surface against organic acid produced by the bacteria present in the plaques drops the pH of the plaque that also forms the growth of the *S.mutans* and some other acid producing bacteria like lactobacilli (Beighton, 2005). Adhesion is the initial step in the formation of biofilm communities. The adhesion material produced by *S.mutans* in the presence of sucrose is a water insoluble polysaccharide called glucan. Glucan production is mediated by extracellular enzyme glucosetransferases and fructosyltransferases and is binds to tooth surface and *S.mutans* with the help of glucan binding proteins present on *S. mutans*

(Steinberg 2000; Bansa and Vickerman, 2003). Persistence of microbial activity in the plaque and formation of dental caries again depends on the general health, sucrose content in the diet and the salivary flow and its components. The saliva plays an important role in lubricating the oral tissue and protects the teeth, oral mucosal surfaces in different ways. Protective functions of saliva are not only depends on its flow and buffering capacity and types of organic and inorganic components present in saliva (sIg A). Finally, "caries to be or not to be", is a complex phenomenon involving internal defense factors, such as saliva, tooth surface morphology, general health, and nutritional and hormonal status, and a number of external factors-for example, sugar in diet, the microbial flora colonizing the teeth, oral hygiene, and fluoride availability (Selwitz *et al.*, 2007).

Among the oral micro flora viridian group of streptococci are present more and act as an opportunistic oral pathogens. of these mutant streptococci plays an important role in the formation of dental and periodontal diseases. Of seven mutant streptococci, *S.mutans* and *S. sobrinus* are frequently isolated from the saliva and the dental plaques of dental caries patients and normal individuals. Among, these two *S. mutans* is more frequently isolated from the saliva and plaque of normal and dental caries individuals. Even *S.mutans* number presence in the saliva and in the plaque is not directly associated with the dental caries and its severity; however, *S.mutans* is invariably isolated from the individuals with dental caries. *S.mutans* proven prime mutans streptococci to initiate the dental plaque formation and that leads to dental caries. As dental caries is a multi-factorial disease influenced by the presence of cariogenic *S.mutans* strains, sucrose in diet, poor oral hygiene, poor general heath and habits of the individuals. Thus,

initiation of dental caries mainly depends on the presence of *S.mutans* with virulent cariogenic factors. Therefore, in this study we have isolated the *S.mutans* from the unstimulated saliva and phenotypic and genotypic characterization to know the types of traits are responsible for the formation of dental caries. Risk factors like age, gender, socioeconomical status and habits on the prevalence of *S. mutans* in an individual. Therefore, in this study we have planned to investigate the prevalence of *S.mutans* in individuals with and without caries, with and without the habit of consuming tobacco, belonging to different age groups and different socio-economic groups.

2. REVIEW OF LITERATURE

2.1. Tooth decay - Dental caries risk and associated Streptococcus species

Tooth decay is one of the most common infectious diseases affecting humans and is a significant health care issue in both developed and developing nations (Mitchell, 2003). This decay is the result of the interaction of the oral microflora (plaque), the tooth surface, nutrition, and the oral environment over time and results in a carious lesion of the tooth enamel (Beighton, 2005; Takahashi and Nyvad, 2008). While in recent years, overall incidences of dental caries have declined in industrialized nations, but are rising in developing nations (Chu and Lo, 2008). Even in the US, some reports show caries incidence among children under 12 years of age, to range from about 40 to 50% of children tested, but as many as 70-85% of children have caries by age 17 (Smith, 2010; Bagramian *et al.*, 2009; Brown and Selwitz, 1995; Bowen, 2002; Edelstein, 2002). Although these figures do represent an overall decline in caries rates in the US over the last 20 years, they still represent a large portion of the population and the rate of decline seems to have reached a plateau (Bagramian *et al.*, 2009). Furthermore, caries prevalence is not evenly distribute across the population and communities with the highest incidences are usually, those in lower socioeconomic groups that have limited access to sufficient oral health care (Bowen, 2002; Featherstone, 2000). Despite the fact that studies show a decline in caries in the United States, tens of dollars are spent in this country each year on treatment of tooth decay, and this figure represents only the fraction of the population that seeks out and can afford treatment (Benjamin, 2010). In other industrialized nations such as China and the UK, caries prevalence in the past decade has

been over 50% in children. In developing nations, where oral health care is significantly less available, caries rates are rising at an alarming rate. Studies done in the past decade in countries such as the Philippines, Peru, Mexico and Taiwan revealed presence of caries in 75 to 90% of children (Bagramian *et al.*, 2009).

In most of the developing countries, the levels of dental caries were low until recent years, but are now tending to increase due to increasing of consumption of sugars and inadequate exposure to fluorides. In contrast, a decline in caries has observed in most industrialized countries over the past 20 years or so because of effectiveness of fluorides coupled with changing living conditions, lifestyles and improved self-care practices. According to Peterson *et al.*, (2005) the prevalence of dental caries among adults is high as the disease affects nearly 85-100% of the population in the majority of countries.

In India, dental caries survey took place in 1939, by Taylor and Day and reported low prevalence of caries in children of Kangra valley of Punjab (Johnson, 1991) and dental caries prevalence was less in Indian children as compared to American children. There after many studies have been conducted in different parts of India, Shourie in 1941 showed 44.6% prevalence of caries in the age group of 5-7 years and an average DEFT (Decay, extracted filled teeth) was 1.37%. In 1985, Tiwari has conducted a survey on decayed, missing or filled permanent teeth (DMFT) in school children. National epidemiological survey done using WHO guidelines and reported the DMFT index in different age groups (WHO, 1983). Dental cares prevalence rate was in North Eastern part of India was 24.7% compared to 88.90% in urban areas of same region. Prevalence

of caries is very low in the Southern parts of India and DMFT indices for Karnataka, Andra Pradesh and Kerala was 0.6, 1.63 and 2.10% respectively.

Hundreds of caries risk factors and indicators has been identified over decades of research (Harris *et al*., 2004). Most commonly used risk factors are salivary factors (e.g. flow rate and buffering capacity), carbohydrate intake, oral hygiene, fluoride exposure, previous caries experience, and socioeconomic characteristics (Tinanoff, 1995; Brathall and Petersson, 2005; Eriksen and Bjertness, 1991). Traditionally, *S. mutans* and *Lactobacillus* counts have been the principal biological factors used for prediction of future caries experience. These salivary tests aid dental professionals in identifying the two extremes in a disease susceptible population but are less effective in predicting caries in moderate risk groups. The accuracy of tests for *S. mutans* in predicting future caries in the whole population is less than 50 % (Reich and Newbrun, 1999).

The discovery that demineralization of tooth enamel is caused by acid produced by bacteria in dental plaque occurred as early as the 1890s and coincided with Koch's postulates and the "germ theory of disease" (Russell, 2009). Since that time there has been some debate between the two main theories of the role of plaque in caries development. The non-specific plaque theory states that the collective activity of most or all plaque bacteria contributes to the increase of plaque acids that leads to caries. The specific plaque theory states that one or a few specific bacteria are responsible for caries development. Microorganisms such as *Streptococcus mutans* and *Lactobacillus* have acquired significant advantages over other oral acidogenic bacteria due to their acidoduric nature.

Streptococcus mutans is widely accepted as one of the most important etiologic agents in the caries development and show that it causes caries in germ-free and specific pathogen-free rat models. While incidences of caries have found without *S. mutans* on the contrary high percentages of *S.mutans* have recovered from non-carious individuals, however *S. mutans* remains the most common species associated with caries (Takahashi and Nyvad, 2008; Kleinberg, 2002; van Houte, 1994; Hamada and Slade, 1980; Nyvad and Kilian, 1990). Although *S. mutans* possesses several properties that promote its cariogenicity, robust biofilm formation in the presence of dietary sucrose is a critical component in the development of caries.

Control and prevention of dental caries depends on the health of an individual, diet, oral hygiene and visit to dentist. Among these, oral hygiene may alone help the individual in controlling and preservation of dental caries, however, person with good oral hygiene, but having bad habits like, consuming or chewing various intoxicants including tobacco may become more susceptible for caries. Tobacco consumption in either form smoking or chewing by individuals experienced more oral diseases and correlation with prevalence of caries. In tobacco consuming persons saliva secretion may affect, however it affects the physiology of the saliva by changing the pH, buffering capacity and immunity. This leads to growth of cariogenic bacteria and enhance the chances of dental caries (Sajith *et al.*, 2006).

Dental caries is progressive diseases that inevitably lead to the eventual loss of a tooth unless a dentist intervened surgically. The conventional method of dealing with dental decay involved detection of the carious lesions, followed by drilling and filling (Fontana and Zero, 2006; Evans and Howe, 2008; Domejean-Orliaguet *et al.*, 2006).

Even though the antibiotics play little role in managing the caries but it is necessary to know the antibiogrm of important cariogenic bacteria / microorganisms which may help in the post surgical treatment and to treat the mild caries cases. Oral health has recently recognized as important to overall health. The report by the surgeon General in 2000 emphasized the importance of preventing oral diseases, specifically dental caries. It known that dental caries is a preventable disease with a combination of "individual, professional, and community measures" (Satcher, 2000).

Even *Streptococcus mutans* universally accepted as prime etiological agent of dental caries, it can be isolated from the saliva and the dental plaque of all age group individuals with or without caries. This clearly indicates that *S. mutans* is present in all individuals and its cariogenity is determined based on its virulence and individual's health and diet. So, it is essential to know the cariogenic virulence characteristics of *S. mutans* by conventional and molecular methods. Thus the literature on the details about dental caries, etiology and their characterizations, antibiogram, and its control measures are reviewed in the following sections.

2.2 Dental Caries:

Dental caries is one of the most common infectious diseases of mankind. It becomes a major health problem in most countries mainly because of its high prevalence. Its consequences upon oral and general health of individuals (Pain, impairment of function, reduced quality of life) were one of the determinant factors for "Oral health" to become a special unit in year 2000, was to reduce Decayed, Missing or Filled permanent Teeth (DMFT). So that it would be no more than 3 DFMT per individual at 12 years of age. This goal achieved by 68% of the countries (WHO report, 2003). However, there is

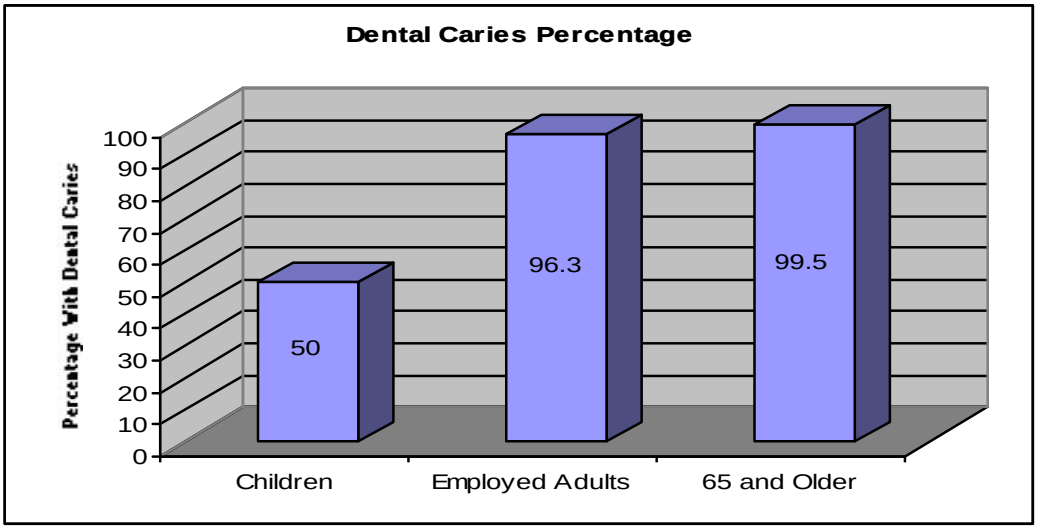
still a very unequal distribution of caries prevalence between countries, not very much related to their developmental stage, but more likely related to the individual socio-economic status. Dental caries is a multifactorial infections disease that does not develop in the absence of dental plaque (dental biofilm). The principal (Cariogenic) bacteria are transmissible and usually acquired in infancy.

Types of caries		**Microorganisms associated**
1.	Pit and Fissure	*S.mutans, S.sanguis* and *Lactobcillus* sps
2.	Smooth surface	*S.mutans, S. salivarius,*
3.	Root surface	*Actnomyces viscosus, A. naeslundii, S.mutans* and *S.sanguis*
4.	Deep dental caries	*Lactobcillus* sps, *A. naeslundii other filamentous rods*

2.2.1. Prevalence of dental caries:

A. International level:

Dental disease is a huge problem worldwide. According to 1993 Public Health Reports **(Figure)** "more than 50% of U.S. children, 96.3% of employed U.S. adults and 99.5% of Americans 65 years and older have experienced dental caries" (Petersen *et al.,* 2005; Petersen, 2003; World Oral Health Report, 2003.)



It is for this reason that dental caries has said to be the most common bacterial infection in humans. In 1992, $38.7 billion spent on dental services in USA and was increase to $203.6 billion in 2000. This growing problem is a large burden on consumers. In fact, tooth decay may be the most expensive because it is so constantly reoccurring because of the present treatment method. Unless patients had professional cleaning and fluoride treatments every two weeks, the disease would not stop. Professionally delivered tooth debridement is so labor intensive that its cost would make it economically unavailable to most individuals. This lack in proper dental care comes mostly from the fact that there are only a few practical preventative methods aimed at controlling the bacterial factors involved in decay. A more practical way to prevent this disease would be for dentists to identify “infected” individuals (which would be almost everyone in the beginning) and then, using antimicrobial treatment, eliminate or suppress the disease-causing bacteria in the mouth, while allowing harmless bacteria to grow in its place.

In the Alma Ata conference in 1978, WHO informed the alarming percentage of population affected for dental health care (Petersen *et al*., 2005). However, until now, this program has depended on social-economic condition and ability of each country. Therefore, the result of this program has varied. WHO reported that dental caries experience in children is relatively high in America (DMFT=3.0) and in the European region (DMFT=2.6) whereas the index is lower in most African countries (DMFT=1.7) and in most developing countries.

The prevalence of dental caries among children and adolescents living in industrialized nations declined in the 1970's and 1980's (Marthaler and Vrbic, 1996; Fontana and Zero, 2006). It was suggest by most experts that regular exposure to fluoride was the most significant contribution to this decline in caries (Petersson and Brathall, 1996; Kidd, 1999). The decline has since stabilized in many countries: however, in some areas, there are reports that the prevalence of caries is again on the rise (Dye and Li, 2007). A report of the Surgeon General that dental caries is the single most common chronic disease of childhood, with a prevalence rate five times greater than that of asthma (U.S. National Institute of dental and craniofacial Research; 2000). The diminished pervasiveness and severity of dental caries in many developed countries, along with an increasing number of dentate elderly retaining their teeth longer, have brought about a noticeably skewed distribution of disease in the population (Hausen and Seppa, 2000; Pitts, 1998). It has been estimated that approximately (60-75%) of the caries occurs in only 20-25% of the population. In addition, findings from the national preventive dentistry demonstration program showed that most severe disease was limited to only 5% of the children (Disney and Zack, 1992). With the earlier high prevalence of dental

caries observed in western civilization between 1950's and 1980's most of society was categorized as having the disease (Moss and Zero, 1995). This led health professionals to utilize a population-based approach in which its goal was to alter the distribution of disease by controlling the underlying determinants of dental caries in the entire population (Rose, 2001; Beck, 1998).

In most developing countries, the levels of dental caries were low until recent years, but prevalence rates of dental caries and dental caries experience are now tending to increase. The main reason for this trend is the increasing consumption of sugars and inadequate exposure to fluorides. In contrast, a decline in caries has observed in most industrialized countries over the past 20 years or so. This result was explain by the effectiveness of fluorides together with changing living conditions lifestyles and improved self-care practices (Petersen *et al*., 2005).

B. National level:

In India, the earliest references of dental status back to 1939, when Taylor and Day reported low prevalence of caries in children of Kangra valley of Punjab (Johnson, 1991). Later in 1940 Day and Tendon carried out an investigation in another group of children in Punjab and reported that dental caries prevalence was less in Indian children as compared to American children. However, Shourie in 1941 for the first time in India conducted a multi-centric epidemiological study in various parts of country in the age group of 5-7 years (Damle, 2002). The results of that study showed caries prevalence of 44.6% with an average DEFT of 1.37. Thereafter, a number of point prevalence studies have conducted in different parts of the country.

In 1985, a national epidemiological survey was conducted by Tiwari and others involving three age groups of 5-6 years, 15-16 years and 30-35 years of general population, using standardized recording methods and the WHO (1983) DMFT/DEFT index(Johnson, 1991). The data available from previous caries prevalence studies indicate that it ranges from as low as 24.7% with deft of 0.6 in rural Abohar in northern part of India (Damle, 2002). There is varied picture of caries prevalence of 88-90%, whereas in rural Sikkim, it is only 22 % (Johnson, 1991). The high prevalence figures in the North Eastern region are possibly due to recent exposure to urbanization. Very few studies have conducted to estimate the caries prevalence in children below 6 years of age in western parts of India. Most of these studies were carry out in urban Mumbai. The prevalence figures of caries in this region were always seen to be high, as reported by Sehgal (1960), Anita (1962) and Tiwari (1985). In southern India, the dental caries prevalence was comparatively low as reported by Gupta *et al*., (1987) and Johnson 1991). The mean DEFT found in Karnataka, Andra Pradesh and Kerela were 0.6, 1.63 and 2.10 respectively.

C. Regional level:

In Gulbarga division, the incidence of dental disease was more (48.0%) among school children than the rural people (35.3%). Dental caries accounted for the highest incidence (21.0%) with 30.7% among school children to 14.9% among the rural people. The overall incidence of gingivitis and abscess was almost the same affecting (9.78%) and (9.46%) of individuals from rural people and school children (Jamshetty, 2004). Among rural people incidence of gingivitis was more (10.5%) than abscess (9.8%), while

among school children abscess accounted for slightly higher incidence (8.8%) than gingivitis (8.5%).

2.2.2. Caries risk factors.

A multitude of caries risk factors and indicators has identified over decades of research. (Harris *et al*., 2004) found 106 risk factors were significantly relating to the prevalence of dental caries. With so many factors, it can seem daunting to decide which variables should be chose for inclusion in the caries risk assessment instrument (CRA). The risk factors selected for research are typically dictate by the purpose of the study, as there are very few standardized CRA instruments available (Featherstone *et al*., 2007). The few CRA models currently in use are recent additions to the discipline and tend to focus on the principal factors associated with caries development, namely diet, microbial pathogens, and host susceptibility factors (Powell, 1998). The University of Toronto's CRA model is congruent with these instruments (e.g. cariogram, CAMBRA- caries management by risk assessment; Featherstone *et al*., 2007). In that, it maintains its focus on the basic caries risk elements that can easily identified in the dental clinic and modified through preventive care practices. The most commonly used caries risk factors and indicators in multifactorial CRA models include levels of cariogenic bacteria (i.e. *Streptococcus mutans* and *Lactobacillus*), salivary factors (e.g. flow rate and buffering capacity), carbohydrate intake, oral hygiene, fluoride exposure, previous caries experience, and socioeconomic characteristics (Tinanoff, 1995; Brathall and Petersson, 2005; Eriksen and Bjertness, 1991).

A. Levels of cariogenic bacteria:

Among the cariogenic bacteria, *Streptococcus mutans* and *Lactobacillus* have acquired significant advantages over other oral acidogenic bacteria due to their acidoduric nature. Not only are *S. mutans* able to survive in an acidic environment, but they have also adapted the ability to increase their rate of acid production, thus driving the pH in the oral cavity lower and forming a cariogenic plaque (Moss and Zero, 1995). Whereas *S. mutans* are the primary initiators of the formation of carious lesions (Loeshce, 1986) *Lactobacillus* contributes substantially to the propagation of the lesion due to their ability to survive at a lower pH than *S. mutans.* In addition, *S. mutans* have evolved the capacity to store energy for occasions when fermentable carbohydrates are scarce in the oral cavity. This incredible adaptation allows oral *S. mutans* levels to remain relatively constant regardless of dietary modifications. Lactobacilli have yet to develop this ability and thus *Lactobacillus* counts are often using to determine a patients' compliance to dietary changes (Hildebrandt, 1995).

Traditionally, *S. mutans* and *Lactobacillus* counts have been the principal biological factors used for prediction of future caries experience (Disney *et al*., 1992; Petersson and Bratthall, 2002; Beck *et al*., 1992; Scheinin *et al*., 1992; Krasse, 1998; Demers *et al*., 1990). Studies have shown that not only are these microorganisms related to the incidence of dental caries. Nevertheless, those children with high levels of these pathogens develop a significantly greater number of carious lesions than children with low levels (Zickert and Krasse, 1982). Nevertheless, salivary levels of *S. mutans* and *Lactobacillus* have been more successful in identifying low risk children than those at an elevated risk for developing dental caries (FDI working Group, 1998; Van Houte, 1993).

The accuracy of tests for *S. mutans* in predicting future caries in the whole population is less than 50% (Reich and Newbrun, 1999). Unfortunately, despite their prevalent use in CRA's, the predictive power of microbiologic tests remains uncertain at the individual level as well (FDI Working Group, 1998; Eriksen and Bjertness, 1991; Van Houte, 1993). With the exception of findings in young children, salivary levels of *S. mutans* have been disappointing with regard to risk assessments (Tinanoff, 1995). Salivary tests for *Lactobacillus* are even less sensitive than tests for caries prediction than *S. mutans*. This is to be expected as *Lactobacillus* is not primarily responsible for the initiation of dental caries, but they are found in large quantities when a considerable amount of carbohydrates have been consumed (Fontana and Zero, 2006). *Lactobacillus* counts are commonly analyzed to reflect dietary changes and the test results can be useful to motivate patients and to monitor changes in oral hygiene, diet, and microbial therapies (Petersson, 2003; FDI Working Group, 1998; Petersson and Bratthall, 2003).

B. Salivary factors:

Human saliva not only lubricates the oral tissues, making oral functions such as speaking, eating and swallowing possible, but also protects teeth and oral mucosal surfaces in different ways. The lubricating and antimicrobial functions of saliva maintained mainly by resting saliva. Stimulation of saliva results in a flushing effect and the clearance of oral debris and noxious agents.

i) Salivary flow rate, Buffering effect and Dental caries:

Probably the most important caries-preventive functions of saliva are the flushing and neutralizing effects, commonly referred to as "salivary clearance" or "oral clearance

capacity" (Lagerlof and Oliveby,1994). In general, the higher the flow rate, the faster the clearance (Miura *et al*., 1991) and the higher the buffer capacity (Birkhed and Heintze, 1989). Reduced salivary flow rate and the concomitant reduction of oral defense systems may cause severe caries and mucosal inflammations (Daniels *et al.*, 1975; Van der Reijden *et al.*, 1996). It must be emphasize that no linear relationship exists among salivary secretion rate, caries activity, and DMFS/DMFT values (Birkhed and Heintze, 1989; Russell *et al*., 1990). The buffer capacity of both unstimulated and stimulated saliva involves three major buffer systems: the bicarbonate (HCO_3), the phosphate, and the protein buffer systems.

These systems have different pH ranges of maximal buffer capacity (Bardow *et al*., 2000), the bicarbonate and phosphate systems having pK values of 6.1-6.3 and 6.8-7.0, respectively. Since most of the salivary buffering capacity operative during food intake and mastication is due to the bicarbonate system (based on the equilibrium HCO_3^- + H+ <=> CO_2 + H_2O), sufficient saliva flow provides the oral cavity with the neutralizing components (Birkhed and Heintze, 1989). The phosphate and protein buffer systems make a minor contribution to the total salivary buffer capacity, relative to the bicarbonate system. An inverse relationship between buffer capacity and caries experience is well established, and salivary flow and buffer effect show an inverse correlation (Heintze *et al*., 1983) with caries susceptibility. Among the elderly, an inverse relationship of salivary buffer capacity in stimulated saliva has established for both enamel (Guivante-Nabet *et al*., 1998) and root caries (Ravald and Birkhed, 1991; Lundgren *et al*., 1998).

(ii) Antimicrobial proteins in Saliva:

a. Innate defense factors:

The innate defense factors identified in saliva have been extensively studied *in vitro,* and they express different antimicrobial properties (Tenovuo and Lumikari, 1991; Tenovuo *et al*., 1991). It is well know that many antimicrobial proteins in saliva interact *in vitro* with each other. The interactions result in additive, synergistic, or inhibitory effects on mutans streptococci, lactobacilli, or fungi. The main oral innate defense factors are the peroxidase systems, lysozyme, lactoferrin, and histatins. *In vitro,* these proteins are known to limit bacterial or fungal growth, interfere with bacterial glucose uptake or glucose metabolism, and promote aggregation and, thus, the elimination of bacteria. It should emphasized that, in addition to the antimicrobial action of both salivary peroxidase and myeloperoxidase systems (Mansson-Rahemtulla *et al*., 1987), one of the main purposes of these systems is to eliminate H_2O_2, Which is highly toxic for mammalian cells (Hanstrom *et al*., 1983; Tenovuo and Larjava, 1984). Especially lysozyme, lactoferrin, and peroxidases are present in measurable concentrations and mainly synthesized in, and secreted via, the major or minor salivary glands, but a smaller amount enters the oral cavity from tissue fluid or polymorphonuclear leukocytes (PMNs) *via* the gingival crevicular fluid (Tenovuo and Lumikari, 1991; Tenovuo *et al*., 1991). During early childhood, the non-immune salivary factors-e.g. lysozyme, salivary peroxidase, and peroxidase generated hypothiocyanite (HOSCN/OSCN') are present at levels similar to those in adults. However, lactoferrin, myeloperoxidase, and to protein are still significantly less abundant (Mandel *et al*., 1983; Tenovuo *et al*., 1987). All non-immune defense factors reach adult levels by the early teenage years (Kirstila *et al*.,

1998) and remain at high concentrations even among elderly people with full dentition. The only positive relationships with caries might be predicted for proteins that promote adhesion or maintain inorganic component homeostasis in the oral cavity (Rudney, 1995).

b. Specific defense factors and dental caries:

The immunoglobulins, IgG, IgM, IgA, and secretary IgA (sIgA), form the basis of the specific salivary defense against oral microbial flora, including mutans streptococci. The most abundant Ig in saliva, as in all other human secretions, is dimeric (sIgA), which is produce by plasma cells located in the salivary glands. Two IgA subclasses are present in saliva; IgA1 forms the major component of Igs, although the relative amount of IgA2 is higher in saliva than in other secretions (Tappuni and Challacombe, 1994). In human beings, IgG, mainly of maternal origin, is the only detectable Ig in the saliva of neonates. Salivary IgA is absent at birth but is readily detectable in infants at the age of only one week (Cole *et al*., 1998). The IgG concentration decreases to non-detectable levels after some months but appears again after tooth eruption (Brandtzaeg, 1989). The formation of specific IgAs in saliva correlates with the colonization of bacteria in the oral cavity. In most children over three years of age, salivary IgAs against mutans streptococci can detect and their amount increases with the length of exposure (Smith and Taubman, 1992). In the oral cavity, Igs act by neutralizing various microbial virulence factors, limiting microbial adherence, and agglutinating the bacteria, as well as by preventing the penetration of foreign antigens into the mucosa. IgGs are also capable of opsonizing bacteria for phagocytes, which are reported to remain active in dental plaque and saliva (Scully, 1980; Newman, 1990). In some studies the Ig levels are correlated with DMFT/DMFS scores (that is past experience of caries), whereas in other studies they are

correlated with the presence of active caries or with the levels of mutans streptococci in the mouth. It must also noted that the presence of active caries lesions may induce the formation of specific IgGs (Challacombe, 1980; Kirstila *et al*., 1998), and that they may remain at a higher level for several weeks or months after eradication of the lesions.

(iii) Other salivary factors:

Saliva serves multiple protective functions against the initiation and progression of dental caries. It assists to clear food particles and bacteria from the oral cavity and it buffers the acids produced by microorganisms in dental plaque (Reich and Newbrun, 1999; Fontana and Zero, 2006). The number of individuals suffering from a reduced salivary flow rate is increasing, especially in the elderly population. Xerostomia (dry mouth) may be the consequence of a variety of conditions including radiation therapy to the head and neck region and medical ailments such as Sjogren's syndrome, Parkinson's disease and uncontrolled diabetes mellitus (FDI Working Group, 1998; Hunter, 1988). Although xerostomia has long been known to be a risk factor for individuals of any age, the elderly are especially susceptible to salivary changes due to the large number of medications they are often required to take (Fure, 1998; Burt and Eklund, 1999). Individuals with chronically reduced salivary function have found to have a significant increase in caries activity. Testing an individual's unstimulated salivary flow rate can accomplished easily in clinical practice and it has a strong predictive validity for assessing caries risk. The stimulated flow rate using paraffin wax, it also customarily measured to conclude if preventive strategies based on salivary stimulation (e.g chewing sugarless gum) will benefit the patient (Reich and Newbrun, 1999; Fontana and Zero, 2006).

A. Carbohydrate intake:

Consumption of sugar and carbohydrates is considered as an important etiological factor in the development of dental caries (Evans *et al*., 2008; Zero, 2004). The role of diet is primarily local in nature rather than systemic as bacteria metabolize carbohydrates and sugar, producing acidic by products that cause the demineralization of the enamel surface. Whether this activity proceeds to a carious lesion depends on various elements, as well as the patient's oral hygiene and exposure to fluoride (Burt and Pai, 2001).There are several dietary elements that need to address when assessing a patient's caries risk level. Whether or not a food is cariogenic depends on a number of factors specific to the individual who eats it, namely the predominant oral bacteria in plaque, salivary flow rate and buffering capacity, and fluoride availability in the oral cavity (Burt and Eklund, 1999). The clinician must also take into account the retentiveness of the food, protective elements in food (e.g. fluoride, calcium, phosphate) the frequency of meals and snacks, sugar-containing non - foods (e.g. lozenges, gum, medications) and patterns of consumption (e.g. sipping, sugared drinks over a long period of time; Reich and Newbrun, 1999; Fontana and Zero, 2006). Assessing diet alone is usually inadequate at predicting caries. Studies in humans have not found a consistent relationship between consumption of cariogenic foods and dental caries experience (FDI Working Group, 1988; Tinanoff, 1995). In a longitudinal study by Burt *et al*.,(1988) the between meal sugar consumption was found to be only marginally related to inter proximal caries increment but not at all related to caries in the pits and fissures.(Burt *et al*., 1988). Dental caries is a mutifactorial disease and thus caries risk is always not directly correlates with fermentable carbohydrate consumption. For example, it found that children developed

very few caries if they had good oral hygiene irrespective of their dietary intake but if oral hygiene was poor, a high sugar intake revealed an increase in caries prevalence (Van Houte, 1994). It is thus more prudent to consider dietary factors in association with other caries factors such as oral hygiene practices and fluoride exposure.

(a) Frequency:

It would be beneficial if clinicians could have more information about which patients are most affected by dietary factors, and how much and how frequently carbohydrate consumption is significant. The World Health Organization has provided some dietary recommendations to decrease caries. Eating or drinking free sugar, more than 4 times per day or at a level of more than 10% of the total energy intake create higher caries risk. In some studies, food "frequency" does not consider the actual frequency of eating events, but only the frequency of choosing a particular food or drink. It seems appropriate to develop a way of estimating the time that substances are in contact with the teeth, since available information about the Microbiology of caries supports the idea that production of acid regularly follows.

(b) Types of carbohydrate:

The type of food or drink has also traditionally evaluated to determine carcinogenicity. It is generally accepted that some sugars like lactose and starchy foods are less cariogenic than sucrose containing foods (Moynihan, 2005). High intakes of different beverages may also indicate caries risk, as shown by Sohn *et al.*, (2006) in children with high carbonated beverage consumption had higher risk than children with high Juice, milk, or water consumption.

The presence of organic compounds in the diet is an area of interest. For example, phenolic compounds from the cranberry fruit are known to inhibit *Streptococcus mutans*, a major cariogenic bacterial species (Gregoire *et al*., 1960-1968) other compounds found in cocoa, green tea, and grape seed are being evaluated for their effect. In addition, citric acid present in many other products is able to erode enamel chemically by its ability to cause desaturation of calcium and hydroxyapatite in saliva. This may also prevent remineralization of tooth structure following demineralization by bacterial acids (Korithoski *et al*., 2005). It is known that many species of *Lactobacillus* are capable of utilizing citric acid as an energy source (Drinan *et al*., 1976), the presence of citric acid causes increased growth of the bacteria and increased production of acid (Konings *et al*., 1997) lactobacilli have been frequently isolated from carious lesions.

The carbohydrates especially easily fermentable refined ones are the main source for production of acids by the cariogenic microorganism. The early studies of Miller showed that when teeth incubated in mixtures of saliva and bread or sugar, Decalcification occurred. There was no effect on the teeth when meat or fat used in place of the carbohydrates. Both cane sugar and cooked starches produced acid but little acid formed when raw starch was substituted (Sivapathasundharam *et.al.,* 2006).

The carcinogenicity of dietary carbohydrates varies with the frequency of ingestion, physical form, and chemical composition, route of administration and presence of other food constituents. Sticky, solid carbohydrates are more cariogenic than those consumed as liquids. Carbohydrates in detergent foods are less damaging to the teeth than the same substances in soft retentive foods, carbohydrates, which are rapidly cleared from the oral cavity by saliva and swallowing, are less conducive to caries. Than those which

are slowly cleared. Polysaccharides are less easily ferments by plaque bacteria than monosaccharides and Disaccharides. Plaque organisms produce little acid from the sugar alcohols, sorbitol and mannitol. Glucose or sucrose fed entirely by stomach tube or intravenously; do not contribute to decay as they are unavailable for microbial breakdown. Meats high in fat, protein or salt reduce the oral retentiveness of carbohydrates.

Refined, pure carbohydrates are more cariogenic than crude carbohydrates complexes with other food elements capable of reducing enamel solubility or possessing antibacterial properties. The etiology of dental caries involves interplay between oral bacteria, local carbohydrates and other tooth surface that may be shown as: Bacteria+sugar+Teeth--- organic acids--- dental caries (Sivapathasundharam *et.al.,* 2006; Newbrun, 1989). The most important substrate in the involvement of *S.mutans* in the caries process is the disaccharide sucrose (Sivapathasundharam *et.al.,* 2006). The role of sucrose in the etiology of dental caries is base on epidemiological grounds and controlled human and animal studies. Sucrose has found to be more cariogenic than any other carbohydrate invariably and induced the smoothest surface type of lesions.

B. Oral hygiene and fluoride exposure:

Topical and systemic fluoride exposure, oral hygiene habits, and diet are often not strong predictive factors for caries development but they are often still included in CRA instruments because they may be prescriptive for the preventive actions recommended. Determining a problem in one or more of these areas will aid the dentist and patient to customize a care plan using these elements to alter other caries risk variables, such as bacterial and salivary factors (Tinanoff, 1995). In order to prevent dental caries, it has

recommended that a constant, low ambient level of fluoride should be maintained in the oral environment (FDI Working Group, 1988; Burt and Eklund, 1999).

Fluoride works via three mechanisms:

(1) Inhibition of demineralization- fluoride becomes incorporated into the enamel hydroxyapatite crystal and reduces enamel solubility in the presence of acids (pre- and post- eruptive).

(2) Enhancement of reminerlization- low levels of fluoride available in the oral cavity enhance remineralization during repeated cycles of demineralization and remineralization in the early stages of the caries process (post-eruptive).

(3) Inhibition of bacterial enzymes- fluoride inhibits glycolysis, the process by which fermentable carbohydrates are metabolized by cariogenic bacteria to produce acid (post- eruptive; Featherstone, 1999).

The considerable reduction in the prevalence of dental caries from 1960 to 1990 related initially to the introduction of fluoride into the public water system and to the subsequent use of topical fluoride through fluoridated toothpaste and professionally applied delivery systems (Evans *et al*., 2008; Featherstone *et al*., 2002). Its use has been shown to prevent and arrest carious lesions and its protective mechanisms allow for more conservative management strategies in the prevention and treatment of dental caries (Fontana and Zero, 2006). When completing a CRA, the several of fluoride must taken into account such as fluoridated drinking water, food and drinks, fluoridated toothpaste and mouth rinse, and professionally applied topical fluoride. As dental caries is a microbial disease so the prerequisite for caries development is the presence of dental plaque on the teeth and unless this biofilm present caries will not occur, regardless of any

other risk factors (Kidd, 1999). Researchers have failed to demonstrate a consistent relationship between dental plaque scores and caries (Hunter, 1988). Not all patients with poor plaque control inevitably develop caries; however, those who clean their teeth infrequently or ineffectively may be at higher risk for developing carious lesions (Fontana and Zero, 2006; Kidd, 1999).

C. Influence of heredity on caries experience:

The influence of hereditary factors in dental caries has well described and it is likely that many of these are associated with MS colonization in the child. It is reasonable to hypothesize that many genetic factors, such as the HLA genes, modulate the host's immunological responses, which in turn, influence MS colonization in the mouth. In addition, these genetic factors may also modulate bacterial colonization through changes in the saliva, tooth and mucosal surfaces (Acton *et al*., 1999; Ozawa *et al*., 2001).

i. Reduced susceptibility of caries:

1. Hereditary fructose intolerance is an autosomal-recessive disorder caused by deficiency of the enzyme fructose-1-phosphate aldolase; the blood glucose level may fall in response to fructose ingestion causing pallor, vomiting, sweating and even coma. Thus, individuals develop a strong aversion to sweets and high proportions are caries free.
2. In primary immune deficiencies relatively low caries experience is probably a result of prolonged antibiotic therapy.
3. Chronic renal failure that occurs in a number of inherited disorders also inhibits caries due to high salivary pH.

4. In congenital chloride diarrhea, an autosomal-recessive disorder, low caries experience has reported. This may be because of high salivary pH as a result of metabolic alkalosis.
5. Growth hormone deficiency has been associated with resistance to caries because of the retarded eruption and consequently increased time for enamel maturation before exposure to oral environment.
6. Turner's syndrome patients also demonstrated low caries experience perhaps through inter-dental spacing.
7. An unusually low caries incidences particularly approximal lesions has been observed in tri-somy 21 (Down syndrome), possibly related to delayed eruption and interdentally spacing.

ii. Increased susceptibility of caries:

1. In dystrophic epidermolysis bullosa, extensive caries is found possibly as a result of poor oral hygiene associated with painful oral blistering following minor trauma.
2. In connective tissue disorders, namely cutis–laxa, Rapp–Hodgkin ectodermic dysplasia and in focal dermal hyperplasia, gross caries found.
3. In Rubinstein–Taybi syndrome, marked caries was found perhaps as a result of poor dental care resulting from small mouth opening, malignant of teeth and mental retardation.
4. Klienfilter's syndrome (47, XXY males) was also associated with increased susceptibility.

D. Socioeconomic characteristics and habits:

The association between regional socio-economic context and dental caries experience may be characterized by various patterns based on individual characteristics, such as gender or age (Tellez *et al.*, 2006; Choi and Lee, 2011; Aida *et al.*, 2008). Biological (genetics, hormones, reproductive history) and anthropological (behavioral) factors, such as culture-based division of labour and gender-based dietary preferences, may also play roles (Madlena *et al.*, 2008; Lukacs, 2011). Additionally, despite the large number of studies that have examined neighborhood influences on individual oral health outcomes, few reported studies have conducted in Asian populations (Doyal and Naidoo, 2010). Neighborhood effects may differ between Asian and Western societies due to differences in social relationships, community formation, and economic development, as well as in individual demographic factors. The independent variables included social and economic conditions such as family income, parental levels of education, number of children, house ownership, household overcrowding (number of persons and number of rooms), presence of clinically detectable dental plaque, gender, age and anthropometric measures, including height and weight.

A disparity in prevalence of dental caries exists across socioeconomic and geographic subgroups in the population. Low-income children have two times greater prevalence of dental caries when compared to other children. In addition, low-income children have a significantly greater amount of untreated decay than other children. The available research, though limited, supports this assumption. A distinct disparity has seen in the survey data in urban and rural areas that dental caries among children and adults to be more prevalent in rural populations than in urban populations. Income level is a major

factor contributing to utilization of access to care. Adults living in poverty are less likely to receive dental care than wealthier. Among people who considered non-poor, 72 percent had a dental visit the year. Among the near poor, the percentage dropped to 48.5 % in 1999. Among the poor, the percentage is even lower at 46.2% having a dental visit the year (Burt, and Eklund, 1999). The prevalence of dental caries was high in the low socio-economic status because of their poor oral hygiene practice, lack of awareness, improper food intake and family status (Sogi and Baskar, 2001).

The risk factors for the dental caries are the health of the individual, diet, oral hygiene and delay in first visit to dentist. Oral hygiene also includes the chewing habit of various intoxicants including tobacco consuming. Tobacco consuming in either form of smoking or chewing individuals experienced more oral diseases. In tobacco smokers the saliva secretion may not affected however, it affects the physiology of the saliva by changing the pH, buffering capacity and immunity. This change enhances the growth of few bacterial species especially the Lactobacilli and Streptococci, become opportunistic pathogens, and increases the dental caries in susceptible individuals (Sajith *et al*., 2006). Association between the cigarette smoking and dental caries is well established in old age peoples (Locker, 1992; Jette *et al.*, 1993). Among middle age group (Axelsson *et al*., 1998) and young adults (Sgan Cohen *et al*., 2000) results are inconsistent. Over all predisposal factors of dental caries are genetic factors, oral hygiene, diet habits and the general health of individuals.

2.2.3. Caries as multifactorial in nature (Cariogram):

The cariogram developed in 1996 was originally conceived as an educational model aiming to demonstrate the multifactorial etiology of dental caries in a simple

manner (Bratthall *et al*., 2004). The Cariogram is a challenge for the biological factor approach is to correctly summarize the complex picture of the various inter-related caries risk factors, so that the dental professional routinely in the clinic can easily use it. A new model for understanding the interactions of various factors was therefore proposed and a graphical model, the cariogram, was drawn up to illustrate the fact that caries can be controlled by several different means. The computer version of the cariogram presents a graphical summary that illustrates a possible overall caries risk scenario. Furthermore, it expresses the extent to which different etiological factors of caries affect the caries risk for a particular individual and provides targeted strategies for those individuals. The cariogram does not specify the particular number of cavites that will or will not occur in the future. Based on this model, an interactive computer program developed in 1997. Changes made to the program included the addition of two more sections to the pie chart-circumstance 'and chance of avoiding caries'. The circumstances sector included factors that did not participate directly in the development of caries but were risk predictors of dental caries, such as past caries experience and systemic disease (Axelsson, 2000).

2.2.4. Diet and Disease:

The process of dental caries has carefully studied from the etiology, microbiology and epidemiology. It is well established that caries caused by bacterial acids, which are the by-products of metabolism of dietary carbohydrates (Wood wards and Walker, 1994). However, in clinical disease, the role of dietary carbohydrates is not too straightforward. Although there is a clear relationship between the amount of sugar consumed and caries in developing countries, the relationship is lacking in industrialized countries. This suggests that there may be additional factors that contribute to caries beyond simply the

amount of dietary carbohydrate. Historic evidence from the Vipeholm study indicates that between meals snacking is a significant contributor to caries (Krasse, 2001). Recent published evidence does not consistently support a link between dietary carbohydrate consumption and increased caries (Marshall *et al.*, 2007).

The association between dietary carbohydrates in beverage and caries is mostly supported by clinical observations but it also weakly supported by evidences. Many dentists associations make their communities aware of significant caries problems caused by carbonated beverages (Mulvany, 2001; Yaghi, 2001). In particular, these dentists report an increase in rampant smooth surface caries occurring in patients with a pop drinking habit (Mulvany, 2001; Yaghi, 2001). Some research supports the idea that high intakes of different beverages may indicate caries risk. Children with high carbonated beverage consumption had higher risk than children with high quantity of juice, milk or water consumed (Sohn *et al.,* 2006). Epidemiological data from adults with a high incidence of caries reports carbonated beverage consumption as a risk factor for caries (Burt *et al*., 2006). Several studies have evaluated diet and caries about obesity. Examination of children from the Iow fluoride cohort showed that carries and obesity coexist in children of low socioeconomic status (Marshall *et al*., 2007). In another study, utilizing Third National Health and Nutrition Examination Survey, (NHANES) data reported no association between caries and obesity in children (Hong *et al*., 2008). Some reported that obesity and overweight status does predict more caries in adolescents and frequent snacking in early childhood can indicate a higher risk for caries later in life (Alm *et al*., 2008).

2.2.5. Caries risk assessment:

Caries Risk Assessment (CRA) is the process of collecting data regarding various factors (e.g. bacterial level) and indicators (e.g. previous caries experience) to predict caries activity in the immediate future (Petersson and Bratthall, 2003).

Formal CRA has been described as a four step-process:

1) Identification of measurable risk factors
2) Development of a multifactorial tool
3) Risk assessment to determine a patient's profile and
4) Application of preventive measures tailored to the risk profile (Moss and Zero, 1995).

It has noted by a couple of researchers that the majority of CRA studies have conducted in children and adolescent population (Powell, 1998; Eriksen and Bjertness, 1991). Only few studies have been concentrating on investigate general caries activity in populations that include younger adults (Orliaguent and Featherstone, 2006; Bader *et al*., 2005; Marthaler *et al*., 1990-1995). This is promising because the younger adult population may express different disease factors due to lifestyle changes they encounter early into adulthood, such as living away from home for the first time and changes in dental care utilization and insurance pattern (Axelsson, 2000).

The multifactorial caries risk assessment instruments are firstly the cariogram is a widely available tool that has been validated (Petersson, 2003) and has received much attention in the discipline of cariology. It has been used extensively to identify caries risk factors for a variety of populations globally (Petersson and Bratthall, 2003; Petersson *et*

al., 2002; Sonbul *et al*., 2008; Miravet *et al*., 2007; Campus *et al*., 2009; Merritt *et al.*, 2001). Secondly, the caries risk assessment form (caries Risk and preventive needs assessment) from the faculty of dentistry at the University of Toronto is a university-developed model utilized by the student in the dental school clinic.

2.3. Aetiology of Dental Caries:

2.3.1. Microbiology of oral cavity:

Bacteria are a ubiquitous, nutritionally and environmentally diverse group of microscopic, unicellular prokaryotic organisms. Each bacterial cell retains its autonomy, that is, its ability to metabolize, grow and reproduce independently of other cells. The first direct evidence of the role of bacteria in causing disease came from the study of anthrax by the German physician Robert Koch (1843-1910). His criteria for proving the capsular relationship between a microorganism and a specific disease known as the Koch's postulates:

A. Oral cavity as a microhabitat:

The digestive system of human begins with oral cavity and is well suited for grinding, churning, chewing, mixing and initial phase of digestion of the food materials. To suit these functions it is made up of both soft and hard tissues comprising tongue, cheeks, palate, oropharyngeal region, gums and teeth. The oral cavity is always kept moist by the continuous secretion of mucous and saliva. Saliva not only helps in the digestion of food but also helps in cleaning the mucous off the surfaces of soft tissues, teeth and gums. The pH of the oral cavity is maintained constant by the buffering action of the saliva.

Because of its complex anatomical structures and varying physiological conditions, the oral cavity provides different types of environmental niches for the growth of varying kinds of microorganisms; it has a constant temperature (35-36 ^{O}C) and pH, an excellent supply of a variety of food particles and differing O/R potential. Under such congenial environment aerobes, facultative anaerobes and obligate anaerobes flourish well.

The microorganisms present in the oral cavity acquired from food and through local and geographic environment. Those microorganisms favoured by the nutritional and physiologic conditions and not inhibited by the mechanical and antagonistic mechanisms of the oral cavity become oral residents. Teeth provide 3 kinds of niches depending on the availability of nutrients, O_2 tensions and hygiene. Facultative anaerobes and aerobes are present in supragingival regions and strictly, anaerobes are present in the sub gingival region. The microbial population that formed on the surfaces of the crowns of teeth is aerobic. It usually utilizes the simple carbohydrates and forms the stick gum like structures (accumulation of dextran). It helps the microorganisms to adhere and grow on the surfaces of teeth and initiate tooth decay. This leads to the formation of microenvironment for the growth of anaerobic microorganisms which enhance the tooth decay leading to dental caries.

The microorganisms present on the surface of tongue are usually aerobic, utilize the food particles left over, and loosely adhere to the surfaces. Most of these microorganisms are always washed away along with saliva many are transient in nature contributed by the food.

Nutrients derived from at least five sources. The food eaten by a person is quantitatively probably the most important nutrient source for the oral flora. Nutrients present in saliva and the gingival crevice fluid, and epithelial cells shed from the oral surfaces are lysed releasing the nutrients. Microbes them self and their products also act as nutrients for the microorganisms present in the supragingival and subgingival plaques, tongue and soft tissues. Host products like serum secretions and epithelial cells in the oral cavity also act as nutrients.

B. Factors affecting the microbial flora of the oral cavity:

Many factors both intrinsic and extrinsic have an effect on the composition and metabolic activities of the micro flora of the mouth (Hardie and Shah, 1982). The main factors that influence the growth maintained in the various niches are nutrients, pH, O_2 tension, Redox potential (Eh), Superoxide radicals, lytic enzymes present in the saliva and mechanical friction like brushing and chewing. The chemical composition of the diet, the physical consistency of its components and the frequency of its presentation are the factors that influence the effectiveness of the diet as a microbial nutrient. This bioavailability of simple sugars makes them cariogenic. Frequently eating of a high sucrose diet is associated with high dental caries activity. The consistency of food also influences the plaque flora. The salivary lactobacilli levels and the levels of intracellular polysaccharide forming bacteria decrease appreciably on low carbohydrate diets. Reduction of caries by replacement of sucrose with non cariogenic sugar substitutes, such as Xylitol, Mannitol, and Sorbitol is observed. These sugars reduce the pH and this will not encourage dental caries. Saliva acts as source of nutrients and buffer for the growth of oral micro flora. The saliva also contains specific antibodies, lysozymes,

lactoperoxidases lactoferrin and high molecular weight adhesions may affect some microorganisms.

The pH of the oral cavity is around 7.0 and maintained by the bicarbonate buffers in the saliva, but the pH of the plaque is acidic (pH 5.0). Similarly reduced end products formed by bacteria lower the redox potential in the dentogingival area as low as -100 mv and O_2 tensions as low as 1%, thus it is considerably supportive for the growth of anaerobic microorganisms, Streptococci grows very well between the pH 5 to 7 and lactobacilli grow well in between pH 4 to 6 (Willium,1988).

Frequent brushing of tooth decreases the adherence of bacteria to the surface, hence reduces plaque formation. Brushing of teeth also reduces the availability of nutrients in the supra gingival region. Use of mouthwash and scaling of plaques present on the supragingival area drastically reduce the congenial environment for the growth of plaque forming microorganisms.

2.3.2. Different groups of etiological agents in oral cavity:

Oral microorganisms were among the first to be observed (Dobell *et al* 1932). Interest in oral microbiology lagged because of not taking the oral diseases as serious as other diseases. This is so because of problems of oral cavity are mainly due to the infection in teeth or in tonsils leading to severe pain, which can control by removal of tonsils or by teeth extraction. To study the microbial flora of oral cavity two methods have adopted. One is direct smearing of the testing material from the oral cavity and second one is culturing of the microorganisms from the samples taken from the different niches of oral cavity and from saliva. The natural niches in oral cavity in which microorganisms inhabited are tongue, saliva, gingival crevices, dental plaques etc.

The predominant cultivable bacteria isolated from the tongue are mainly microaerophils. These are Streptococci (38%), *Veillonella* (14.5%), *Diphtheriods* (20.4%), Micrococci and Staphylococci (7%), Bacteroides (5%), *Neisseria* (2.5%), *Vibrio* (2%), *Fusobacterium* (1%), unidentified gram-negative rods (3%) and gram-positive cocci (2%). The Streptococci isolated from the tongue are predominantly *Streptococcus salivarious* (21%-55%) and *Streptococcus milleri* and *Streptococcus mitis* (1-15%) (Gibbons, 1972; Mejare and Edwardsson, 1973; Kolenbrander, 2000) spirochetes were not isolated.

Quantitative and qualitative microbial determinations of plaque obtained from young subjects have been reported to have a mean total microscopic count of 2.5×10^{11} organisms per gram (wet weight). Anaerobes accounted for 4.6×10^{9} and aerobic count was 2.5×10^{9} per gram (wet weight). Important microorganisms identified from the plaque are facultative streptococci (27%), facultative diphtheroids (23%), anaerobic diphtheroids (18%), Peptostreptococci (13%), *Veillonella* (6%), *Bacteroides* (4%), *Fusobacterium* (4%), *Neisseria* (3%) and *Vibrio* (2%).

Noncariogenic plaque contains the following microorganisms *Actinomyces*, *Streptococcus sanguis*, *Streptococcus mutans*, and *Lactobacillus*. On the other hand cariogenic plaque contains *Streptococcus mutans, Actinomyces, Streptococcus sanguis* and *Lactobacillus* in the decreasing order. Human dental plaque was found to contain bacteriocin forming streptococci (Kelstrup *et al*., 1969). Bacteriocin typing used for identifying *Streptococcus mutans,* and *Streptococcus sanguis*, isolated from the plaque.

Suprogingival plaque growing at the gingival margin of teeth influences the development of subgingival plaque niche become anaerobic; this type of environment encourages the establishment of anaerobic bacteria and the formation of calculus (Kopczyk and Conroy, 1968; Mandel, 1974). In the initial stage of calculus development streptococci and actinomycetes are predominant and filamentous microorganisms are at low concentration as it develops well filamentous bacteria are increased and streptococci decreased (Howell, 1965). As many as 34 species of microorganisms are isolated from the human supragingival and subgingival calculus and 18 organisms are involved in the calcification. Gram positive facultative cocci (29%): Staphylococci, Enterococci, *S. mutans*, *S. sanguis*, *S. mitis*. Gram positive anaerobic rods (20%): *Actinomyces bifidus*, *Actinomyces israelii*, *Actionomyces odontolyticus*, *Propinobacteria*, Leptotrichea, Corynebacteria. Gram negative anaerobic rods (16%): *Bacteroides melaninogenicum, Bacteroides orials, Fusobacterium nucleoticum.* Gram negative anaerobic cocci (10%): *Veillonella alcalescensand Veillonella parcula.* Gram positive anaerobic cocci (7%): Peptostreptococci. Gram negative facultative cocci – *Neisseria* and Spiral organisms (2%): *Treponema oralis, and Borrelia vincentii* (Socranzky, 1970).

2.4. Role of Mutans Streptococci in Dental Caries:

The old theories of dental caries include the worm theory, humor theory, vital theory, chemical theory, parasitic theory and septic theory. The more recent theories include proteolytic theory, proteolytic chelation theory and acidogenic or chemoparasitic theory (Sivapathasundaram *et al.*, 2006; Newbrun, 1989). The most accepted theory is the Millers' chemo-parasitic theory or acidogenic theory, which implicates carbohydrates, oral microorganisms and acids as the main factors in the caries process

(Sivapathasundaram and Raghu, 2006; Samaranayake, 2002; Newbrun, 1989). The role of microorganisms or bacteria has extensively studied. Miller (1890) isolated many microorganisms from the human oral cavity, some acidogenic and some proteolytic and showed that caries is caused by a variety of microorganisms and bacterial communities that accumulate on the teeth surface known as dental plaque. Several organisms have found capable of inducing caries lesions when used as mono-contaminants in gnotobiotic rats. This include the mutans group of streptococci, *Streptococcus salivarius, S. mitior, S. milleri, S. oralis, S. sanguis*(different strains); *Peptostreptococcus intermedius, Lactobacillus acidophilus, L.casei , Actinomyces Viscosus and Actinomycetes Neaslundii* (Sivapathasundaram *et al*., 2006; Loesche, 1986; Van Houte, 1994).

2.4.1. Role of *S. mutans* in caries pathology:

Streptococcus mutans and *Lactobacillus* sps are active microorganisms contributing to dental caries. The essential role of *S.mutans* is in the initial process of caries while *Lactobacillus sp* is correlated with the active caries episode (Van Houte, 1993). As many as 34 bacterial spices have been isolated from the plaque. Among the total flora associated with teeth, S. mutans accounts for 30%. Of these *S. mutans* and *S. sobrinus* are mainly found in human dental plaque and are strongly associated with development of dental caries (Choi *et al.,* 2008) however *S. salivarius* present in saliva and is not usually isolated from the caries sites. It is reported that caries incident in children with both *S. mutans* and *S. sobrinus* was four times higher than in those with *S. mutans* alone (Okada *et al.,* 2005). However, the *S.mutans* is the major species of mutans streptococci isolated from the human oral cavity acquired before the emergence of the primary dentition and later it helps in the initiation and progress of caries lesions (Whily

and Beighton, 1998; Wan *et al.*, 2001; Marsh, 1994). The initiation of caries development mainly depends on the capacity of production of water insoluble glucan with the help of the glucosyl transeferases (GFTs) in the presence of sucrose by cariogenic bacteria. However several bacteria possess these enzymes but few like *S. mutans* and *S. sobrinus* and *S. dowani* having capacity to produce water insoluble glucan and acidoduric in nature. *S.mutans* becomes prominant among oral mutans streptococci because of its ability to survive, produce acids and grow at low pH (5-7).

2.4.2. Importance of *S.mutans* in caries formation:

The characteristics of *S.mutans* which, make it prime odontopathogen in causing caries are as follows (Samaranayake, 2002).

- It is the first colonizer in mouth of infants immediately after eruption of teeth and disappearing from the mouth following extraction of teeth: thus provides it with the advantages of being the early colonizer in the absence of competition.
- It can easily transmit and infect infants from their parents or from other individuals with whom they have frequent contact since organisms not found free living in nature and have only been isolated from humans and certain animals.
- As it is acidogenic it can produce acidic environment which is necessary for demineralization and it can effectively thrive in that acidic environment. Therefore, it is acid uric (acid loving).
- Significant correlation exists in humans between *S.mutans* count in saliva and plaque with the prevalence and incidence of caries.
- *S.mutans* can be isolated from precise sites on the tooth surface before the development of caries.

- Positive correlation between progression of carious lesion and *S.mutans* count.
- Most effective streptococcus in experimental caries in animals (Rodents and non-human primates)
- Produces water soluble and insoluble extracellular polysaccharides from sucrose by consolidating microbial attachments and possibly producing a barrier against neutralizing and protective effects of saliva.
- Ability to transports sugars rapidly in competition with other plaque bacteria even at low pH.
- Ability to initiate and maintain microbial growth, metabolism and acid production in sites with low pH.
- Efficient and rapid metabolism of sugars to lactic and other organic acids.
- Can attain the critical pH for enamel demineralization more rapidly than other common plaque bacteria.
- Produces intracellular polysaccharides which, can act as a food store for use when dietary carbohydrates are low.
- Immunization of animals with *S.mutans* significantly reduces the incidence of caries.

A fundamental problem with prediction about the cariogenic role of different acidogenic plaque organisms is that aciduric organisms are not necessarily cariogenic and acid tolerant. Actinomyces strain proved to be non-cariogenic in contrast *to S.mutans* strain with higher acidogenicity and acid tolerance. *In situ* determination of the pH of the plaques mass formed by the test organisms showed that *S.mutans* had a much greater pH lowering potential. In contrast to these *in-vivo* results, prolonged exposure of enamel *in-*

vitro to acids formed in an actinomyces plaque from sugar leads to caries development. This suggest that, under the conditions prevailing in the human mouth, the frequency and intensity of bacterial acid production in plaque must be exceed a critical level before alternating periods of the demineralization and remineralization leads to net demineralization. Such conditions prevailing in human mouth can be modified by modification of Keyes factors and alter the caries process, but this is presently possible only in the enamel caries especially pit and fissure caries, where by modifying the pit and fissure with sealants the host factors (Tooth) can be modified and not in case of smooth surface caries. This can modify only by modifying the intake of carbohydrates, especially fermentable disaccharides by which carries producing *S.mutans* strain produce the acids, which cause demineralization of enamel (Van Houte, 1994).

2.4.3. Prevalence of *S.mutans* in saliva:

Streptococcus mutans belongs to the group of Alfa hemolytic streptococci. Mutans streptococci related to the occurrences of early demineralization, i.e., initial phases of the development of caries (Loesche, 1986). In the concentration of 10^4-10^5 CFU/ml in saliva, *S. mutans* is able to colonize clean, smooth surface of a tooth. The presence of mutans streptococci on tooth surfaces increases the possibility that caries would develop thereon. The mean levels of *S.mutans* in saliva at all ages when colonization was first detected the mean *S.mutans* levels in pre-term infants (1400 CFU/mL) were similar to those in full term infants (1695 CFU/mL; Wan and Tudehope, 2001b). *S. mutans* colonization was higher in infants from families of low total annual income, and infants with mothers who have only a primary only a primary school education, at 9 months ($p < 0.01$), 12 months ($p<0.03$), and 15 months of age ($p< 0.05$).

However, at 24 months of age, this trend reversed and the *S. mutans* colonization rate was higher in infants of high socio-economic status ($p < 0.03$). As is the case before tooth eruption, *S.mutans* colonization after tooth eruption is influence by both maternal and infant factors. It is now well-recognized that the mother is usually the primary source of *S.mutans* for infection of her child (Kohler and Bratthall, 1978; Li and Caufield, 1995), and poor maternal oral hygiene and dietary habits increase the likelihood of transmission of the infection from mother to child. Several infant factors contribute significantly to the colonization of *S.mutans*. Thus, with tooth eruption, the colonization rate of the infants increases as their ages increase. Under certain circumstances, however, oral environmental changes may modify the composition and metabolic activities of the bacterial consortium leading to predominance of pathogens (Marsh, 2006). High counts of mutans streptococci (MS), *S. mutans* and *S. sobrinus*, have been associated with higher prevalence of coronal caries in temporary and permanent dentitions. (Lemons and Burne, 2005; Liljemark and Bloomquist, 1996; Tanzer and Thompson, 2001; Thenishch and Steurer, 2006) and also root caries (Preza *et al.*, 2008). In spite of the widely acknowledged association between increased levels of MS and higher caries experience, other studies have argued against this association (Giacaman *et al.*, Padilla, 2010; Helderman *et al.*, 1996). Colonization and pathogenicity of these facultative gram-positive cocci derive from their capacity to synthesize extracellular polysaccrides (Kreth *et al.*, 2008). Indeed, adult subjects colonized by biofilm-forming *S.mutans* strains have higher caries experience than individuals without these types of strains (Jalil, 1995).

2.4.4. Role of adhesion in *Streptococcus mutans*:

It is clearly accept that the development of caries depends on the interaction of saliva. Protective and noxious factors that are part of the acquired pellicle and the plaque in addition to the balance between the microbial cariogenic and non-cariogenic populations within the plaque and the physicochemical characteristics of the enamel, dentine and cement that make hydroxyaptite more or less vulnerable to acid attack (Hicks *et al.,* 2003). The acquired pellicle is a biologic layer resulting from the selective adsorption of salivary components on the dental enamel surface (Vitorino *et al.,* 2004), Mostly (98%) made up of salivary proteins, which act as substrate for fixation of cariogenic bacteria such as *S. mutans*. Interactions between these bacteria and the proteins in the acquired pellicle are part of a very complex process that has been the subject matter of studies in recent years. Various studies of salivary proteins have been identified, some of them as responsible for the initial binding of *S.mutans* to the tooth's surface. Nevertheless, the potential differences between the salivary components of individuals with natural resistance to dental caries and individuals who are susceptible of caries have not yet clearly established. Hydroxyapatite (HA), has been used for many years as an enamel model for studying salivary proteins, as its surface is similar to that of the tooth (Rui, *et al.,* 2004; Proctor *et al.,* 2005; Vitorino Rui *et al.,* 2005; Lendenman *et al.,* 2000) the adhesion of *S. mutans* to HA between the with and without caries groups. The average value of adhesion in the without caries group was 35.12 dpm/10^7 cells/ml, while in the with caries group it was 24.02 dpm/10^7 cells/ml, 27% higher in incubations with whole saliva from individuals in the without caries group.

2.5. Isolation of and characterization of S.mutans from Saliva:

2.5.1. Taxonomy of *S. mutans* and mutans streptococci:

Streptococcus mutans was first isolated from carious lesions by Clarke *et al*., as early as 1924, but it was not until the 1960's that taxonomical advances allowed researchers to match strains of oral streptococci recovered from human carious lesions (and similar strains recovered from rodent caries) with the original *S. mutans* isolate from 1924 (Loesche, 1986; Hamada and Slade, 1980). Nevertheless, even at that point, the taxonomy of the oral streptococci remained difficult and has remained in a state of flux ever since. *Streptococcus mutans* was the name given to all oral streptococci that were isolated from carious lesions that could ferment mannitol and sorbitol, that produced extracellular glucans and were cariogenic in rodent models of caries. They called as "mutans" due to their appearance on gram stains where they resembled mutant versions of streptococci, possessing a smaller and more oval appearance (Loesche, 1986). The original *S. mutans* strains encompassed a variety of serotypes (a-h). Eventually, as DNA analysis improved,20 *S. mutans* strains were reassigned into several species: *S. mutans, S. sobrinus, S. criceti, S. downei, S. ferus, S. macaccae, S. ratti,* and *S. hyovaginalis* (Facklam, 2002; Whiley and Beighton, 1998), and collectively designated as the mutans streptococci (MS). The most common human species *S. mutans; S. sobrinus* is also common in humans, but less so than *S. mutans*. *S. criceti* and *S. ratti* are only rarely recovered from humans.

Scientific classification of *Streptcococcus mutans* (Clarke 1924)**.**

Kingdom	**:** Bacteria	**Phylum**	**:** Firmicutes
Class	**:** Bacilli	**Order**	**:** Lactobacillales

Family : *Streptococcaceae* **Genus** : *Streptococcus*

Species : *Streptococcus mutans (S. mutans)*

2.5.2. Isolation of mutans streptococci and *S.mutans*:

Mutans streptococci, in particular *Strptococcus mutans* and *S. sobrinus* are the most cariogenic microorganisms in human (Loesche, 1986). Even though *S. sobrinus* is correlated with caries incidence, but its role in the initiation of caries not clear. To know the types of mutant streptococci involved in the caries formation can done by isolating the microorganisms from the different sites of oral cavity for that selective media are required. MS agar is commonly use to isolate mutans streptococci in addition to other streptococcus species. For the selection of mutans streptococci, Mitis salivarius-sucrose bacitracin (MSB) agar is used (Gold *et al.*, 1973). *S. mutans* produces characteristic colonies when incubated anaerobically for 48 hours on MSB agar.

2.5.3. Virulence factors *S. mutans*:

Strptococcus mutans possesses a variety of virulence factors that enable it to establish colonization, accumulates large numbers on the tooth surface, utilize a wide array of carbohydrate sources, produce acid and thrive at low pH. Experiments have demonstrated that *S. mutans* was more acidogenic than other non-mutans oral streptococci and lactobacilli strains. It also showed the most rapid growth at lower pH than any of these bacteria (van Houte, 1994; Harper and Loesche, 1984;Takahashi *et al.*, 1997). The trait that further separated *S. mutans* from other oral plaque bacteria was the ability to accumulate large numbers in the presence of dietary sucrose. This required both adhesion to the tooth surface or to plaque colonies, and cohesion among the dividing cells (Marsh, 2004; Kolenbrander *et al.*, 2010).

A. Adherence and Accumulation factors:

S. mutans adhere to the tooth enamel or to plaque surface by two mechanisms. These are sucrose-independent adherence and sucrose-dependent adherence. As previously stated, most people harbor *S. mutans* but this does not always or immediately lead to caries (Gibbons *et al.,* 1986). Interactions of *S. mutans* with the enamel pellicle and with primary colonizers enable residence in the plaque biofilm, in small numbers, until conditions are favorable for rapid accumulation, such as host consumption of Sucrose (Mitchell, 2003). Although they do not bind to pellicle proteins very efficiently, *S. mutans* possesses multiple surface adhesion proteins. There is a high degree of specificity in the sites that *S. mutans* binds and the primary colonizers that they associate with, which surpasses general ionic, hydrophobic or van der Wall's forces (Gibbons, 1984). These adhesion events involve cell surface adhesins of *S. mutans* and interactions with the acquired pellicle and primary colonizing bacteria. The most important adhesin is the P1 antigen (also known by the following Names: antigen I/II, SpaP, Pac, MSL-1, antigen B (Matsumoto-Nakano *et al.,* 2008)) which is encoded by the *spaP* gene and binds to salivary glycoproteins (Bowen *et al.,* 1991). Cell wall associated protein A (WapA) is a small, immunogenic, cell wall protein that promotes sucrose-independent intercellular aggregation in *S. mutans*. WapA expression is repressed in the presence of sucrose, which suggests that it may enable *S.mutans* cells to bind to a biofilm in the absence of sucrose or glucan (Zhu *et al.,* 2006). Primary colonizers, like *S. gordonii*, express several proteins that bind *S. mutans.* While these may not directly considered as *S. mutans* virulence proteins, they attach to receptors on the *S. mutans* cell wall and help facilitate initial binding to the biofilm. Sucrose independent adhesion of *S. mutans* allows

the species to become part of the plaque biofilm but does not normally allow for the accumulation necessary for caries development.

(i). GTF Enzymes:

Glucan production via *S. mutans* glucosyltransferase (GTF) enzymes occurs by splitting sucrose and polymerizing glucose moieties into either water soluble- or water insoluble glucan extracellular polysaccharide. This critical process enables *S.mutans* to create robust biofilms and become cariogenic (Hamada and Slade, 1980). Several studies using glucosyltransferase (*gtf*) mutant strains of *S. mutans* have shown a decrease in the cariogenicity of the bacteria and the ability to adhere to smooth surfaces or form biofilms *in vitro* (Michalek *et al.,* 1975b; Tanzer *et al.,* 1974; Hirasawa *et al.,* 1980a; Hirasawa *et al.,* 1980b).

Wild type *S. mutans* strains do not accumulate large numbers and are not cariogenic when grown in ample concentrations of sugars other than sucrose (Gibbons, 1984). Sucrose-dependent adhesion to glass surfaces has shown to mediated by GtfI and GtfSI (Tsumori and Kuramitsu, 1997; Ooshima *et al.,* 2001). *S.mutans* makes three Gtf enzymes: GtfI, encoded by glucosyltransferase B (*gtfB*); GtfSI, encoded by *gtfC*; and GtfS, encoded by *gtfD*. GtfI and GtfSI primarily synthesize water-insoluble glucan that is comprised mainly of α1-3, glycosidic linkages with varying degrees of branching linkages, while GtfS synthesizes water-soluble glucan that is mostly linear α1-6 linked glucose molecules (Banas and Vickerman, 2003). Gtfs also called glucan-sucrase enzymes, are found in other oral streptococci, lactococci, lactobacilli, and *Leuconostoc mesenteroides* and have very well conserved sequences and structure. Gtf enzymes are finding both extracellularly and cell wall bound, and there is evidence that Gtfs avidly

bind the enamel pellicle (Koo *et al.*, 2010; Hannig *et al.*, 2008; Schilling and Bowen 1988; Vacca-Smith and Bowen, 1998). Kuramitsu *et al*., (1978) showed that cell wall-associated Gtfs were derived from extracellular Gtfs that became bound to glucan coating the cell wall of *S. mutans* (Kuramitsu and Ingersoll, 1978), Germaine *et al.*,1976). Proposed multiple Gtfs binding to cell wall-associated glucan promoted aggregation while de Stoppelar *et al.*, using aggregation deficient mutants, showed evidence of a possible protein that could act as a glucan surface receptor (Germaine and Schachtel, 1976; de Stoppelar *et al.*, 1971). Using affinity chromatography, several researchers set out to find a possible glucan receptor on the surface. Along with the Gtfs, they discovered non-Gtf glucan-binding proteins (Banas and Vickerman, 2003; Kuramitsu and Ingersoll, 1978).

(ii) Glucan binding proteins:

The first of these glucan-binding proteins, GbpA, was isolated by Russell along with 2 Gtf enzymes, on a dextran affinity column and was resolved at 74 kD on an SDS-PAGE (Russell, 1979). Sequence analysis of the GbpA (then known as Gbp) protein revealed that the processed protein was actually 59 kD and contained a glucan-binding domain with repeats that were similar to the Gtfs of *S. mutans* and other oral streptococci (Banas *et al.*, 1990). Russell's group showed a role for GbpA in *S. mutans* adhesion to glass surfaces.

GbpB was isolated by Smith *et al.*, (1994) also by affinity chromatography, and was estimated to be 59 kD by SDS-PAGE. This protein was immunologically distinct from GbpA and shown to be quite antigenic in humans and rodents. Later work by Mattos-Graner *et al*. (2006) showed that GbpB's dextran-binding properties may be

somewhat weak and showed a possible role for the protein in cell wall synthesis or cell division. It should noted though, that GbpB is an essential gene that is positively regulated by the VicRK system under stress and that the amount of extracellular GbpB production correlated with biofilm growth in a select group of clinical isolates (Mattos-Graner *et al.,* 2001). Dextran-dependent aggregation is a property that *S. mutans* displays under certain stressful conditions, such as sub-inhibitory antibiotic concentrations or the presence of ethanol.

Sato *et al.*, (1997b) isolated GbpC, from a dextran-dependent aggregation (DDAG) deficient mutant strain of *S. mutans.* GbpC observed to be a cell wall anchored protein. Among the oral streptococci, proteins capable of promoting dextran dependent aggregation are also termed glucan-binding lectins (Ma *et al.,* 1996). Sequence analysis revealed that the GbpC shared some homology with the major streptococcal surface protein P1. P1-like proteins on oral streptococci may possess glucan-binding abilities but confirmatory data does not exist in the literature.

Fourth as Gbp, GbpD, was also discovered and isolated based on sequence analysis of the complete, annotated sequence of *S. mutans* UA159 strain. GbpD possesses the amino acid repeats similar to those in the glucan-binding domains of GbpA and the Gtfs (Shah and Russell, 2004). Experiments using mutants with an inactivated *gbpD* gene, showed a possible role in aggregation and smooth surface adhesion for GbpD. The GbpD was shown to have lipase activity and binds lipoteichoic acid of *S. sanguis*. This could have a function in interspecies competition in plaque biofilms (Shah and Russell, 2004).

(iii) Bacteriocins:

Bacteria to establish colonization in a particular niche produce bacteriocins. In biofilm formation, one of the early mechanisms of "biological warfare" utilized by bacteria is the release of anti-microbial peptides called bacteriocins. *S. mutans* produces at least four bacteriocins (called mutacins), three of which (Mut-I, II and III) are lantibiotics that kill a wide range of Gram-positive bacteria, and one (Mut-IV) that is a non-lantibiotic that kills closely related streptococcal species such as *S. sanguinis* and *S. gordonii* (Kreth *et al.*, 2006; Hamada and Ooshima, 1975). Several of these mutacin genes are activated by population density through quorum sensing (two-component systems). While they may not directly contribute to the virulence or cariogenicity of *S. mutans*, they are an important tool utilized by the bacteria for killing off competing oral streptococcal species when trying to establish colonization of the tooth.

B. Acidogenicity and Aciduricity:

Besides their adhesive and cohesive properties, equally important aspects of *S. mutans* virulence are its abilities to produce and tolerate large quantities of acid. Acidogenicity of *S. mutans* obtained through the ability of these bacteria to metabolize a wide variety of carbohydrate. Sequence analysis of *S. mutans* UA159 revealed 14 phosphoenol pyruvate-sugar phosphotransferase (PTS) systems, 4 ATP-binding cassette (ABC) transporters, as well as a possible galactoside-pentose hexuronide (GPH) translocation (Ajdic and Pham, 2007). PTSs work by transporting and phosphorylating sugars using phosphoenolpyruvate (PEP) and a set of cell wall-bound enzymes. In addition to transport and metabolism of various sugars, some components of PTSs act to regulate other carbohydrate metabolic systems so that the bacteria preferentially select the most rapidly metabolizable sugars (Vadeboncoeur and Pelletier, 1997). There is also a

multiple sugar metabolism (MSM) system which most likely uses one of the ABC tranporters that is capable of recognizing and metabolizing several different sugars (Russell *et al.,* 1992). Therefore, *S. mutans* is able to rapidly transport, metabolize and possibly regulate metabolism of glucose, fructose, sucrose, lactose, galactose, mannose, cellobiose, β-glucosides, trehalose, maltose/maltodextrin, raffinose, ribulose, melibiose starch, isomaltosaccharides, sorbose mannitol and sorbitol. Through glycolysis, *S. mutans* is able to produce pyruvate from these sugars, and has all necessary enzymes for pyruvate metabolism. Lactic acid is the major by-product of these fermentations, especially when 2, 6, glucose is abundant but formate, acetate and ethanol are also produced (Ajdic *et al.,* 2002).

The acid that *S. mutans* produces is what ultimately leads to dental caries. However, the extent to which this species can withstand the low pH, helps create sets it apart from most other plaque species. Acid tolerance in *S. mutans* is due to a robust acid tolerance response (ATR), which includes several genes that are up-regulated in response to low pH. The main contributing factors to this response are the F-ATPase proton pumps that are capable of acting in low pH in *S. mutans.* In fact, *S. mutans* can continue to ferment carbohydrate at pH levels where it has ceased to grow. The continued generation of ATP drives the proton pumps (Quivey *et al.,* 2001; Kuhnert *et al.,* 2004). Several oral bacteria utilize an arginine deaiminase system (ADS) to produce ammonia from urea and arginine that raises the pH of the surrounding media. *S. mutans* contains genes encoding part of an ADS system but not arginine deaiminase. Ajdic *et al*. (2002) proposed that *S. mutans* may use a different enzyme in this pathway (Ajdic *et al.,* 2002; Quivey *et al.,* 2001). Other components of the general stress response also contribute to *S. mutans* acid

tolerance such as RecA (DNA Recombinase A) and Smn (*S. mutans* exonuclease) DNA repair enzymes, Ffh (protein secretion and possible assembly), and DagK (fatty acid synthesis), which are all up-regulated under acidic conditions (Quivey *et al.,* 2001).

The up-regulation of fatty acid synthesis genes agrees with the observation by Fozo *et al.,* that *S. mutans* has a greater proportion of long chain mono-unsaturated fatty acids in the cytoplasmic membrane under low pH conditions. The chaperonins DnaK and RopA are up-regulated in *S. mutans* under acidic conditions as is HtrA protease and Clp ATPases (Cotter and Hill, 2003; Len *et al.,* 2004). The ATR of *S. mutans* appears to be controlled in part by the ComCDE quorum sensing two-component signaling system. Low pH has been shown to up-regulate a number of other genes that have roles in carbohydrate metabolism, protein folding and fatty acid synthesis (Len *et al.,* 2004b; Welin *et al.,* 2003). The properties that make *S. mutans* acidogenic, like the ability to ferment and regulate metabolism of a wide array of carbohydrates, coupled with the aciduric properties, namely the ATR, provide *S. mutans* a selective advantage within dental plaque leading to higher proportions of *S. mutans* and other similarly acidogenic and acid-tolerant species (Kreth *et al.,* 2008). They are not only able to out-compete other members of the oral flora, including some oral streptococcal species, for nutrients but the acidic environment is unfavorable to these other species. These properties also lead to demineralization of tooth enamel. At this point, the ecology of the plaque transformed into one that substantially increases the risk of developing dental caries.

C. *S. mutans* biofilm architecture:

The highly acidogenic and aciduric properties of *S. mutans* must linked with its ability to adhere to the tooth surface and form a biofilm in order to fully recognize its cariogenicity. The strong association of dietary sucrose with caries highlights the important contribution that Gtf enzymes make to *S. mutans* virulence. Research by (Mattos-Graner *et al*.,2000) using clinical isolates of *S. mutans* from caries-positive and caries-free children showed a strong correlation between the ability to synthesize water-insoluble glucan *in vitro*. Both in colonization on a tooth and in caries evidences exist which suggests that the two-component system Cov RS, that is responsible for biofilm formation has been shown to transcriptionally regulate *gtf* genes as well as *gbpC* (Biswas and Biswas, 2006; Biswas *et al.,* 2007; Idone *et al.,* 2003). Given the role of extracellular glucan in *S. mutans* sucrose-dependent adhesion, biofilm formation and cariogenicity, it is natural to speculate upon the roles of *S. mutans* proteins capable of binding glucan. Since the discovery of Gbps there have been several studies aimed at determining whether they play a role in *S. mutans* cariogenicity. Experiments involving deletion of genes encoding Gbps have shown that they influence adhesion, aggregation, biofilm architecture and cariogenicity of *S. mutans*, even as multiple studies of *gbpA* mutants have yielded opposing conclusions regarding the nature of that influence. No systematic study using a single, defined model of caries has carried out to investigate the role of each Gbp. There is a great deal of evidence suggesting a relationship between adhesive and aggregative properties of *S. mutans* and caries. These properties have shown to alter the architecture of an *in vitro* biofilm in a *gbpA* knockout strain of *S. mutans*. Differences

in adhesion and aggregation caused by elimination of Gbps may affect the cariogenicity of *S. mutans*.

2.6. Chemotherapy of Dental Caries and ast of *S.mutans:*

2.6.1. Treatment of dental caries:

Historically, it thought that dental caries was progressive disease that inevitably led to the eventual loss of a tooth unless a dentist intervened surgically. The conventional method of dealing with dental decay involved detection of the carious lesions, followed by drilling and filling (Fontana and Zero, 2006; Evans and Howe, 2008; Domejean-Orliaguet and Featherstone, 2006). Although treating dental caries by restorative means will offer relief from pain and restore function to the tooth, it will likely not prevent the lifelong continuation of the disease process and will undoubtedly allow recurrent decay necessitating further surgical interventions (Kutsch and Kutsch, 2006; Kutsch *et al*., 2007). Restoration of the carious lesions removes area of cariogenic microorganisms but it does not alter the risk level of the patient. Research demonstrates that placing dental restorations contributes very little to the management of the caries disease process as there is no measurable effect on the cariogenic bacterial load in the mouth once restorative procedures are completed (Jenson and Young, 2007; Wright and Caulfield, 1992).

A great deal of dental work is focused on treating the symptoms of this bacterial infection rather than focusing on the causative factors. Restorations, by themselves, are incapable of modifying the etiological factors of dental caries in order to eradicate caries forming bacteria (Pitts, 1998; Young *et al.,* 2007). When health professionals are dealing with other systemic disease, measures to eradicate the causes of the disease utilized such

as immunization and antibiotics. Dental professionals need to consider dental caries in the same manner and treat the disease rather than just the clinical manifestations of the disease; it is believe that our current understanding of the caries disease process is strong enough to accomplish this (Featherstone and Stewart, 2003). Medical management of dental caries is not only possible, but it has shown to provide superior outcomes to surgical intervention alone. It demonstrated that patients treated via a caries risk assessments and medical model approach had developed significantly fewer new carious lesions then patients treated solely with the conventional surgical approach (i.e. drill &fill). A more practical way to prevent this disease would be for dentists to identify "infected" individuals (which would be almost everyone in the beginning) and then, using antimicrobial treatment, eliminate or suppress the disease-causing bacteria in the mouth, while allowing harmless bacteria to grow in its place.

2.7. Molecular Studies of *S. Mutans:*

Typing of isolates applied in epidemiological studies to determine bacterial occurrence and modes of transmission. Typing of isolates is also performed for evaluation of whether certain strains are associated with specific clinical disease conditions and to characterize the heterogeneity of infection, i.e., whether one colonizes subjects or multiple types of the microorganism. Evaluation criteria for typing methods include type ability (ability to give an outcome for every isolate included), reproducibility (ability to give the same result when repeating the analysis) and discriminatory power (ability to differentiate between unrelated strains; Arbeit, 1999). Two main types of epidemiological typing systems for microorganisms are available, the phenotypic and the genotypic methods.

2.7.1. Phenotyping:

Traditional methods of characterizing bacterial isolates have relied on measurement of characteristics expressed by the microorganisms, such as bacteriocin production and sensitivity to bacteriocins, serotype, complicated biochemical properties, antibiotic resistance and bacteriophage typing (Maslow and Mulligan, 1996).

A. ***Bacteriocin typing:***

One of the first epidemiological typing systems for oral streptococci was bacteriocin typing. Bacteriocins are proteinaceous substances produced by the bacteria that inhibit the growth of other, mostly closely related, bacteria. The typing is performed by measuring the inhibiting effect on bacterial growth of certain indicator strains, and by measuring the sensitivity of the bacteria to be typed to bacteriocins from other strains (Jack *et al*., 1995). Heterogeneity among strains of mutans streptococci within one individual was first show by bacteriocin typing (Kelstrup *et al*, 1970).

B. ***Serotyping:***

Using Ouchterlony immunodiffusion, Bratthall demonstrated five serological groups of mutans streptococci (Bratthall, 1970). Eight serotypes were subsequently recognized (Perch *et al*., 1974; Beighton *et al*., 1981). The classification is based on cell-wall carbohydrate antigen. Serotyping by immunodiffusion, immunofluorescence or immunoelectrophoresis has been widely applied for typing of mutans streptococci. The mutants group of streptococci is one of the five groups in viridian streptococci (Whiley and Beighton, 1998). Mutants streptococci consists of seven species that can be classified into 8 serotypes: *S.mutans* (serotypes c, e and f) *S.sobrinus* (serotype d & g), *S.*

criceti (serotype a) *S.downei* (serotype h) *S.ferus* (serotype c) *S.macacae* (serotype c) *S. ratti* (serotype b; Facklam, 2002; Whiley and Beighton, 1998). Two species *S.sobrinus* and *S.mutans* are consistently isolated from the caries patients, especially serotype c of *S.mutans*.

C. *Biotyping:*

In 1974, Shklair and Keene divided mutans streptococci into five biotypes (a-e) based on fermentation characteristics, arginine hydrolysis and bacteriocin sensitivity, and they reported that these biotypes corresponded with the serotypes reported in 1970. Their later scheme (Shklair and Keene, 1976) also includes serotypes *f* and *g*.

Among other phenotypic methods are cellular fatty acid analysis, whole-cell protein analysis and multilocus enzyme electrophoresis (MEE). MEE based on the relative electrophoretic mobility of metabolite cellular enzymes. MEE has successfully applied in studies with many organisms, but only one report using MEE in strain identification of mutans streptococci has been published (Gilmour *et al*., 1987). Phenotypic typing is, in most cases, relatively inexpensive to perform and typeability of mutans streptococcal isolates is high, however, reproducibility and discriminatory power are usually somewhat poorer (Arbeit, 1999).

2.7.2. Genotyping:

Molecular typing methods have a higher discriminatory ability and reproducibility since these methods do not examine the gene expression but rather the DNA of the microorganisms to be studied (Arbeit, 1999; Olive and Bean, 1999). Among these typing methods are plasmid analysis, restriction endonuclease analysis (REA), Restriction

Fragment Length Polymorphism (RFLP) (including ribotyping), pulsed field gel electrophoresis (PFGE) and arbitrarily primed polymerase chain reaction (AP-PCR).

A. Plasmid analysis:

Plasmids are an extrachromosomal DNA molecule that encodes many properties, including antimicrobial resistance, many virulence traits and hydrocarbon metabolism (Madigan *et al*., 1997a). Plasmid analysis was the first DNA-based technique applied in epidemiological studies on mutans streptococci (Caufield *et al*., 1982). Because plasmids are infrequently detected in mutans streptococci, in only 5% of strains (Hamada and Slade, 1980b), plasmid analysis is not applicable to typing of these bacteria.

B. Restriction endonuclease analysis (REA):

In restriction endonuclease analysis (REA), bacterial chromosomal DNA cut with restriction endonucleases and DNA fragments are separate by gel electrophoresis. The restriction endonucleases are enzymes that cut the DNA chain at specific recognition sequences. The restriction enzymes are now days synthetically fabricated, but were originally isolated from bacteria, with their original function being defense against other bacteria. After separation by gel electrophoresis, gels stained with ethidium bromide and detected under UV light, whereby the banding patterns obtained for different strains are compared. Often the process results in fingerprints with many bands, thus the interpretation of the REA profiles can be complicated. REA has applied for evaluation of relatedness of mutans streptococcal isolates (Caufield and Walker, 1989; Kulkarni *et al.*, 1989).

C. Restriction fragment length polymorphism (RFLP):

After cleaving the chromosomal DNA of the microorganisms to be studied, the separation products can be labelled with either DNA or RNA probes in the Southern blot technique (Southern, 1975). The use of a probe derived from the *Escherichia coli* ribosomal operon introduced by Grimont and Grimont in 1986. They had discovered that variations of the genes encoding ribosomal ribonucleic acid (rRNA), and variations in sites flanking those loci, could serve as a means of typing strains since ribosomal sequences are highly conserved. The term ribotyping was introduced in 1988 (Stull *et al.*, 1988), and ribotyping of mutans streptococci has been applied since 1993 (Saarela *et al.*, 1993) mainly in studies on transmission of mutans streptococci and stability of infection (Alaluusua *et al.*, 1994).

D. Pulsed field gel electrophoresis (PFGE):

In pulsed field gel electrophoresis, a variation of agarose gel electrophoresis, the orientation of the electric field across the gel is changed periodically ("pulsed") thus, larger bacterial DNA fragments can be analysed than by REA (Arbeit, 1999). PFGE considered as a "gold standard" of molecular typing methods, with excellent discriminatory power and reproducibility (Arbeit, 1999; Olive and Bean, 1999). This method not been applied for typing of mutans streptococci.

E. Arbitrarily primed polymerase chain reaction (AP-PCR):

Of the genotyping methods thus far applied to mutans streptococci, AP-PCR is perhaps the least laborious (Olive and Bean, 1999). AP-PCR can perform with a very small sample volume. For the polymerase chain reaction, the template annealed to one or more short primers (typically 9-10 bp) at low stringency. Amplification results in an array

of DNA fragments, often termed random amplified polymorphic DNA (RAPD) that can be resolved by gel electrophoresis. AP-PCR requires no previous knowledge of the DNA to be analyzed (Welsh and McClelland, 1990; Williams *et al*., 1990). A limitation of the method is that it is very sensitive to even minor variations in technical factors such as temperature, Mg^{2+} concentration and polymerase source. Inter laboratory comparison of typing results is impeded by only a fair reproducibility (Arbeit, 1999), and only isolates processed simultaneously and fingerprints obtained concomitantly can be compared. This fair reproducibility is however, complemented by a good discriminatory power (Arbeit, 1999). AP-PCR typing has shown to be well applicable to typing of mutans streptococci (Sarela *et al*., 1996; Li and Caufield, 1998).

F. Sequencing of Gtf genes, gtf A,-B,-C:

Several studies have showed genetic heterogeneity among *streptococcus mutans* strains (Sarela and Coufield, 1998; Gronroos and Alaluusua, 2000). One of the important characteristics of *S.mutans* in promoting caries development is the ability to adhere firmly to the tooth surface in the presence of sucrose. This adherence is mediated mainly by the enzymatic action of the glucosyltransferase (GTF) enzymes ((Alaluusua *et. al.*, 1997; Loeshe, 1986; Kuramitsu, 1993) these enzymes are considered fundamental for the virulence of *S.mutans* in the pathogenesis of dental caries (Yamashita *et al.,* 1993). Oho *et al,* (2000) have constructed primers and established conditions for the amplifications of (gtf) genes.

G. Mutacin genes mut-I, -II, -III, Analysis by PCR:

The ability of *streptococcus mutans* to produce mutacins, combined with the production of other virulence factors such as lactic acid, may contribute to the

pathogenesis of this bacterium. Mutacin are peptide or protein antibiotics that are mainly bactericidal for other bacteria of the same or closely related species, as well as for other gram positive micro-organism and are likely to confer an ecological advantage in diverse bacterial communities, such as the dental biofilm (Parrot *et. al.*, 1990; Balakrishnan *et al.* 2002). Some studies have demonstrated that the mutacin activity of *S.mutans* could relate to the prevalence of this species in the dental biofilms, saliva and dental caries (Berkowtz and Jordan 1995; Hillman *et al.,* 1997). Strains based on their bactericidal activity divides mutacins into four types 1,2,3,4. The antimicrobial spectrum of mutacin 4 is specifically against members of the mitis-group of oral streptococci, while that of mutacin 1, 2 and 3 is broader (Qi, *et al.* 1999 b, 2001; Novak *et al.*, 1994).

2.8. Importance of Present Study and Objectives:

Among the oral diseases, dental caries considered a preventable disease. It can prevent by minimizing the frequency of ingesting simple carbohydrate and beverages and regular oral hygiene measure to remove dental plaques. Even then, all appropriate preventable measures taken; many individuals have shown increased susceptibility to dental caries. The most commonly used caries risk factors and indicators in multifactorial Caries Risk Assessment (CRA) models include levels of cariogenic bacteria (i.e. *Streptococcus mutans* and *Lactobacillus*), salivary factors (e.g. flow rate and buffering capacity), carbohydrate intake, oral hygiene, fluoride exposure, previous caries experience, and socioeconomic characteristics (Brathall and Petersson, 2005). North East part of Karnataka comes under socioeconomically backward region. Peoples are unaware of oral cavity hygiene and its consequences on their health. Poverty, illiteracy, change in diet, poor oral hygiene and bad habits enhance the prevalence oral diseases in this region.

As tobacco consumption in any from not directly correlate with prevalence of dental caries but indirectly it effects on the saliva secretion and physiology of the saliva by changing the pH, buffering capacity and immunity.

The main factor for dental caries is the diet, oral hygiene and cariogenic bacterial load. As *Streptococcus mutans* generally considered as one of the principle aetiological agents of dental caries, even though, it has also been isolated from the saliva of caries free individuals. Therefore, it becomes necessary to know the diversity and virulence traits of *S. mutans* isolated from individuals with caries and without caries, by phenotypic and molecular characterization. Only few reports are available in this aspect especially from the Indian context. The risk of dental caries does not depend on the quantity of *S.mutans* load in the oral micro-flora but the cariogenic factors of the organisms and other host factors augments initiation of dental caries. Therefore, we have undertaken the present study with the main aim of isolation of *S.mutans* from with and without caries subjects of all age groups and their phenotypic and molecular characterization and their response to various antibiotics with the following objectives.

OBJECTIVES:

1. Isolation of *S.mutans* from saliva samples collected from individuals with and without caries and with or without habits of tobacco chewing.
2. Characterization of *S.mutans* isolates by conventional methods.
3. Antibiogram typing and MIC determination.
4. Molecular characterization of the *S.mutans* isolates for their cariogenocity and survivability in dental plaque.

3. MATERIALS AND METHODS

3.1. SOURCES AND SUBJECTS OF THE STUDY:

3.1.1. Plan of the study:

The study was planned to investigate the prevalence of the bacterium present in the oral cavity (saliva) which plays a principle role in the initiation of dental caries. Factors affecting the prevalence of bacterium and its virulence in caries risk individuals.

3.1.2. Sources of the study:

We selected two dental college hospitals in Gulbarga namely, Nijalingappa Dental College and Hospital and Al-Bader's Dental College and Hospital, for the collection of saliva samples from subjects for this study.

3.1.3. Subjects included in this study:

The study comprised of a total 254 subjects, which included both the caries active and caries free individuals with or without tobacco chewing habit volunteers attending the outpatient department (OPD) of two dental colleges and hospitals of Gulbarga city. They were selected in such a way that they belonged to different sex, age and socio-economic background.

3.1.4. Collection of the samples:

Unstimulated 2–3 ml of saliva sample was collected into a prelabeled sterile wide mouthed plastic capped bottles from the volunteer individuals with or without caries and the collected saliva samples were immediately transported to the laboratory and processed for the enumeration of mutant streptococci and isolation of *S. mutans.*

3.2. MICROBIOLOGY OF SALIVA SAMPLES:

3.2.1. Enumeration of mutant streptococci in the saliva samples:

The saliva collected from each individual processed for the total count of mutans streptococci and *S.mutans* by serially diluting 1 ml of well-mixed saliva up to 10^{-5} dilutions in sterile normal saline. From the 10^{-2} to 10^{-6} dilutions, 0.5 ml from each tube were inoculated on to Mitis Salivarius Bacitracin (MSB) agar media by spread plate method and plates were incubated in an anaerobic jar for 48 hrs. The plates were observed for the growth and the colonies were counted and the bacterial load in the collected saliva sample was calculated.

3.2.2. Isolation of *S.mutans* from the saliva samples:

Enumeration and isolation of S. *mutans* from the collected saliva samples of each individual was performed by pour and streak plate methods using Mitis Salivarius Bacitracin (MSB) agar medium. For pour plate method, 0.5 ml of serially diluted saliva tubes from 10^{-2} to 10^{-5} dilutions was taken and for streak plate method two loops of undiluted saliva sample was inoculated on to the MSB agar medium. The characteristic colonies of *S mutans* were identified and counted. Among this well-demarked colony was picked up and re-streaked on fresh MSB medium and thus isolated pure culture of *S.mutans* was stored on the MSB slants at -20^{o} C with glycerol for further experiments.

Composition of mitis-salivarius bacitracin (MSB) agar medium:-

Component	*gms/litre*.
Casein enzymatic	15.00
Hydro lysate peptic	5.00
Digest of animal tissue	1.00
Dextrose	50.00
Sucrose	50.00
Dipotassium phosphate	4.00
Tryphan blue	0.075
Crystal violet	0.0008
Water	1000.0 ml
Agar	15.00

Final pH = 7.0 ± 0.2

3.3. Characterization of *S.mutans* isolates from the Saliva Samples:

Pure culture isolates of *S.mutans* from the saliva samples were characterized by following conventional identification methods referring standard microbiology laboratory manuals.

3.3.1. Cultural characteristics of *S.mutans* isolates:

Pure culture of streptococcal isolates from the saliva sample were re-streaked on the MSB agar plates and were incubated anaerobically for 48 hrs and the colony with distinct characteristic morphology of *S.mutans* was observed. The single colony was subcultured on MSB agar slants and growth was stored with sterile glycerol at -20^{o} C.

3.3.2. Morphological characteristics of *S.mutans* isolates:

Part of *S.mutans* colony on the MSB medium was picked up and made thin smear on optically plane microscopic slide and smear was air dried and fixed by slight heating over the Bunsen burner flame. The heat fixed smear was Gram stained and observed for the characteristic staining property and the morphology of the isolate. *S. mutans* are gram positive cocci in small chains without spores and capsule.

3.3.3. Biochemical characteristics of *S. mutans* isolates:

The colony showing distinct cultural morphological characteristics of *S.mutans* was subcultured and growth was used for the performance of biochemical tests. Rapid biochemical identification kit from HI-Media was used along with conventional biochemical tests like fermentation of mannitol and sorbitol, hydrolysis of arginine and esculin and Voges Proskauer. Based on the cultural, biochemical and morphological characteristics the isolates were identified up to the species level.

The rapid biochemical identification kit was purchased from the Hi-media Pvt. Ltd., Mumbai and other biochemical tests were prepared and performed according to the procedures described in the standard microbiology laboratory manuals. The failure of Lancefield extracts of *S. mutans* to react with streptococcal group D antisera, the formation of characteristic gelatinous deposits in 5% sucrose broth, and acid formation in mannitol broth are the three major criteria for recognizing *S. mutans* (Fachlam, 1974).

The binomial nomenclature of *Streptococcus mutans* will continue to denote a *Streptococcus* which ferments mannitol, usually ferments sorbitol and raffinose,

produces glucan from sucrose, and is not an enterococcus. Mutans streptococci group consists of four important bacteria present in the oral cavity associated with dental caries. To distinguish these from one another we performed biochemical tests including sugar fermentation, Voges Proskaeur, Esculin hydrolysis and arginine hydrolysis tests.

Streptococcus mutans subsp mutans (Clarke, 1924) is a Gram-positive coccus, usually found in pairs or chains, ferments mannitol, sorbitol, raffinose and inulin, glucan is produced from sucrose can grow aerobically but CO_2, required for optimum growth and ammonia is not produced from arginine.

S. mutans subsp. Sobrinus is least related to *S. mutans* subsp. mutans. It is a Gram- positive coccus in pairs and chains. Mannitol, inulin, and usually sorbitol are fermented and raffinose is not fermented. Glucan is produced from sucrose. Ammonia is not produced from arginine. Grows in air but CO_2 is required for optimum growth.

Subspecies	Genetic group	DNA base content % (G+C)	Cell-Wall carbohydrates	Other distinguishing characters
S. mutans subsp. Mutans	I	36-38	Glucose rhamnose	Slow glycolysis of fructose when grown on glucose; some strains Lancefield E
S. mutans subsp. Rattus	II	41-43	Galactose Rhamnose	Produces ammonia from arginine
S. mutans subsp. Sobrinus	III	44-46	Glucose, Galactose rhamnose	Does not ferment raffinose
S. mutans subsp. Cricetus	IV	42-44	Glucose, Galactose rhamnose	Does not grow in air

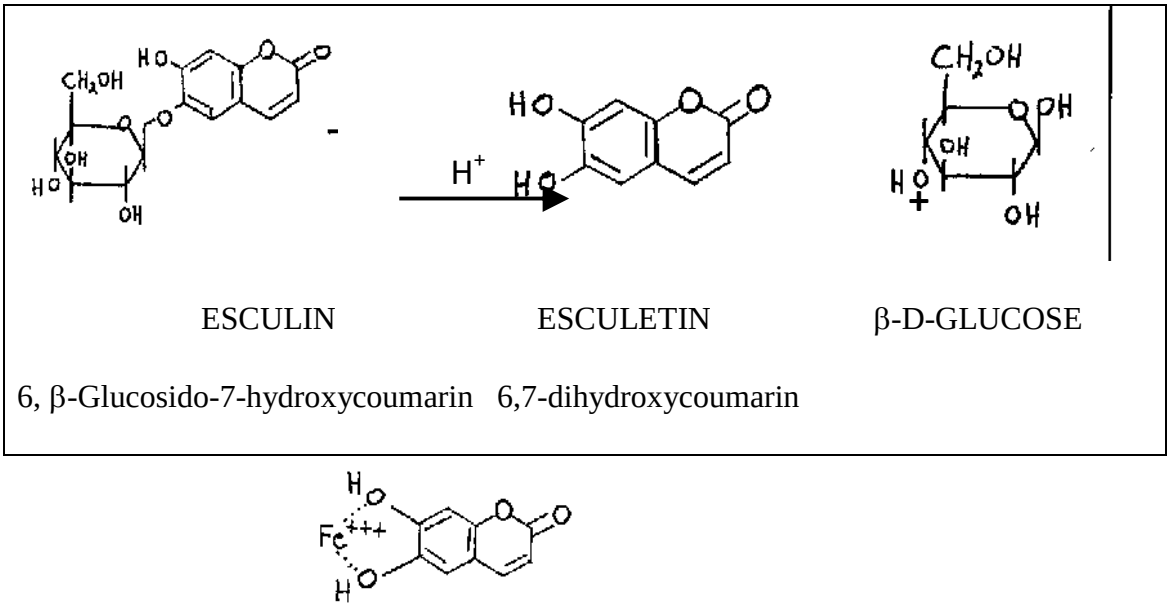
A. <u>Hydrolysis of Arginine:</u>

Streptococcus rattus is similar to other ADS-positive oral bacteria in that it is relatively acid sensitive in the absence of arginine and has not been linked to caries development in humans. However, *S. rattus* is a member of the mutans group of streptococci and is most closely related to *S. mutans*, which is noted for exceptional cariogenicity and acid tolerance. In fact, a major factor distinguishing *S. rattus* from *S. mutans* is the ability to catabolize arginine via the ADS. By initiating a molecular characterization of the *arc* operon of *S.rattus* FA-1, a clearer understanding of the evolution and diversity of the mutans streptococci in relation to pH adaptation will be obtained.

B. Hydrolysis of Esculin:

Ability of bacteria to hydrolyze esculin in the presence of bile is commonly used for the presumptive identification of group D streptococci and enterococci, all of which are positive. Group D streptococci and enterococci include opportunistic pathogens such as *Enterococcus faecalis*, *Enterococcus faecium*, and *Streptococcus bovis.*

Bile esculin medium contains esculin and peptone for nutrition and bile to inhibit Gram-positive bacteria other than Group D streptococci and enterococci. Bile esculin azide agar uses sodium azide to inhibit Gram-negative bacteria, but we don't include the sodium azide due to safety concerns. Since we used isolated Gram positive cultures, it's unnecessary. Ferric citrate is added as a color indicator. Esculin is a glycoside (a sugar molecule bonded by an acetyl linkage to an alcohol) composed of glucose and esculetin. These linkages are easily hydrolyzed under acidic conditions. Many bacteria can hydrolyze esculin, but few can do so in the presence of bile. Organisms that split the esculin molecules and use the liberated glucose to supply energy needs release esculetin into the medium. The free esculetin reacts with ferric citrate in the medium to form a phenolic iron complex, which turns the agar slants dark brown to black. An agar slant that is more than half darkened after no more than 48 hours' incubation is bile-esculin positive. If less than half the slant has darkened, the result is negative.



ESCULIN ESCULETIN β-D-GLUCOSE

6, β-Glucosido-7-hydroxycoumarin 6,7-dihydroxycoumarin

Control: esculin^{+}: *Enterococcus faecalis*

C. Voges Proskaeur (acetoin produced from glucose):

MR-VP medium contains glucose and peptone. Most of the bacteria oxidize glucose for energy; however the end products vary depending on bacterial enzymes. Both the MR and VP tests are used to determine what end products result when the test organism degrades glucose. If organism produce acid, pH become below 4.4 and methyl red gives red color but it produces more neutral products from glucose e.g. ethyl alcohol, acetyl methyl carbinol in this neutral pH the growth of the bacteria is not inhibited. The bacteria thus begin to attack the peptone in the broth, causing the pH to rise above 6.2. At this pH, methyl red indicator is a yellow color (a negative MR test).

The Voges-Proskauer (VP) test determines whether a specific neutral metabolic intermediate, acetoin, has been produced instead of acid from glucose. Acetoin is the last

intermediate in the butanediol pathway. The reagents used for the VP test are Barritt's A (alpha-napthol) and Barritt's B (potassium hydroxide). When these reagents are added to a broth in which acetyl methyl carbinol is present, they turn a pink-burgundy color (a positive VP test). This color may take 20 to 30 minutes to develop. If the bacterium produces acetyl methyl carbinol, then they will be positive for VP test.

3.4. ANTIMICROBIAL SUSCEPTIBILITY TEST FOR *S.MUTANS* ISOLATES:

3.4.1. Antimicrobial susceptibility profile of *S.mutans* isolates:

All the *S.mutans* isolates were subjected for antibiotics susceptibility testing against 11 – frequently used antibiotics in this area and elsewhere for the treatment of dental caries following CLSI-2006 guidelines. All the 11 antibiotics discs were purchased from the Hi-media Pvt. Ltd., Mumbai. The method adopted for the antibiotics susceptibility testing is the Kirby-Baurers agar disc diffusion assay using Mueller-Hinton agar. The discs were dispensed on the pre-inoculated agar plates with the test isolate *S.mutans* and the plates were incubated anerobically at 37^{o} C using the anaerobic jar. The zone of inhibition was measured with zone measuring scale provided by Hi-media. The inhibition zone size was interpreted according to the CLSI-2006 guidelines. The name, abbreviations and concentration per disk of 11 antibiotics used in this study are mentioned below.

Name of the antibiotics	Concentration per disc	Name of the antibiotic	Concentration per disc
Amoxycillin (Ac)	30 Mcg	Ampicillin (A)	10 mcg
Chloramphenicol (C),	30 mcg	Ciprofloxacin (Cf)	5 mcg
Erythromycin (E)	15 mcg	Gentamicin (G)	10 mcg
Levofloxacin (Le)	5 mcg	Penicillin – G (P)	10 units
Streptomycin (S)	10 mcg	Tetracycline (T)	30 mcg
Vancomycin (Va)	30 mcg		

3.4.2. Analysis of multi drug resistance among isolates:

After recording the zone of inhibition of each antibiotic for all *S.mutans* isolates, the inhibition zone size was interpreted according to the national committee of clinical laboratory standards criteria. The number of antibiotics to which each isolate of *S. mutans* is resistant was recorded. According to CLSI-2006 guidelines the isolate resistant to 3 or more antibiotic is considered as Multi Drug Resistant (MDR) strain (isolate). The MDR pattern of *S.mutans* isolates was calculated and prevalence of MDR isolates from with caries and without caries individuals is also recorded.

3.4.3. Analysis of Minimum Inhibitory Concentration (MIC) for *S.mutans* isolates:

Hi–comb consists of a strip made of an inert material, with and extensions that carry the disc of 4 mm, resembling the "**touch of a comb**". Towards the stem of the strip, MIC reading scale in ug/ml is given along with the Hi-media code. A defined concentration of antibiotics is loaded on each of the disc so as to form a gradient, when

placed on agar plate. Hi-comb (Based on the principle of dilution and diffusion) consists of a gradient that covers a continuous range of 16 twofold dilutions on 2- different strips as per the conventional MIC method. When applied to the agar surface. The gradient remains stable after diffusion and the zone of inhibition created takes the form of an ellipse. After 48 hrs of incubation at 37^{o} C in an anaerobic atmosphere, the MIC value was determined as the lowest concentration of antimicrobial agent that inhibited visible growth of an organism.

Minimum inhibitory concentration (MIC) for Amoxycillin, Ciprofloxacin, Chloramphenicol, Erythromycin and Tetracycline was performed using Hi-Comb MIC test strips. Antibiotic discs and strips were procured from Hi-Media Laboratories Pvt. Ltd., Mumbai.

Name of the Antibiotic		**Range of Antibiotic concentration (**mcg**)**
1.	Amoxycillin	0.001 mcg to 240 mcg
2.	Ciprofloxacin	0.01 mcg to 240 mcg
3.	Chloramphenicol	0.001 mcg to 240 mcg
4.	Erythromycin	0.001 mcg to 240 mcg
5.	Tetracycline	0.01 mcg to 240 mcg

3.5. MOLECULAR STUDIES:

3.5.1. PCR amplification of *gtf* B gene:

A. **PCR amplification of Glycosyltransferase gene (*gtf* B):** Adherence substances produced in the presence of sucrose by the glucosyltransferase (GTF) enzymes are considered fundamental for the virulence of *S. mutans* in the pathogenesis of dental caries (Yamashita *et al.,* 1993). There are 3 *gtf* genes named as A,B,C; of these *gtf* B is present in all *S.mutans* strains and is taken as a taxonomic marker to distinguish *S. mutans* from the other streptococci. In this study the 517 bp region off *gtf* B was amplified using the following primers by simple PCR (Oho *et al.,* 2000):

*gft*B Forward 5'- ACTACACTTTCGGGTGGCTTGG-3'

*gft*B Reverse 5'-CAGTATAAGCGCCAGTTTCATC-3'.

B. **Conditions for amplification** (Oho *et al.,* 2000): For the PCR running standardized the condition used by the Oho *et al.*,2000; in the Clinspire Technolgy, Ballery, Karnataka. The conditions applied for the amplification of *gft* B gene using eppendorf gradient PCR machine are as follows:

C. Conditions for *gft* B amplification:

Initial denaturation step : at 95^{o} C for 5 min.

For each cycle (toal 30 cycles)

Followed by denaturation : 95^{o} C for 30 seconds,

Annealing at : 59^{o} C for 30 seconds

Extension at : 72^{o} C for 1 min

Final elongation at : 72^{o} C for 10 min and stand at 10 ^{o} C

D. Visualization of amplified products: PCR products were purified using Sephadex G tubes and analyzed by electrophoresis in 1.5% agarose gel using TBE buffer along with amplified DNA products. After electrophoresis the agarose gel was stained by immersing in the 2.0 ug/ml ethidium bromide solution and the DNA bands were observed under UV transilluminator and Photographed.

3.5.2. PCR amplification of mutacin genes:

A. Mutacin: Mutacins are protein antibiotics that are mainly bactericidal and are helping *S. mutans* to confer an ecological advantage in diverse bacterial communities like dental biofilm (Balakrishnan *et al.,* 2002). Mutacin activity of *S. mutans* could be related to the prevalence of this in dental biofilm, saliva and dental caries (Hillman *et al.,* 1987). Mutacins are classified into four types, I, II, III and IV. The antimicrobial spectrum of mutacin IV is specific against other mitis group of oral streptococci, while that of mutacins I, II and III is broader (Qi *et al.,* 2001; Kamiay, *et al.,* 2004).

B. Primers: We used the following primers for amplification of Type-I (750bp) and Type- II (444bp) by PCR.

Mutacin – Type-I (Qi *et al.,* 1999b)

Forward 5'-AGTTTCAATAGTTACTGTTGC-3'

Reverse 5'- GCCAAACGGAGTTGATCTCGT-3'

Mutacin – Type-II (Novak *et al.,* 1994)

Forward 5'- AACGCAGTAGTTTCTTTGAA-3'

Reverse 5'- TTCCGGTAAGTACATAGTGC-3'

C. **PCR conditions for mutacin gene amplification** (Qi *et al.,* 2001; Kamiay *et al.,* 2005):

Initial denaturation step : at 94^{o} C for 5 min.

For each cycle (toal 35 cycles)

Followed by denaturation : 94^{o} C for 45 seconds,

Annealing at : 52^{o} C for 1 min

Extension at : 72^{o} C for 2 min

Final elongation at : 72^{o} C for 7 min and stand at 10^{o} C

D. **Visualization of amplified products**: PCR products were purified using Sephadex G tubes and analyzed by electrophoresis in 1.0% agarose gel using TBE buffer (Tris/borate/EDTA buffer, pH 8.0) along with amplified DNA products 250 bp DNA ladders (marker) was also loaded in the separate well after mixing with loading dye. After electrophoresis the agarose gel was stained by immersing in the 1.0 ug/ml Ethidium bromide solution and bands were observed under UV transilluminator and photographed.

3.5.3. Adherence study of *S.mutans* isolated from caries and caries free individuals:

(Hamada and Torii, 1978 and Napimoga *et al.*, 2004):

One of the main characteristics of mutans streptococci is their ability to produce jelly like substances when they were incubated in the medium containing 1 to 5% of sucrose. However, *S.sobrinus* makes the medium jelly but *S. mutans* produces polysaccharide (biofilm) which gets attached to the inner surface of the culture tube and most of the bacteria attached to the surface firmly compared to the other mutans streptococci. The adherence capacity of the *S. mutans* isolates from both with and without caries individuals was determined turbidimetrically. Isolates were grown in BHI broth at 37^{o} C in an anaerobic atmosphere for 20 h. Aliquots of 100 µl cells (OD at 550 nm 0.8–1.0) were then transferred to fresh BHI broth containing 1% sucrose and grown under the anaerobic at 37^{o} C conditions at an angle of 30^{o} C for 24 h. Then culture tubes were vigorously mixed in a vortex mixer for 5 seconds and non-adhering cells were transferred to fresh tubes. Aliquots of 3 ml potassium phosphate buffer (0.05 M, pH 7.0) were added to the first tube and agitated for 5 seconds: the released cells were transferred to a third tube. The second and third tubes were centrifuged for 5 min at 5000 g and the pellets were re-suspended in the same buffer. All tests tubes were then sonicated for 30 seconds or vertexed for 5 min and OD of suspended cells at 550 nm was measured by spectrophotometer. The percentage of the adhered cells was calculated by dividing the cell density of adherent cells by the values of total cell density.

Percentage of adherence cells = OD of third tube/OD of 2^{nd} + 3^{rd} tubes X 100.

4. RESULTS.

4.1. ISOLATION AND CHARACTERIZATION OF *S.MUTANS* FROM SALIVA SAMPLES OF INDIVIDUALS WITH AND WITHOUT CARIES:

Overall, 254 samples were collected from the individuals attending the OPD of two dental college and hospitals in Gulbarga. These samples included individuals with or without caries and also with or without habits of chewing any tobacco or tobacco containing materials. The subjects included in this study are of all age groups, both sexes, and belonged to socioeconomically different sections (**Tables -1 to 3; Fig.1**).

A. **Enumeration of bacterial load in the saliva collected**. The mutans streptococcal load of the saliva was determined by serial dilution and spread plate technique using and mitis-salivarius bacitracin (MSB) agar media. Overall, the total bacterial load is high in the samples from habitual individuals with caries, followed by the non-abituals with or without caries individuals and least in habitual individuals without caries (**Table-4**).

B. **Isolation of *S.mutans* from the saliva**: The *S.mutans* in the saliva was isolated by inoculating diluted and undiluted saliva on mitis-salivarius bacitracin (MSB) agar medium and plates were incubated at 37^{o} C under anaerobic condition(using anaerobic Jar) for 48 hours and characteristic colonies were picked up and sub cultured on the same fresh medium(**Fig.-2**). Isolation rate of *S.mutans* was also very high in the individuals with caries compared to without caries (**Table-4**).

C. **Characterization of isolated *S. mutans* by conventional methods:** The isolated streptococci on mitis salivarius bacitracin agar plate, were further characterized

by morphological (grams staining), sets of biochemical and sugar fermentation tests (**Figs-3-5**). The the test results are summarized in the **Table-5**.

4.2. Prevalence of *S. mutans* in the Saliva of Individuals with and without Caries:

4.2.1. Over all incidence of *S. mutans* isolates:

A total of 120 (47.24%) *S. mutans* was isolated from 254 saliva samples collected without stimulant from with caries and caries free individuals with and without habitual (**Table-6**). Incidence rate was very high (57.04%) among the individuals with caries as compared to individuals without caries (34.82%). Incidence of S. *mutans* in non habituals was observed to be 55.03% (82/149) which is much higher than that in the individuals with habits (36.19%; 38/105), however it is maximum (63.46%) in the habitual males with caries compared to any group in this study. We observed higher (60.71%) incidence of *S. mutans* in habitual individuals with caries and lowest (08.16%) in habitual individuals without caries. Over all higher incidents observed in female (51.54%) than in male (44.58%), however it is reverse among genders with caries individuals (**Fig. 6)**.

4.2.2. Incidence of *S.mutans* in the individual with caries:

Over all isolation rate of S. *mutans* from individuals with caries including both habitual and non-habitual was observed to be 57.04%. Incidence was slightly higher 60.71% (34/56) in the individuals with tobacco chewing habit compared to that observed (54.65%; 47/86) in individuals without tobacco chewing habits (**Table-7).** Prevalence

rate of *S. mutans* was higher in males (58.88%; 53/90) than females (53.83%; 28/52) and highest incidence of (63.46%) was in males with habits (**Fig.7**).

4.2.3. Incidence of *S. mutans* in individual without caries:

The incidence rate of *S. mutans* from the individuals without caries including both habitual and non-habitual is shown in the **Table-8** and was found to be 34.82% (39/112). The majority (55.55%) of *S .mutans* isolates were from the individuals without habits of tobacco chewing compared to tobacco chewing individuals (08.06%). We observed that the incidence rate of *S. mutans* was almost double in females (48.88%) than in males (25.37%; **& Fig.8**).

4.2.4. Prevalence of *S.mutans* isolates among habitual and non-habitual individuals:

The isolation rate of *S.mutans* in the habitual and non-habitual individuals is represented in the **Table-9**. According to table, higher rate of isolation of *S.mutans* is from the saliva samples of non-habituals (55.03%) compared to habituals (36.19%) among both with caries and caries free individuals. Maximum of 55.17% incidence of *S.mutans* observed in females among non habitual individuals. Gender wise analysis of results reveals higher incidence in males (37.89%) than in females (20%) of habituals however it is reverse in non-habituals but difference is less than 1%. However, overall incidence was more in female than in males **(Table-9; Fig. 9).**

4.3. Prevalence of *S. mutans* according to age groups:

4.3.1. Age group wise incidence of *S. mutans* in individuals with caries:

Individuals included in this study were divided in to 5 groups based on their age. The incidence of *S. mutans* in different age groups is given in **Table-10**. According to the table the rate of incidence increased with the increase in age, the maximum (62.90%) of incidence was observed in the age group of 36-45 years as compared to 40% in younger age group below 5 years. In the same age group highest (80%) incidence was observed among the caries individuals with habitual compared to non habitual (54.76%), where the maximum (61.11%) incidence is in the age group of 26-35 years. Very less (44.44% to 61.11%) difference in percent incidence rate was observed among the 5 age groups of non-habitual individuals with caries compared to wide range (33.33% to 80%) of percent incidence observed among the habitual individuals with caries. *S.mutans* isolation rate was high (63.46%) in males compared to female (25%) in habitual but it reverse in the non habitual individuals with caries (**Table-10; Fig.10**).

4.3.2 Age group wise incidence of *S.mutans* in caries free individuals:

The results of rate of isolation of *S.mutans* in different age groups of caries free individuals with and without habituals is presented in **Table-11& Fig.11**. The table reveals that the incidence of *S. mutans* gradually increased with increase in age, from 20% in age group below 15 years to 45.71% in age group of 36-45 years. Age group wise incidence rate ranged from 33.33% to 66.6% in non-habitual individuals without caries. Among caries free individuals, higher (55.55%) incidence was observed in non-habituals compared to habituals (08.16%). Incidence rate of *S. mutans* was slightly higher in males than in females of non-habitual and is reverse in the habitual among without caries individuals (**Fig. 11**).

4.3.3 Age group wise and gender wise incidence of *S.mutans:*

Table-12 presents the overall results of age group wise and gender wise incidence of *S.mutans* from individuals with and without caries. From the table overall rate of incidence is higher (51.54%) in females than in males (44.58%). Age group wise highest percent of incidence in females is in the age group of 26-35 years whereas in males highest in the age group of 36-45 years. Interestingly lowest incidence was observed in the age group of below 15 years in both sexes. In all age groups, incidence of *S. mutans* in females is more than in males except in the age group of 36-45 (**Table-12; Fig.12**).

4.3.4. Age group wise and gender wise incidence of *S.mutans*:

Over all age group wise incidence of *S. mutans* gradually increased with increased with age in all age groups. In habitual individuals highest of 51.61% in the age group of 36-45 years followed by 38.70% in 16-25 years age group. Whereas incidence was almost 60% in the age groups of 26-35 and 36-45 years in the individuals without habits. The incidences are higher in the age group of 36-45 years among the individuals with and without caries (**Table-13; Fig.13**).

4.4. Antimicrobial Susceptibility Testing of *S.mutans* Isolates:

Antimicrobial susceptibility testing was performed for all the 120 *S.mutans* isolates against the 11 antibiotics frequently prescribed to treat dental caries and oral cavity diseases.

4.4.1. Resistance among *S.mutans* isolates to various antibiotics:

In addition to participation in the etiology of dental caries, *S.mutans* produces bacteremia, a systematic infection or sub-acute endocarditis and hemorrhagic stroke resulting from dental treatments. Adequate treatment for these infections requires the determination of the antimicrobial susceptibility of the *S. mutans* strains isolated from the oral cavity. Hence the antibiogram of all isolates of *S. mutans* strains was performed against 11 frequently prescribed antibiotics for dental caries **(Fig. 14)**. Percent resistance of *S. mutans* isolates from individuals with and without caries against various antibiotics tested in this study is given in the **Table-14 & Fig. 15.**

Overall results in the table-14 shows that a maximum of 95% of isolates were resistant to penicillin G, however 100% of *S.mutans* isolates from caries individuals were

resistant to penicillin G compared to only 84.61% of isolates from without caries individuals. In contrast, isolates from without caries individuals showed slightly higher percent of resistance than the isolates from with caries individuals. Chloramphenicol resistance was exhibited by 63.33% isolates, followed by 58.33% against amoxycillin and 54.16% to ampicillin. Whereas lowest incidence of 23.33% was observed against ciprofloxacin on the contrary higher resistance rate was observed against vancomycin (39.16%) and incidence of resistance to the remaining antibiotics was in the range of 31 to 39% (**Tables-15&16**).

The results of percent resistance of *S.mutans* isolates from individuals with and without habits against various antibiotics tested in this study are given in **Table-17; Fig.16.** Overall results in the table-17 shows that a maximum of 95% of isolates were resistant to penicillin G, however 97.36% of *S.mutans* isolates from habitual individuals were resistant to penicillin G compared to 93.90% of isolates from without habitual individuals. Among the *S.mutans* isolates from the habitual individuals, highest resistance to penicillin G followed by chloramphenicol (65.78%), ampicillin (63.15%), amoxycillin (52.63%), vancomycin (44.71%) and least of 26.31% to ciprofloxacin, streptomycin, and tetracycline. In contrast, isolates from without habits individuals showed lower percent of resistance to all antibiotics except levofloxacin (40.24%), tetracycline (39.02%) and Streptomycin (34.14%) and lowest resistance was observed to be 21.95% to ciprofloxacin. High resistance rate observed to vancomycin (44.71%) among the isolates from habitual individuals compared to 36.58% resistance to the *S.mutans* isolates from non habitual individuals. 100% resistance to penicillin G was observed in both sexes of habituals with caries and only in females among without caries (**Table-18**) in contrast

only nonhabituals with caries showed 100% resistance to penicillin G (**Table-19**). Overall, *S. mutans* isolates showed lower resistance to ciprofloxacin, lowest of 12.5% of resistance in the isolates from female individuals belongs to non-habitual without caries group.

4.4.2. Gender wise resistance among *S.mutans* isolates to various antibiotics:

The resistance patterns of isolated from both sexes showed not much variation to individual antibiotics, however very high incidence of 41.50% in females compared to males (23.88%) to streptomycin. While to ciprofloxacin it is reversed (28.35% in males and 16.98% for females). Gender wise incidence for remaining antibiotics observed less difference between two sexes (**Table-20; Fig.17**).

4.4.3. Multi drug resistance of *S.mutans* isolates:

Results of Multi drug resistance (MDR) pattern for all the *S.mutans* isolates in this study is represented in **Table-21**. All the *S.mutans* isolates in this study were multi drug resistant (MDR) and were resistant to a minimum of 3 antibiotics. None of the *S. mutans* isolates neither resistant nor susceptible to all 11 antibiotics tested. Maximum of 26.66% of isolates were resistance to 5 antibiotics, 21.66% to 7 antibiotics and 19.16% to 6 and 4 antibiotics. Less than 5% of the isolates showed resistance to 8 and 9 antibiotics. Maximum percent of isolates from the individuals with caries showed resistance to high number of antibiotics (6, 7 and 8) compared to *S. mutans* isolated from individuals without caries (4, 5 and 6 antibiotics; **Table-21**). However, maximum isolates from habitual individuals showed maximum resistance of 28.9% to 4 antibiotics and 30.48% to 5 antibiotics among non-habituals, while remaining isolates showed almost same percentage to 6, 7 and 8 in both habitual and non habitual individuals (**Table-22**).

4.4.4. MIC of commonly used antibiotics *against S.mutans* isolates:

Table-23 shows the minimum inhibitory concentrations (MIC) of commonly used 5 antibiotics *against S.mutans* isolates. Only ten *S.mutans* isolates representing minimum of 3 each from with caries, with or without habitual and without caries non-habitual individuals were used for the determination of MIC against 5 (amoxycillin, ciprofloxacin, erythromycin, chloramphenicol and tetracycline) most frequently prescribed antibiotics. The results are shown in **Table-23; Fig.18**. Overall higher side of MIC was depicted by *S.mutans* isolated from with caries habitual and without caries non-habitual individuals compared to lowest MIC range among in the isolates with caries non-habitual individuals. MIC range was maximum (25 times) from 0.01 µg/mL to 0.25 µg/mL against ciprofloxacin and tetracycline compared 2.5 to 10 times against remaining antibiotics. Lowest range of MIC of 2.5 times (0.1 µg/mL to 0.25 µg/mL) to erythromycin, 5 times (0.1 µg/mL to 0.5 µg/mL) to amoxycillin and 10 times (0.1 µg/mL to 1.0 µg/mL) to chloramphenicol was observed.

4.5. MOLECULAR STUDIES OF *S.MUTANS* ISOLATES:

As dental caries is a microbial disease so the prerequisite for caries development is the presence of dental plaque on the teeth and unless this biofilm present caries will not occur, regardless of any other risk factors (Kidd, 1999). So, initiation and progress of the dental caries mainly based on the survivability of *S.mutans* in saliva and especially in the dental plaque (biofilm) by producing adherence substances like glucan and bacteriocins like mutacins. In order to understand and know the cariogenicity of the isolated *S.mutans,* we selected total of 17 *S.mutans* isolates10 form without caries and 7 from

with caries individuals for molecular typing of *gtf-B* and mutacin genes by PCR amplification.

4.5.1. Determination of glucosyltransferase (*gtf*) genes from *S. mutans* isolates:

Water insoluble substance is required to form biofilm or dental plaque that helps in harboring and adhering of dental caries forming microorganisms. Adherence substances produced in the presence of sucrose by the action of glucosyltransferase (GTF) enzymes, which are considered as fundamentals for the virulence of *S. mutans* in the pathogenesis of dental caries. Glucosyltransferases are of 3 types and are produced by *gtfA, gtfB* and *gtfC* genes. Of these, *gtfB* is present in all *S.mutans* strains and is the main enzyme, which helps in the production of water insoluble glucan responsible for adherence of cells to tooth surface. All isolates in the Fig.19 showed single band corresponds to 517 bp indicates the presence of *gftB* gene. From the table-24, it is clear that all the 17 *S. mutans* isolates from with caries and careies free individuals showed a single band. Presence of *gtf-B* gene in the isolates also confirms the identification of the isolated bacteria as *S.mutans.*

4.5.2. Determination of mutacin genes of *S.mutans* isolates:

Mutacins are protein antibiotics that are mainly bactericidal and are helping *S. mutans* to confer an ecological advantage in diverse bacterial communities like dental biofilm. Mutacin activity of *S. mutans* could be related to the prevalence of this in dental biofilm, saliva and dental caries. Therefore, mutacin could play an important biological role in the formation and composition of dental biofilm, due to their synergetic and antagonistic activity. In this study we have, selected *S.mutans* isolates from both with

caries and caries free individuals, to know the types of mutacin genes present. Using specific primers for *mut* I and *mut* II genes and genomic DNA from *S.mutans* isolates amplified 750 bp and 444bp amplicons by simple PCR method. Amplicons for *mut* I and *mut* II genes generated by each isolate is shown in **Fig.20**. All *S. mutans* isolates from caries individuals express the *mut* I (750 bp) compared to only 70% of isolates from caries free individuals tested for molecular study. However, *mut* II (444 bp) gene is not amplified in all the isolates tested. Profile of *mut* I and *mut* II genes of each isolate is given in **Table-24.**

4.5.3. Adherence study of *S. mutans* isolates from individuals with caries and without caries:

Adherence property of *S.mutans* is mainly dependent on the extracellular production of adhesive substances that express on the bacterium surface itself and this is one of the characteristics of cariogenic (pathogenic) bacteria. This adherence substance produced in the presence of sucrose by the glucosyltransferase (GTF) enzymes is considered fundamental for the virulence of *S. mutans* in the pathogenesis of dental caries. *S.mutans* in the presence of sucrose produces this enzyme which converts sucrose to water insoluble glucan (WIG) which is directly proportional to the plaque formation *in vivo* and biofilm *in vitro*. Significant association between the presence of *gtfB,* production of adhesive substances and percentages of growing bacteria adhering to glass surfaces in the presence of sucrose has been observed.

The results are shown in **Table-25.** The percent adherence of *S mutans* isolates from with caries individuals is higher (39.56%) than the *S. mutans* isolated from without caries (19.11%) individuals These results clearly indicate that *S.mutans* isolated from with caries individuals are having more capacity to form dental plaques than from caries free individuals.

5. DISCUSSION

The dental profession has to deal with one of the most widespread of all human maladies. Dental caries (Harris, 2004), is not self-limiting, like the common cold, nor amenable to treatment with a simple course of antibiotics, like an ear infection in children (McDonald and Dean, 2004). Humanity has been plagued by the persistence of this unique disease since pre historic times (Kuriakose and Joseph, 1999). This disease can aptly be termed as a scourge of modern civilization and no nation or continent has escaped from the ill effects. If has been rightly called "- the last epidemic" (Chawla, 1986).

The global distribution of dental caries presents a varied picture. In the recent years most of the nations with previously low-caries prevalence are experiencing an unprecedented increase in caries prevalence. This has been attributed to lack of dietary and oral hygiene discipline; on the other hand, delayed first dental visit has been one of the causes. Several other countries show a decrease in the presence of dental caries and improvement in oral health care. This decline in caries may be due to the use of fluorides, oral health education programmes and increased oral hygiene awareness (Chawla, 1986). In India, the earliest references of dental caries states back to 1939, when Taylor and Day reported low prevalence of caries in adults of Kangra valley, Punjab (Johnson, 1991). Later in 1940, Day and Tandon reported that dental caries prevalence was less in Indian children as compared to American children. However, Shourie in 1941 for the first time conducted a multicentre epidemiological study in the age group of 5-7 years, which showed caries prevalence of 44.6% (Damle, 2002). Since then, a number of point prevalence studies have been conducted in different parts of the country.

Dental caries occurs as a result of the interaction of a dietary substrate, microbial pathogens, and host factors. Between meal carbohydrate frequency, which is a dietary indicator and stimulated salivary flow rate, both indicators of host susceptibility are significant factors in caries development. Contrarily, bacterial counts (i.e., S*treptococcus mutans)* were not found to be significantly associated with caries. However, when studied in groups, caries experience has found to relate to *S.mutans* counts. Therefore, bacterial counts by themselves are poor predictors of caries activity at the individual level (Burt and Eklund, 1994). This indicates that bacterial counts alone cannot predict future caries experience very accurately and thus we must rely on the overall risk score (i.e. the interactions of multiple factors to asses risk level).

Microbial counts can be an important element to consider when customizing a caries management plan that corresponds to the patient's individual needs (e.g. Prescribing an antimicrobial rinse such as chlorhexidine; Featherstone *et al.*, 2006). Dental caries is a slowly progressing disease that takes two years for an initial lesion to progress through the enamel and requires in the region of three to four years to extend into the dentin (Benn, 1994; Johansson, 1998). Dental caries develop under precise oral conditions and these circumstances are more likely to change during studies of longer duration due to lifestyle or behavioral modifications (Fontanna and Zero, 2006; Powell, 1998).

Among the biological factors mutans streptococci play an important role in the dental caries formation. Of these *S. mutans* is a principle bacterium in the initiation of biofilm on the smooth surface of teeth, which leads to formation of dental caries,

depending on the diet, habits and general health of an individual. Even when the individual is susceptible to the formation of dental plaque and all other factors are favorable, the formation of caries again depends on the careogenity of *S. mutans*. Therefore, a microbiologist has to emphasize on the isolation, characterization and cariogenity of an aetiological agent by conventional, molecular biological methods and antibiotic susceptibility testing.

In the present study, thus we included the individuals of all age groups with caries and without caries and with and without tobacco chewing habit. Over all 254 saliva samples were collected without stimulant from different individuals attending dental colleges and hospitals of Gulbarga city. Collected saliva samples were used for the enumeration of mutans streptococci and isolation of *S.mutans*. Results on frequency of total mutans streptococci and *S. mutans* revealed that high number of mutans streptococci were detected from individuals with caries (10^6 cfu/ml of saliva) compared to without caries individuals (10^5 cfu/ml). The *S. mutans* count was also higher in individuals with caries (7.1×10^4 cfu/ml of saliva) compared to without caries individuals (8.6×10^3 cfu/ml). Non-habituals of with caries individuals showed similar count of *S.mutans* compared to less in habituals of without caries individuals.

Streptococcus mutans in the concentration of 10^4-10^5 CFU/ml in saliva is able to colonize clean, smooth surface of a tooth. The presence of mutans streptococci on tooth surfaces increases the possibility that caries would develop thereon. This indicated that the presence of *S.mutans* in the oral cavity does not lead to caries. Bacterial counts of *S.mutans* ranged from 10^5 -10^7 cfu/ml in stimulated saliva (Napimoga *et al.*, 2004). Under certain circumstances, however, oral environmental changes may modify the composition

and metabolic activities of the bacterial consortium leading to predominance of pathogens (Marsh, 2006). High counts of mutans streptococci (MS), *S. mutans* and *S. sobrinus*, have been associated with higher prevalence of coronal caries in temporary and permanent dentitions (Lemons and Burne, 2005; Liljemark and Bloomquist, 1996; Tanzer and Thompson, 2001; Thenishch and Steurer, 2006) and also root caries (Preza *et al.*, 2008). In spite of the widely acknowledged association between increased levels of MS and higher caries experience, other studies have argued against this association (Giacaman *et al.*, 2010; Helderman *et al.*, 1996). Colonization and pathogenicity of these facultative gram positive cocci derive from their capacity to synthesize extracellular polysaccharides (Kreth *et al.*, 2008). Indeed, adult subjects colonized by biofilm-forming *S.mutans* strains have higher caries experience than individuals without these particular types of strains (Jalil, 1995).

In the present study, a total 120 *S.mutans* were isolated from 254 saliva samples. The overall incidence of *S.mutans* was 47.24% and isolation rate of *S.mutans* was significantly higher (57.04%) in the individuals with caries compared to the individuals without caries (34.82%). Frequency of isolation of *S.mutans* was very less (8.16%) in the saliva of habitual individuals without caries. This clearly indicates that the habitual individuals secrete less saliva and does not contain *S.mutans* in contrast individuals with caries without habit of chewing tobacco. Higher incidence of *S.mutans* in the saliva of habitual individuals with caries may be due to continuous secretion of pathogens from the dental caries. It is also reported that saliva secretion is reduced among long term tobacco chewing individuals. *S.mutans* is highly implicated in dental caries (liljemark and Bloomquist, 1996; Burne *et al*., 1997). However, in the present study we were not able

detect in about 43% of the individuals with caries. This may be due to the fact that the numbers of this microorganism may be below the detection limits of the current cultivation techniques. Another explanation could be the presence of other microorganisms (Lactobacilli and / or actinomyces) that dominate the dental caries. Contrastingly, *S.mutans* detection rate was only about 35% in the individuals without caries.

S.mutans was isolated frequently from both subjects with and without caries. This species was clearly associated with carious lesions and was also found at the healthy enamel sites of subjects of without caries. *S.mutans* was found at levels greater than other species in both caries and without caries subjects from healthy sites and carious dentin. Similar findings have been reported for a pediatric population as well where *S.mutans* is not present in all samples and others bacterial species dominate (Paster, 2008).

The number of *S. mutans* in saliva/dental plaque is not so important, but its role as potent pathogens is important in the caries process (Chhour and Hunter, 2005). The possibility of interactions with the oral condition or dietary factors is interesting. There was not an increase of *S.mutans* with the increase in the number of surfaces with caries or with plaque levels. The exposure time did not affect the levels of *S.mutans*. The level of *S.mutans* did decrease with increasing amounts of dietary carbohydrates. Theoretically, the species of *S.mutans,* while being acid tolerant is not the most acid tolerant of all cariogenic species. Lactobacilli tend to be more aciduric and actually lactobacilli have been found to increase with increasing carbohydrates. Above all else, the identification of bacteria responsible for the healthy state and the diseased state is important for the ultimate end point.

In our study, age group wise incidence of *S.mutans* results revealed that incidence rate increases with increase in age. Maximum incidence (56-70%) was observed in the age group of 36-45 years and lowest of 32.00% in the age group of below 15 years, however no incidence has been found in the older age group (46-60 years). This variation may be due to sample number was very small (2 subjects only). Over all incidence rate is higher in females and trend was same in first 3 age groups, however, it was slightly more in male than females in the age group of 36-45 years. Our study clearly indicates that among the adults, above 30 years of age is at high risk for getting caries.

Dental caries is considered a preventable disease. Prevention requires minimizing the frequency of ingesting simple carbohydrate foods and beverages, regular oral hygiene measures to remove plaque and to introduce topical fluoride in the form of toothpaste. It is recognized that even when the appropriate preventable measures are taken, some individuals have increased susceptibility to dental caries. The oral pathogens are not also restricted to the oral cavity alone; they may enter the internal organs through the blood stream, and can cause life threatening systemic diseases. In addition to *S.mutans* participation in the etiology of dental caries, it also produces bacteremia, a systematic infections or sub-acute endocarditis and strokes resulting from dental treatments (Ullman *et al.*, 1988). Therefore, the recent focus on microbial factors in caries control indicates that clinicians desire better therapies and preventive measures for their patients. An antimicrobial therapy in the form of chlorhexidine varnishes and rinses is not yet proven effective (Gold, 2008).

Adequate treatment for these infections requires the determination of the antimicrobial susceptibility of the *S.mutans* strains isolated from the oral cavity. Therefore, in this study antibiogram for all *S.mutans* isolates was performed against 11 frequently prescribed antibiotics used to treat dental caries. Our results revealed that more than 50% of isolates were resistant to beta lactum antibiotics with highest resistance of 95% against penicillin G. Followed by 63.33% of isolates exhibited resistance to chloramphenicol and to vancomycin it was 39.16%, which is much higher than ciprofloxacin (23.33%). On the other hand, 100% of *S.mutans* isolates from caries individuals were resistant to penicillin- G compared to only 84.61% of isolates from without caries individuals. Nevertheless, it is reverse in case of ciprofloxacin. Majority of reports on incidence of resistance to various antibiotics were very low compared to our results, study from Iran reporting none of their *S.mutans* isolates were resistance to vancomycin but 1.1% of the isolates were resistance to imipenem (Fani *et al.,* 2007).

All the *S. mutans* isolates in this study were multidrug resistant (MDR) and are resistant to a minimum of 3 antibiotics. Maximum of 26.66% of isolates were resistant to 5 antibiotics followed by 21.66% to 7 antibiotics and 19.16% to 6 and 4 antibiotics each. Less than 5% of isolates were resistance to 8 and 9 antibiotics. Maximum percent of isolates from with caries individuals showed resistance to higher number of antibiotics (7, 5 and 6) compared to *S. mutans* isolated from without caries individuals which showed a maximum of 30.76% to 5 antibiotics and 20% each to 6 and 4 antibiotics. Multidrug resistance among *S. mutans* isolates has been widely reported (Fani *et al*., 2007). This problem has been shown to be on the rise worldwide. New antimicrobial substances of plant or bacterial origin will thus have to be developed in order to treat bacterial

infections. According to Fox, more efficient antibiotics will have to be sought continually because of the capacity of microorganisms to survive their action (Fox, 1997).

Constant expose to many antibiotics used in the routine treatment of many diseases, the bacteria become resistance or increases its minimum inhibitory concentration in course of time. Now, it becomes routine to check the MIC for bacterial pathogens, which are not susceptible to the normal doses of drugs. Therefore MIC for amoxycillin, ciprofloxacin, chloramphenicol, erythromycin, and tetracycline was carried out by using Hi-comb MIC test strips against *S.mutans* isolates following the CLSI (2006) guidelines. Results revealed that higher range of MIC's are exhibited by *S.mutans* isolated from individuals with caries habituated and without caries non-habitual individuals, compared to lower MIC range in the isolates with caries non-habitual individuals. MIC- range was maximum (25 times) against ciprofloxacin and tetracycline compared 2.5 to 10 times against remaining antibiotics and lowest MIC range of 2.5 times against erythromycin. The MIC for all the isolates of this study was found to be similar to those observed by other authors (Liebana *et.al.,* 1991; De La Higuera *et al.,* 1999; Gamboa *et al.,* 2004; Fani *et al*., 2007). Our observations differ from the report of Jarvinen *et al.,* (1993) who reported slightly higher MIC for tetracycline (1 ug/ml) and lower MIC's to amoxycillin and erythromycin (0.13 ug/ml).

Adhesion can occur by a variety of mechanisms and generally involves reversible and irreversible states. The first stage of attachment is reversible and is usually mediated by hydrophobic interactions, electrostatic interactions, or van der Waals forces (Dune, 2002). Irreversible attachment is mediated by specific host cell or bacterial cell receptors. Adhesion proteins and receptors are common for clinically relevant biofilm forming

bacteria and contribute to this initial attachment, as well as, facilitate co-aggregation of bacteria within a species or of different species (O' Toole *et al*., 2000; Characklis, 1990). The *S. mutans* adherence to smooth surface of tooth and formation of biofilm depends on the production of water insoluble polysaccharide glucan in presence of sucrose and specific surface proteins are essential.

The adherence efficiency of the *S.mutans* isolated from different individuals to the smooth surface like glass *in vitro* in the presence of sucrose was carried out turbidimetrically (Hamada and Torii, 1978; Napimoga *et al.,* 2004). The results revealed that the *S.mutans* isolates from with caries individuals showed more adherence capacity than isolates from without caries individuals. This higher percentage of *S.mutans* isolates from caries individuals attaching to glass surface suggests the role of adhesion in pathogenesis of *S. mutans* in the cariogenic activity and strain variations among the *S.mutans* isolated between the individuals with and without caries. Recently biofilm formation *in vitro* is another method used for the testing the adherence efficiency of *S.mutans* with or without sucrose and many other components also involved in the formation of heterogeneous biofilm (Li *et al*., 2001 & 2002).

S. mutans property of adherence is mediated mainly by the production of water insoluble polysaccharide glucan and expression of specific surface proteins. The glucosyltransferase (GTF) enzymes mediate glucan production in *S.mutans* (Loeshe, 1986; Kuramitsu, 1993; Alaluusua et al., 1997; Sarela and Coufield, 1998; Gronroos and Alaluusua, 2000). The aim of this present study was to evaluate the genotypic diversity of *S.mutans* in caries free and caries active subjects and to compare some virulence traits between strains isolated from these two groups. Some isolates of *S.mutans* from with

caries and without caries, individuals were considered for the *gtf*-B identification by PCR amplification using specific primers designed by Oho *et al*., (2000) to amplify a 517 bp region.

We observed that all the *S.mutans* isolates were positive for the amplification of the 517 bp region of the *gtf*-B gene. The results revealed that all *S.mutans* are isolates containing *gtf*-B gene, this suggesting that the isolated streptococci are *S.mutans* having a capacity to produce glucan in the presence of sucrose. However, formation of biofilm, and the survivability of *S.mtuans* in the biofilm depends on many other factors like mutacin, and adhesive protein production.

The ability of *S. mutans* to produce mutacins, combined with the production of other virulence factors such as glucan and acid production may contribute to the pathogenesis of this bacterium. Mutacins are peptide or protein antibiotics that are mainly bactericidal for other bacteria of the same or closely related species, and are likely to confer an ecological advantage in diverse bacterial communities, such as the dental biofilm (Parrot *et al*., 1990; Balakrishnan *et al*., 2002). Studies have demonstrated that the mutacin activity of *S. mutans* could be related to the prevalence of this species in the dental biofilm, saliva and dental caries (Berkowtz and Jordan, 1995; Hillman *et al*., 1997). Based on their bactericidal activity against different bacteria mutacins are divide into four types 1, 2, 3, and 4 (Qi, *et al*., 1999a & 2001).

In the present study, we determined types of mutacins produced by *S. mutans* isolates, from individuals with and without caries by PCR amplification of *mut* I and *mut* II genes, using the specific primers. All the *S.mutans* isolates from individuals with caries

showed positive result for *mut* I, amplification of 250 bp region. Whereas, 40 % of *S.mutans* isolates from without caries individuals did not show the amplicon for *mut* I. None of the *S.mutans* isolates from both the individuals with or without caries did not show amplicons (750 bp) for *mut* II. Presence of *mut* I gene in all *S.mutans* isolates from with caries individuals indicates mutacin is also one of the virulence factor in cariogenic streptococci and its presence in *S.mutans* isolated from without caries individuals indicates they are in risk of getting dental caries. Mutacin production by *S. mutans* helps in the transmission from mother to child (Gronroos *et al*., 1998). According to Kamiya *et al*.(2005) mutacin could play an important role in the regulation and composition of dental biofilms due their synergetic and antagonistic activity. Therefore, wide spectrum mutacins (*mut* I, *mut* II and *mut* III genes) may be essential for colonization and stabilization of *S. mutans* (cariogenic species) mainly in the stable niche of highly complex microbial activity (Napimoga *et al*., 2005).

Finally, our study suggests that prevalence of dental caries is not restricted to children but is equally prevalent in all ages, in both sexes, socioeconomically poor and slightly higher in tobacco chewing individuals. Higher percentage resistance of *S.mutans* isolates to vancomycin is challenging to the dentists in treating the oral diseases. Therefore, we suggest routine testing of MIC along with antibiotic susceptibility testing. As dental caries is a microbial disease, until and unless biofilm (plaque) is not present caries will not occur, regardless of any other risk factors (Kidd, 1999). Detection of cariogenic bacteria in oral cavity genotyping of *S.mutans* isolates from oral cavity is essential for the prediction of dental caries risk in an individual.

6. SUMMARY AND CONCLUSION

6.1. SUMMARY:

Dental caries is one of the most common infectious diseases affecting humans and is a significant health care issue in both developed and developing nations (Mitchell, 2003). In most developing countries, the levels of dental caries were low until recent years, but are now tending to increase due to increase in consumption of sugars and inadequate exposure to fluorides and lack of oral hygiene. In contrast, a decline in caries has been observed in most industrialized countries over the past 20 years or so because of effectiveness of fluorides coupled with changing living conditions, lifestyles and improved self-care practices. According to Peterson *et al.,* (2005) the prevalence of dental caries among adults is high as the disease affects nearly 85-100% of the population in the majority of countries. While in recent years overall incidence of this disease has declined in industrialized nations, caries rates are rising in developing nations (Chu and Lo, 2008).

Tooth decay is the result of the interaction of the oral micro flora (plaque), the tooth surface, nutrition, and the oral environment, over a time result in a carious lesion of the tooth enamel (Beighton, 20005 ; Takahashi and Nyvad, 2008). Caries is a process of cavity formation in which a mass of cariogenic and non cariogenic, bacteria like *S.mutans, S.sanguis, S.salivarius, Lactobacillus sps,* Actinomyces with the utilization of carbohydrates like sucrose produce acids. These organic acids act and dissolve the mineral content of the teeth that leads to cavity.

Among the oral diseases, dental caries is considered as a preventable disease. It can be prevented by minimizing the frequency of ingesting simple carbohydrates and beverages and regular oral hygiene measure to remove dental plaques. Even with all appropriate preventable measures taken, many individuals have shown increased susceptibility to dental caries. The most commonly used caries risk factors and indicators in mulitfactorial Caries Risk Assessment (CRA) models include levels of cariogenic bacteria (ie., *Streptococcus mutans* and *Lactobacillus*), salivary factors (e.g. flow rate and buffering capacity), carbohydrate intake, oral hygiene, fluoride exposure, previous caries experience, and socioeconomic characteristics (Brathall and Petersson, 2005). As this part of Karnataka comes under socioeconomically backward region, awareness and maintenance of oral hygiene is poor and many people are having the habit of chewing various intoxicants including tobacco. Tobacco consuming individuals have experienced more oral diseases. Though there is no direct correlation between tobacco consumption and prevalence of dental caries however, it effects the saliva secretion and physiology of the saliva by changing the pH, buffering capacity and immunity.

The main factors for dental caries are the diet, oral hygiene and cariogenic bacterial load. Generally *Streptococcus mutans* is considered as one of the principle aetiological agent of dental caries, however, it is also isolated from the saliva of caries free individuals (Gamoba *et al.,* 2004). Therefore, it becomes necessary to know the diversity and virulence traits of *S. mutans* isolated from individuals with caries and without caries, by phenotypic and molecular characterization (Napimoga *et al,* 2004; Kamiya *et al.,* 2005; Al-Ahmed *et al.,* 2006). It is also important to study the antibiogram of *S. mutans* isolates for an effective treatment; otherwise, they may cause life

threatening systemic diseases like endocarditis and hemorrhagic stroke when they enter the main stream through blood during tooth extraction and in diseased conditions. Therefore, we have undertaken the present study with the main aim of isolation of *S. mutans* from with and without caries subjects of all age groups and their phenotypic and molecular characterization and their response to various antibiotics. The summary of the thesis is given in the following paragraphs.

For this study, we selected 254 individuals belonging to different genders and age groups with or without dental caries, and with or without tobacco chewing habits. The unstimulated saliva samples were collected in to a sterile container from the individuals attending dental college hospitals at Gulbarga and were transported to the laboratory immediately. Enumeration of mutans streptococci and isolation of *S.mutans* were performed by inoculating on Mitis salivarius bacteriacin (MSB) agar medium and incubating anerobically at 37^{o} C for 48 hrs. Isolated streptococci were identified as *S.mutans* based on cultural characters on the MSB medium, morphological characters (grams staining) and sets of biochemical and sugar fermentation tests. Frequency of total mutans streptococci and *S. mutans* was calculated. Higher number of mutans streptococci were detected from individuals with caries (10^6 cfu/ml of saliva) compared to without caries individuals (10^5 cfu/ml). The *S. mutans* count was also higher in individuals with caries (7.1×10^4 cfu/ml of saliva) as compared to without caries individuals (8.6×10^3 cfu/ml). Non-habituals of with or without caries individuals showed similar count of *S. mutans* compared to less in habituals of without caries individuals.

Total saliva samples collected included 142 with caries and 112 were from without caries individuals among these 105 were from habitual and 149 from non-

habitual individuals. Of the 254 samples 157 were collected from males and remaining 97 from females. Prevalence of *S.mutans* isolates in different groups of individuals was calculated. Over all incidence was (47.24%) and incidence among the caries individuals was higher of (57.04%), when compared to (34.82%) among individuals without caries; however, incidence in habitual individuals (36.19%) was lower than non-habituals (55.03%). Gender wise incidence was higher in females (51.54%) than males (44.58%). Incidence of *S. mutans* in the caries individuals of habitual and non-habitual was (57.04%) which was slightly higher (60.71%) in the individuals with habitual compared to in non-habituals (54.65%). Isolation rate of *S.mutans* was higher in males than females in habitual individuals. Prevalence of *S.mutans* among individuals without caries was less (34.82%). Maximum isolates of *S.mutans* were from the individuals without the habit of tobacco chewing compared to tobacco chewers. Over all incidence rate of *S.mutans* was almost double in females (48.88%) than males (25.37%; Tables-8 & 9).

Age group wise analysis of incidence of *S.mutans* among individuals with caries increased with the increase in age. Maximum incidence was observed in the age group of 36-45 years as compared, highest of 80% incidence was observed in the age group 36-45 years among the caries individuals with habitual compared to non habitual, where the maximum (61.11%) incidence was in the age group of 26-35 years. *S.mutans* isolation rate was very high (63.46%) in males compared to females (25%) in habitual but not much differences in the non-habitual with caries. On the contrary, incidence of *S. mutans* among caries free individuals in both habitual and non-habituals also increased with increase in age from 20% to 45.71%. Age group wise higher incidence rates ranged from 33.33% to 66.6% in non-habitual individuals compared to habituals without caries

(Table-11)**.** In all age groups, incidence of *S. mutans* among females was more than in males except in the age group of 36-45. *S. mutans* isolation rate was maximum of (56.66%) in females observed in the age group of 26-35 years whereas for males in the age group of 36-45 years (57.62%). Interestingly lowest incidence was in the age group below 15 years in both sexes (Table-12). Among the 5 age groups considered in this study higher incidence was observed in the age group of 36-45 years of both habitual and non-habituals individuals and gradual increase of incidence with increase in age in both groups. Higher range of incidence from 40.00% to 62.90% was observed in caries individuals compared to 20.00% to 45.71% among without caries individuals (Table -13).

With an aim of providing information on the resistance pattern of antibiotics and MIC for *S. mutans* isolates to dentists for better and effective treatment of dental caries and to avoid systemic infections by these bacteria, antimicrobial susceptibility testing was performed for all 120 *S. mutans* isolates against 11 frequently prescribed antibiotics to treat dental caries and oral cavity disease. Percent resistance of *S. mutans* isolates to various antibiotics was calculated. Maximum of 95% of *S. mutans* isolates were resistant to penicillin G and 100% resistance for *S. mutans* isolates from caries individuals compared to only (84.61%) of isolates from without caries individuals. In contrast, overall isolates from without caries individuals showed slightly higher percent of resistance than the isolates from with caries individuals. (63.33%) of isolates, showed resistance to chloromphenicol, followed by (58.33%) against amoxycillin and (54.16%) to ampicillin. Where as, lowest incidence of 23.33% was against ciprofloxacin, but higher resistance rate was observed to vancomycin (44.73%) isolated from habitual individuals and incidence of resistance to the remaining antibiotics was in the range of 31.66% to

38.33% (Tables-14 & 17). All *S. mutans* isolates from both sexes of habitual individuals with caries and only females of habitual individuals without caries showed 100% resistance to penicillin G (Tables -15 &16). Overall *S.mutans* isolates showed lower resistance to ciprofloxacin, lowest of 12.5% resistance in the isolates from female individuals belonging to non-habitual without caries (Table-19).

All the *S.mutans* isolates in this study were multi-drug resistant (MDR) and are resistant to a minimum of 3 antibiotics. None of the *S.mutans* isolates were neither resistant nor susceptible to all the 11 antibiotics tested. Maximum of (26.66%) of isolates were resistant to 5 antibiotics, (21.66%) to 7 antibiotics and 19.16% to 6 and 4 antibiotics each. Less than 5% of the isolates showed resistance to 8 and 9 antibiotics. Majority of isolates from with caries showed resistance to higher number of antibiotics (6, 7 and 8) compared to isolates from without caries (4, 5 and 6 antibiotics). However, isolates from habitual individuals showed resistance of 28.9% to 4 antibiotics and 30.48% to 5 antibiotics among non-habituals, while remaining isolates showed almost same percentage to 6, 7 and 8 in both habitual and non-habituals (Tables-21 & 22).

MIC against amoxycillin, ciprofloxacin, erythromycin, chloramphenicol and tetracycline was performed using selected *S.mutans* isolates using HI-Comb MIC test strips procured from HI-Media Laboratories Pvt. Ltd., Mumbai. Overall, higher side of MIC was showed by *S.mutans* isolated from with caries habitual and without caries non-habitual individuals. MIC range was maximum (25 times) from 0.01 ug/ml to 0.25 ug/ml against Ciprofloxacin and Tetracycline compared to 2.5 times to 10 times against remaining antibiotics. Lowest range of MIC of 2.5 times (0.1 ugml to 0.25 ug/mL) to

Erythoromycin, 5 times (0.1 ug/mL to 0.5 ug/mL) to Amoxycillin and 10 times (0.1 ug/mL to 1.0 ug/mL) to Chloramphenicol was observed.

Molecular studies performed for the selected *S.mutans* isolates from with and without caries individuals to type the glycan producing glucosyltransferase (*gtf*) genes, mutacin producing genes and adherence ability of *S.mutans*. We selected *gtf* B that encodes glucosyltransferase that synthesizes water insoluble glucan for amplification in *S.mutans* isolates by simple PCR method using gene specific primers. All the *S.mutan*s isolates from with or without caries individuals showed a single band corresponding to 517 bp region. Presence of *gtf B* gene in the isolates also confirms the identification of isolated strains as *S.mutans* and its cariogencity.

Mutancins are small protein antibiotics that are mainly bactericidal and are helping *S. mutans* to confer an ecological advantage in diverse bacterial communities like dental biofilm. Mutacin activity of *S.mutans* would relate to the prevalence of this in dental biofilm, saliva and in dental caries. All the *S.mutans* isolates from individuals with caries showed positive result for *mut* I (250 bp, amplicon) compared to only 60 % of *S.mutans* isolates from without caries individuals. None of the *S.mutans* isolates from both with and without caries individuals did not show amplicons for *mut* II gene. Presence of *mut* I gene in all *S.mutans* isolates from with caries individuals indicates mutacin is also one of the virulence factor in cariogenic streptococci and its presence in some of *S.mutans* isolated from without caries individuals indicates they are in risk of getting dental caries.

Adherence property of *S.mutans* mainly depends on the extracellular production of adhesive substances that express on cell surface and this is one of the characteristics of cariogenic (pathogenic) bacteria. Glucan an adherence substance synthesized by glucosyltranferase (GTF) enzyme of *S.mutans* in the presence of sucrose is considered as one of the virulence factors and is directly proportional to the plaque formation *in vivo* and biofilm *in vitro*. Significant association between the presence of *gtfB*, production of adhesive substances and percentages of growing bacteria adhering to glass surfaces in the presence of sucrose has been observed. Results showed that percent adherence of *S.mutans* isolated from with caries individuals was higher (39.56%) than the *S.mutans* isolated from without caries (19.11%). These results clearly indicate that *S.mutans* isolates from with caries individuals are having higher capacity to form dental plaques than isolates from caries free individuals.

6.2. CONCLUSIONS:

1) Average load of mutant streptococci and *S.mutans* in saliva was high among individuals with caries than without caries individuals correspondingly isolation rate of *S.mutans* was higher in the individuals with caries as compared to without caries.

2) Overall incidence of *S.mutans* in this study was less than 50% and was higher among females than in males. Highest incidence of *S.mutans* was observed in the age group of 35-46 years and lowest in the age group below 15 years.

3). Maximum (more than 50%) of *S.mutans* isolates showed resistance to beta lactum antibiotics followed by 40% to vancomycin, while lowest to ciprofloxacin (23.33%). All the isolates of this study are multidrug resistant (more than 3

antibiotics). Maximum isolates from with caries individuals showed resistance to higher number of antibiotics (6, 7 and 8) compared to isolates from without caries individuals (4, 5 and 6 antibiotics).

4) Higher range of MIC was observed for *S.mutans* isolates from with caries habitual and caries free non-habitual individuals compared to lowest MIC range in the isolates from with caries non-habitual individuals.

5) Amplicons of *gtf* B gene from all *S.mutans* isolates tested showed single band on agarose gel corresponding to 517 bp region. Study suggested that isolated streptococci are *S.mutans* and cariogenic in nature.

6) Higher adherence capacity of *S.mutans* isolates from with caries individuals to glass surface and presence of *mut* I gene in all *S. mutans* isolates from caries individuals suggesting the role of *gtf* B and *mut* I in the cariogenicity of *S.mutans.*

7) From the molecular studies of *S.mutans* isolates from saliva collected from caries individuals exhibited higher cariogenic virulence factors than *S.mutans* from caries free individuals. Our study emphasizes the presence of virulence factors in the cariogenic strains of *S.mutans* as essential to initiate the dental caries in the susceptible individuals of any age group.

8) Further research on the formation of biofilm in animal models and in *in vitro* by the *S.mutans* isolated from different niche of oral cavity of caries and caries free individuals of different geographical areas is required to confirm the exact role of *gtf a*nd *mut* genes in the cariogenesis of *S.mutans*.

9) Molecular study of virulent factors of *S.mutans* isolates from different geographical areas is essential to design and produce an effective multivalent

vaccine to prevent not only dental caries but also the prevention of life threatening diseases caused by *S.mutans*.

10) Lastly, our study also concludes that presence of cariogenic *S.mutans* is essential to initiate caries process in susceptible individuals with poor oral hygiene, sugar in diet and socioeconomically poor.

REFERENCES

Aas JA, Paster BJ, Stokes LN, Olsenl, Dewhirst FE. (2005). Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol*, 43**(11):** 5721-5731.

Acton R, Dasanayake AP, Harrison R, Li Y, Roseman JM, Go R. (1999). Associations of MHC genes with levels of caries-inducing organisms and caries severity in African-American women. *Hum Immunol;* **60:**984-989.

Aida J, Ando Y, Oosaka M, Niimi K, Morita M:(2008). Contributions of social context to inequality in dental caries: a multilevel analysis of Japanese 3-year-old children. *Community Dent Oral Epidemiol,* **36(2):**149–156.

Ajdic, D., McShan, W.M., McLaughlin, R.E., Savic, G., Chang, J., Carson, M.B. (2002). Sugar transporters using microarrays", *Journal of Bacteriology,* 189**; (14):**5049-5059.

Ajdic, Pham. V.T (2007). "Global-Transcriptional analysis of *Streptococcus mutans* sugar transporters using micro-array." *Journal of Bacteriology*, **(189):**5049-5059.

Al-Ahmed, Napimoga, M.H., Kamiya, R.H., Hofling, J.F., Mattos-Graner, (2004). Genotypic Diversity and Virulence traits of *Streptococcus mutans* in caries-free and caries-active individuals. *J.Med Microbiol*. **(53):**67-75.

Alaluusua S, Alaluusua SJ, Karjalainen J, Saarela M, Holttinen T, Kallio M, Hölttä P, Torkko H, Relander P, Asikainen S. (1994). The demonstration by ribotyping of the stability of oral *Streptococcus mutans* infection over 5 to 7 years in children. *Arch Oral Biol;* **39:** 467-471.

Alaluusua S, Kleemola-Kujala E, Nyström M, Evälahti M, Grönroos L.(1987). Caries in the primary teeth and salivary *Streptococcus mutans* and lactobacillus levels as indicators of caries in permanent teeth. *Pediatric Dentistry;* **9:** 126-130.

Alaluusua S. (1993). Salivary counts of mutans streptococci and lactobacilli and past caries experience in caries prediction. *Caries Res;* **27(suppl 1):** 68-71.

Alauusua S, Granross, L, Zhu X, Saarela M, Matto J, Asikainen S, Fukushima K. (1997). Production of glucosyltransferase by clinical mutans streptococcal isolates as determined by semiquantitative cross-dot assay. *Arch oral Biol* **(42**):417-422.

Alm A, Fahraeus C, Wendt L, Koch G, Andersson-Gare B, Birkhed D. (2008). Body adiposity status in teenagers and snacking habits in early childhood in relation to approximal caries at 15 years of age. *Int J pediatr Dent,* **18:**189-196.

Andreasen Jo, Andreasen Fm. (2002). Dental trauma. In: Pinc, C. editor. Community oral health. London: *Elsevier Science Limited*; 94-99.

Arbeit RD. (1999). Laboratory procedures for the epidemiologic analysis of microorganisms. *In: Manual of clinical microbiology.* Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, eds. *7th ed. ASM Press:* Washington, D. C. 116-137.

Aution Gold J. (2008). The role of chlorhexidine in caries prevention. *Operative Dent* **33(6**):710-716.

Axelsson P, Lindhe J, Nystrom B. (1991). On the prevention of caries and periodontal disease. Results of a 15 year longititudinal study in adults. *J. Clin Periodontol,* **18(3):** 182-9.

Axelsson P, Paulander J, Svardstrom G, Tollskog G, Nordensten S.(1993). Integrated caries prevention: effect of a needs- related preventive program on dental caries in children. County of Varmland, Sweding: results after 12 years. Caries Res. 27 (**1)**:83-94.

Axelsson P. Diagnosis and risk prediction. Illinois: *Quintessence Publishing CO. Inc;* (2000). External modifying factors involved in dental caries: 91-150.

Bader JD, Perrin NA, Maupome G, Rindal B, Rush WA (2005). Validation of a simple approach to caries risk assessment. *J Public Health Dent;* **65(2):**76-81.

Bagramian, R.A., Garcia-Godoy, F. Volpe, A.R. (2009), "The global increase in dental Caries. A pending public health crisis ", *American Journal of Dentistry*, 22 **(1):** 3-8.

Balakrishan M, Simmonds, R.S., Killian, M and Tagg, J.R. (2002). Different bacteriocin activities of *Streptococcus mutans* reflect distinct phylogenetic – lineages. *J Med Microbiol.* **(51):** 941-948.

Banas, J. A. & Vickerman, M. M. (2003). Glucan-binding proteins of the oral streptococci. *Crit Rev Oral Biol Med 14*, 89–99.

Banas, J.A. & Vickerman, M.M. (2003). "Glucan-binding proteins of the oral streptococci", Critical reviews in oral biology and medicine: an official publication of the American Association of Oral Biologists, **14 (2):** 89-99.

Banas, J.A., Russell, R.R. Ferretti, J.J. (1990). "Sequence analysis of the gene for the glucan-binding protein of *Streptococcus mutans* Ingbritt", *Infection and immunity;* **58(3):** 667-673.

Bardow A, Moe D, Nyvad B, Nauntofte B (2000). The buffer capacity and buffer systems of human whole saliva measured without loss of CO2. *Arch Oral Biol* **45:**1-12.

Beck JD, Weintraub JA, Disney JA, Graves RC, Stamm Jw, Kaste LM, Bohannan HM. (1992). University of North Carolina caries Risk Assessment Study: Comparisons of high risk Prediction, any risk prediction, and any risk etiologic models. *Community Dent Oral Epidemiol ;* **20(6):**313-21.

Beck JD. (1998). Risk revisited. *Community Dent Oral Epidemiol,* **26(4):**220-5.

Beighton D, Russell RR, Hayday H.(1981). The isolation and characterization of *Streptococcus mutans* serotype h from dental plaque of monkeys (Macaca fascicularis). *J Gen Microbiol ;* **124:** 271-279.

Beighton, D. (2005). The complex oral microflora of high-risk individuals and groups and its role in the caries process. *Community Dent. Oral Epidemiol*; **33:248**–255.

Benjamin, R.M. (2010). "Oral health: the silent epidemic ", *Public health reports (Washington, D.C.: 1974)*; 125**(2**1):58-159.

Benn DK. (1994). Radiographic caries Diagnosis and monitoring. *Dentomaxillofac Radiol,* **23(2):**69-72.

Berkowitz, R.J. and Jordan, H.V. (1975). Similarity of bacteriocins of *Streptococcus mutans* from mother and infants. *Arch Oral Biol***. (20):**725-730.

Birkhed D, Heintze U (1989). Salivary secretion rate, buffer capacity and pH. In: Human saliva: *clinical chemistry and microbiology.* Tenovuo J, editor. Boca Raton, FL: CRC Press**. (1):** 25-73.

Biswas, I., Drake, L. & Biswas, S. (2007). "Regulation of gbpC expression in *Streptococcus mutans*", *Journal of Bacteriology;* **189(18):** 6521-6531.

Biswas, S. & Biswas, I. (2006). "Regulation of the glucosyltransferase (gtfBC) operon by CovR in *Streptococcus mutans*", *Journal of Bacteriology;* 188 **(3):** 988- 998.

Bönecker, M., & Cleaton-Jones, P. (2003). Trends in dental caries in Latin American and Caribbean 5-6 and 11-13-year-old children: a systematic review. *Community Dentistry and Oral Epidemiology,* **31,** 152-7.

Bowen, W. H. (2002). Do we need to be concerned about dental caries in the coming millennium? *J. Am. Dent. Assoc.* **133:**1405–1407.

Bowen, W.H., Schilling, K., Giertsen, D., Pearson, S., Lee, S.F., Bleiweis, A., Beeman, D (1991). "Role of a cell surface-associated protein in adherence and dental caries'. *Infection and immunity,* **(59):** 4606-4609.

Brandtzaeg P (1989). Salivary immunoglobulins. In: Human saliva: *clinical chemistry and microbiology.* Tenovuo J, editor. Boca Raton, FL: CRC Press. **(2):** 1-54.

Bratthall D, Hansel Petersson G, (2005). Cariogram- a Multifactorial risk assessment model for a multifactorial disease. *Community Dent Oral Epidemiol;* **33 (4):**256-64.

Bratthall D. (1996) Dental caries: Intervened-Interrupted-Interpreted: Concluding remarks and Cariography. *Eur J.Oral SCi*; **(104):** 486-491.

Bratthall, D. (1970). Demonstration of five serological groups of streptococcal strains resembling *Streptococcus mutans. Odontol. Revy* (**21):**143-152.

Brown, L.J. & Selwitz, R.H. (1995). "The impact of recent changes in the epidemiology of Dental caries on guidelines for the use of dental sealants", *Journal of public health Dentistry;* 55**(5**): 274-291.

Brown, L.J., Wall, T.P., & Lazar, V. (2000). Trends in total caries experience: permanent and primary teeth. *Journal of the American Dental Association,* **131,** 223-231.

Bryun, L.E. (Ed). (1984). Antimicrobial Drug resistance Academic Press, *Inc. Orlando,* Fla.

Burne (1997). Analysis of gene expression in *Streptococcus mutans* in biofilms in Vitriol.*Adv.Dent Res***. (11):** 100-109.

Burt BA, Eklund SA, Morgan KL, Larkin FE, Guire KE, Brown LO, Weintraub JA. (1988). The effect of sugar intake and frequency of ingestion on dental caries increment in a three –year longitudinal study. *J Res Dent;* **67(11):**1422-9.

Burt BA, Eklund SA. (1994). Dentistry, Dental practice and the community. *5th ED. Philadelphia:* W.B. Sauders Company; Dental caries; **P (2)** 12-36.

Burt BA, EKlund SA. (1999). Dentistry, dental practice, and the community. 5th ed. Philadelphia: W. B. Saun ders Company; Dental caries; 212-36.

Burt BA, Kolker JL, Sandretto AM, Yuan Y, Sohn W, Ismail Al (2006). Dietary patters related to caries in a low-income adult population. *Caries Res* **40(6):**473-480.

Burt BA, Pai S, (2001). Sugar Consumption and caries risk: a systematic review. *J Dent educ;* **65910:**1017-23.

Campus G, Cagetti MG, Sacco G, Benedetti G, Strohmenger L, Lingstrom P.(2009). Caries risk profiles in Sardinian schoolchildren using Cariogram. *Acta Odontol Scand,* **27:** 1-7.

Carvalho, J.C., Van Nieuwenhuysen, J.P., & D'Hoore, W. (2001). The decline in dental caries among Belgian children between 1983 and 1998. *Community Dentistry and Oral Epidemiology,* **29:** 55-61.

Castro, L., M.G. Bergeron, S. Chamberland. (1993). Characterization of multi-resistant strains of *Nisseria gonorrhaeae* isolated in Nicaraguc. *Transm Dis.* **(20);** 314-320.

Caufield PW, Walker TM. (1989). Genetic diversity within *Streptococcus mutans* evident from chromosomal DNA restriction fragment polymorphism. *J Clin Microbiol;* **27:** 274-278.

Caufield PW, Wannemuehler Y, Hansen JB. (1982). Familial clustering of the *Streptococcus mutans* cryptic plasmid strain in a dental clinic population. *Infect Immun;* **38:** 785-787.

Challacombe SJ (1980). Serum and salivary antibodies to *Streptococcus mutans* in relation to the development and treatment of human dental caries. *Arch Oral Biol* **25:**495-502.

Chhour KL, Nadkarni MA, Byun R, Martin E, Jacques NA, Hunter N. (2005). Molecular analysis of microbial diversity and advanced caries. *J clin Microbiol.* **43(2):**843-849.

Choi Y, Lee SG. (2011). Does regional socioeconomic context affect the dental caries experience? A multilevel study of Korean adults. *Eur J Oral Sci;* 11**9(4):**294–300.

Chu, C.H. & Lo, E.C. (2008). "Promoting caries arrest in children with silver diamine Fluoride: a review ", *oral health & preventive dentistry;* 6**(4):**315-321.

Clark K.(1924). On the bacterial factor in the aetiology of dental caries. *Br J Exp Pathol;* **5:**1417.

Clarke, J. Kilian (1924). "On the Bacterial Factor in the etiology of Dental Caries". *British Journal of Experimental Pathology* **5**: 141–7. PMC 2047899. //www.ncbi.nlm.nih.gov/pmc/articles/PMC2047899/.

Clinical and Laboratory Standards institute, Performance standards for antimicrobial Susceptibility testing 17th. *Informational supplement.* Clinical and laboratory standards institute. (2007).

Clinical Laboratory Standard (CLSI-2006). Performance Standard for antimicrobial Susceptibility Testing. 16th Informational Supplement. CLSI document. M100-S16.

Cole MF, Bryan S, Evans MK, Pearce CL, Sheridan MJ, Sura PA, *et al.* (1998). Humoral immunity to commensal oral bacteria in human infants: salivary antibodies reactive with *Actinomyces naeslundii* genospecies 1 and 2 during colonization. *Infect Immun* **66:**4283-4289.

Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M. (1997). Bacterial Biofilms in nature and disease. *Annu Rev Microbiol,* (**41):**435-464.

Cotter, P.D. & Hill, C. (2003). "Surviving the acid test: responses of gram-positive bacteria to low pH *Microbiology and molecular biology reviews: MMBR;* 67**(3):**429-53.

Dample SG, (2002). Epidemiology of dental caries in India. IN: *Pediatric Dentistry. Arya publishing House, New Delhi:* 75-96.

Daniels TE, Silverman S Jr, Michalski JP, Greenspan JS, Path MCR, Sylvester RA, et al, (1975). The oral component of Sjogren's syndrome, *Oral Surg,* **39:**875-885.

Davies J. (1996). Bacteria on the rampage. Nature (London), **(383):** 219-221.

Day CDM, Tandon GC. (1940). The incidence of dental caries in the Punjab. *BR Dent J:* **69:**381.

Dela, Higuera, A., Gutierrez, J., Liebana, J., Garcia Mendoza, A., Castilo, N. (1999). A new biotyping method for *Streptococcus mutans* with the API ZYM System. Clinical Microbiology infection. **(5):** 88-91.

Dobell, C. (1932). Antony van Leeuwenhoek and his "little animals". Harcourt Brace & Co, New York. :217-255.

Dom L, Haffajee AD, Socransky SS. (1999). Microbial etiological agents of destructive periodontal disease. *Periodontolgy*, **5:**78-111.

Domcjean-orliaguet S, Gansky SA, Featherstone JD. (2002). Caries risk assessment in an education environment. *J Dent Edu;* 70 **(12):** 1346-54.

Domejean-Orliaguet S, Gansky SA. Featherstone JD. (2006). Caries risk assessment in an educational envi ronment. *J Dent Educ;* 70**(12):**1346-54.

Doyal L, Naidoo S. (2010). Why dentists should take a greater interest in sex and gender. *Br Dent J;* 209**(**7**):**335–337.

Drinan DF, Tobin S, Cogan Tm (1976). Citric acid metabolism in hetero and homofermentative lactic acid bacteria. *Appl Environ Microbiol* 31**(4):**481-486.

Dunne, W.M., Jr (2002). "Bacterial adhesion: Seen any good biofilms lately? " *Clinical microbiology reviews*, **(5):**155-166.

Dye BA, Tan S, Smith V, Lewis BG, Barker LK, Thornton-Evans G, Eke PI, Beltrian-Aguilar ED, Horowitz Am, Li CH.(2007). Trends in oral health Status: United States, 1988-1994 and 1999-2004. *Vital Health Stat 11.* **;(248):**1-92.

Eriksen HM, Bjertness E. (1991). Concepts of health and disease and caries prediction: a literature review. *Scand J Dent Res;* 99 **(6):** 476-83.

Evans RW, Pakdaman A, Dennison PJ, Howe ELC. (2008). The caries management system. An evidence-based preventive strategy for dental practitioners. Application for adults. *Aust Dent J;* 53**(1):**83-92.

Facklam, R. (2002). "What happened to the streptococci: overview of taxonomic and nomenclature changes ", *Clinical microbiology reviews;* 15**(4):** 613-630.

Fani MM. Kohantebs., Dayahi, M. (2007). Inhibitory activity of garlic (Allium Sativum) extract on multi-drug-resistant *Streptococcus mutans*. *J .Indian Soc Pedod Prevent Dent. December*. 164-168.

FDI Working Group. Review of methods of identification of high caries risk groups and individuals. Federation Dentaire Internationale Technical Report No. 31. *Int Dent J. 1998 Sep;* **38(3):**177-89.

Featherstone JDB, Adair SM, Anderson MH, Berkowitz RJ, Bird WF, Crall JJ, Den Besten PK, Donly KJ, Glassman P, Milgrom P, Roth JR, Snow R, Stewart RE.(2003). Caries management by risk assessment: Consensus statement. *J Calif Dent Assoc;* 31**(3):**257-69.

Featherstone JDB, Adair SM, Anderson MH, Berkowitz RJ, Bird WF, Crall JJ, Den Besten PK, Donly KJ, Glassman P, Milgrom P, Roth JR, Snow R, Stewart RE.(2002) Caries management by risk assessment: Consensus statement, *J Calif Dent Assoc*. **(3):**257-69.

Featherstone JDB. (1999). Prevention and reversal of dental caries: role of low level fluoride. *Community Dent oral Epidemiol;* **27(1):**31-40.

Featherstone, J.D. (2000). "The science and practice of caries prevention", *Journal of the American Dental Association (1939),* 131 **(**7**)**: 887-899.

Featherstone, J.D. (2006). "The continuum of dental caries evidence for a dynamic disease process ", *Journal of dental research*, **83**:39-42.

Flemming HC, Wingender (2010). The biofilm matrix. *Nat Rev Microbiol;* **8:** 623-633.

Fontanna M. Zero DT. (2006). Assessing Patients caries risk. *J. AM Dent Assoc;* 137**(9):**1231-9.

Fox, J. (1997). Antibiotic resistance on the rise globally. *ASM News,* **(63):** 655.

Fredy Gomboa, Mabel Estupinan, Adriana Galindo. (2004). "Presence of *Streptococcus mutans* in saliva and its relationship with dental caries.' Antimicrobial susceptibility of the isolates. Antimicrobial Susceptibility of the isolates. *Unversitas Scientiarum, Pontificia Universidad, Javerianan*. **(9):** 23-27.

Fure S. (1998). Five- year incidence of caries, salivary and microbial conditions in 60- 70- and 80 year old Swedish individuals. *Caries Res;* 32**(3):**166-74.

Germaine, G.R Schactele, C.F. (1976). "*Streptococcus m*utans dextran sucrose; mode of interaction with molecular weight dextran and role in cellular aggregation'. *Infection and immunity,* **(13):** 365-372.

Giacaman RA, Araneda E, Padilla C. (2010). Association between biofilm-forming isolates of mutans streptococci and caries experience in adults. *Arch Oral Biol;* **55:** 550-554.

Gibbons, R. J. (1972). Presence of an invertase-like enzyme and a sucrose permeation system of *Streptococcus mutans. Caries Res***. 6:**122-131.

Gibbons, R.J. (1984). "Adherent interactions which may affect microbial ecology in the mouth", *Journal of dental research;* 63**(3):** 378-385.

Gibbons, R.J., Cohen, L. & Hay, D.I. (1986). "Strains of *Streptococcus mutans* and *Streptococcus sobrinus* attach to different pellicle receptors", *Infection and immunity;* 52**(2)**555-561.

Gilmour MN, Whittam TS, Kilian M, Selander RK. (1987). Genetic relationships among the oral strep tococci. *J Bacteriol;* **169:** 5247-5257.

Gold O, G., Jordan, H.V. and Van Houte J. (1973). A Selective medium for Streptococcus mutans. *Arch Oral Biol* (**18)**:1357-1364.

Gregoire S, Singh AP, Vorsa N,Koo H (2007). Influence of cranberry phenolics on glucan synthesis by glucosyltransferases and *streptococcus mutans* acidogencity. *J App Microbial **103:***1960-1968.

Grimont F, Grimont PAD. (1986). Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann Inst Pasteur/Microbiol;* **137B**: 165-175.

Gronroos, L and Alaluusua S. (2000). Site specific oral colonization of mutans streptococci detected by arbitrarily primed PCR fingerprinting. *Caries Res*. **(34):** 474-80.

Gudachun RA, Weinburg, A. (2001). Role of bacteria in health and disease of periodontal tissue. *Periodontology.* **40:** 50-76.

Guivante-Nabet C, Tavernier JC, Trevoux M, Berenholc C, Berdal A (1998). Active and inactive caries lesions in a selected elderly institutionalized French population. *Int Dent J* **48:**111-122.

Gupta AK, Tewari A, Chawla HS. (1987). Assessment of treatment needs of dental caries and gingival Disease of South Indian population and correlation with specific risk factors, Thesis, Punjab university, Chandigarh.

Hamada, S and Torii, M. (1978). Effects of sucrose in culture media on the location of glucosyltransferase of *Streptococcus mutans* and cell adherence to glass surface. *Infect Immun.* **(20):** 592-599.

Hamada, S. & Ooshima, T. (1975). "Production and properties of bacteriocins (mutacins) From *Streptococcus mutans*", *Archives of Oral Biology;* 20 **(10)**:641-648.

Hamada, S. & Slade, H.D. (1980b). "Biology, immunology, and carcinogenicity of *Streptococcus mutans*", *Microbiological reviews;* 44**(2)** 331-384.

Hannig, C., Ruggeri, A., Al-Khayer, B., Schmitz, P., Spitzmuller, B., Deimling, D., Huber, K., Hoth-Hannig, W., Bowen, W.H. & Hannig, M. (2008). "Electron microscopic detection and activity of glucosyltransferase B, C, and D in the in situ formed pellicle", *Archives of Oral Biology;* 53**(11):** 1003-1010.

Hansel Petersson G, Fure S, Bratthall D. (2003). Evaluation of a computer – based carries risks assessment program in an elderly group of individuals. *Acta Odontol Scand;* 61**(3):**164-71.

Hansel Petersson G, Twetman S, Bratthall D. (2002). Evaluation of a computer program for caries risk assessment in school children. *Caries Res;* **36(5):**327-40.

Hanstrom L, Johansson A, Carlsson J (1983). Lactoperoxidase and thiocyanate protect cultured mammalian cells against hydrogen peroxide toxicity. *Med Biol;* **61:**268-274.

Harper, D.S. & Loesche, W.J. (1984). "Growth and acid tolerance of human dental plaque bacteria", *Archives of Oral Biology;* 29 **(10**). 843-848.

Harris N, Garcia- Godoy F. Primary Preventive Dentistry. *6th Edition. Julie Levin Alexander Pearson education, INC:* (2004).

Harris R, Nicoll AD, Adair PM, Pine CM. (2004). Risk factors for dent al caries in young children: a systematic review of the literature. *Community Dent Health;* 21 **(1 Suppl):**71-85.

Hausen H, KarKKainen S, Seppa L. (2000). Application of the high-risk strategy to control dental caries. *Community Dent Oral Epidemiol*; 28**(1):**26-34.

Heintze U, Birkhed D, Bjorn H (1983). Secretion rate and buffer effect of resting and stimulated whole saliva as a function of age and sex. *Swed Dent J;* **7:**227-238.

Hicks J, García-Godoy F, Flaitz C. (2003). Biological factors in dental caries: Role of saliva and dental plaque in the dynamic process of demineralization and remineralization (part 1). *J Clin Pediatr Dent;* **28:**47-52.

Hildebrandt GH. (1995). Caries risk assessment and prevention for adults. J Dent Educ; *59***(10):**972-9.

Hillman, J. D., J. van Houte, and R. J. Gibbous. (1970). Sorption of bacteria to human enamel powder. *Arch. Oral Biol.* **15:**899.

Hillman, J.D., Dzuback, A.L. and Andrews, S.W. (1987). Colonization of the human oral cavity by a *Streptococcus mutans*. *J Dent Res*. **(66):** 1092-1094.

Hirasawa, M., Kiyono, H., Babb, J.L., Shiota, T., Michalek, S.M. & McGhee, J.R. (1980a). "Virulence of *Streptococcus mutans*: in vivo reversion of low-virulence mutant results in partial displacement and pathogenesis", *Infection and immunity;* 27**(3)**:1003-1011.

Hirasawa, M., Kiyono, H., Shiota, T., Hull, R.A., Curtiss, R., 3rd, Michalek, S.M. & McGhee, J.R. (1980b). "Virulence of *Streptococcus mutans*: restoration of pathogenesis of a glucosyltransferase-defective mutant (C4)", *Infection and immunity;* 27**(3):** 915-921.

Hong L, Ahmed A, McCunniff M, Overman p, Mathew M. (2008). Obesity and dental caries in children aged 2-6 years in the United States: National health and nutrition examination survey 1999-2002. *J Pub Health Dent* 68**(4):**227-223.

Howell AJ, Jordan HV, Georg LK, Pine L (1965). Odontomyces viscosus, gen. nov., spec. nov., a fila-mentous microorganism isolated from periodontal plaque in hamsters. *Sabouraudia,* 4**(2):** 65–68.

Hunter PB. (1998). Risk factors in dental caries. *Int Dent J;* 38**(4):**211-7.

Idone, V., Brendtro, S., Gillespie, R., Kocaj, S., Peterson, E., Rendi, M., Warren, W., Michalek, S., Krastel, K., Cvitkovitch, D. & Spatafora, G. (2003). "Effect of an orphan Response regulator on *Streptococcus mutans* sucrose-dependent adherence and cariogenesis", *Infection and immunity;* 71 **(8)** 4351-4360.

Jack RW, Tagg JR, and Ray B. (1995). Bacteriocins of gram-positive bacteria. *Microbiol Rev;* **59:** 171-200.

Jalil RA. (1995). Correlating *Streptococcus mutans* counts in saliva with plaque amount, gingival inflammation and caries experience in school children. *Singapore Dent J;* **20:** 16-20.

Jenson L, Budenz AW, Featherstone JDB, Ramos-Gomez FJ, Spolsky VW, Young DA. (2007) Clinical protocols for caries management by risk assessment. *J Calif Dent Assoc.* **(35):** 714-23.

Jette, A.M., H.A. Feldman, S.L. Tennsted, T. (1993). Tobacco use: a modifiable risk factor for dental disease among the elderly. *Am. J. Public Health;* 83**(9):** 1271-1276.

Johansson H. (1998). Caries development from 11 to 22 years of age: a prospective Radiographic study. *Caries Res;* 32**(1):**10-6.

Johnson NW. (1991). Dental caries: Markers of high and low risk groups and individuals. Cambridge University Press, Cambridge-1991.

Kamiya R, U., Napimoga, M.H., Rosa, R.T., Hofling, J.F Goncalves, R.B. (2005). Mutacins production in Streptococcus mutans genotypes isolated from caries-active and caries-free individuals, *Oral Microbiol Immunol*, **20:** 20-24.

Keene HJ, Shklair IL. (1974). Relationship of *Streptococcus mutans* carrier status to the development of carious lesions in initially caries free recruits. *J Dent Res.* **53:**1295

Kelstrup J, Richmond S, West C, Gibbons RJ. (1970). Fingerprinting human oral streptococci by bacteriocins production and sensitivity. *Arch Oral Bio1;* **15:** 1109-1116.

Kelstrup, J. and Gibbons, R.J. (1969): Bacteriocins from Human and Rodent Streptococci, *Arch Oral Biol* **14:**251-258.

Keyes PH. (1960). The infectious and transmissible nature of dental caries. Findings and implications. *Arch Oral Biol;* **1:**304-20.

Kidd EA. (1999). Caries management. *Dent Clin of North Am; 43*(**4):**743-64.

Kirstila V, Hakkinen P, Jentsch H, Vilja P, Tenovuo J. (1998). Longitudinal analysis of the association of human salivary antimicrobial agents with caries increment and cariogenic micro-organisms: a two-year cohort study. / *Dent Res* **77:**73-80.

Kleinberg, I. (2002). "A mixed-bacteria ecological approach to understanding the role of the oral bacteria in dental caries causation: an alternative to *Streptococcus mutans* and the specific-plaque hypothesis".*Critical reviews in oral biology and medicine: an official publication of the American Association of Oral Biologists;* 13**(2)** 108-125.

Koch, G. & Poulsen, S. (2006). Pediatric dentistry-a clinical approach. *Blackwell, ISBN 10-87-16-12271-2*, **Denmark,** 119, 134.

Kohler B, Bratthall D. (1978). Intrafamilial levels of *Streptococcus mutans* and some aspects of the bacterial transmission. *Scand J Dent Res;* **86**:35-42.

Kolenbrander, P.E. (2000). Oral microbial *communities*: biofilms, interactions, and genetic systems. *Annual Review of Microbiology* **54:** 413-437.

Kolenbrander, P.E., Palmer, R.J.,Jr, Periasamy, S. & Jakubovics, N.S. (2010). "Oral multispecies biofilm development and the key role of cell-cell distance ", *Nature reviews. Microbiology.*

Konings WN, Lolkema JS, Bolhuis H, Van Veen HW, Poolman B, Driessen AM (1997). The role of transport processes in survival of lactic acid bacteria: Energy transduction and multidrug resistance. *Antonie van Leuwenhoek,* **71:**117-128.

Koo, H., Xiao, J., Klein, M.I. & Jeon, J.G. (2010). "Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms ", *Journal of Bacteriology.*

Kopczyk R, Conroy C (1968). The attachment of calculus to root planed surfaces. *Periodontics* **6:**78-83.

Korithoski B, Krastel K, Cvitkovitch DG. (2005). Transport and metabolism of citrate by *Streptococcus mutans*. *J Bacteriol;* 187**(13):**4451-4456.

Krasse B. (2001). The Vipeholm dental caries study: recollections and reflections 50 years later. *J Dent Res* 80**(9):** 1785-1788.

Kreth, J., Merritt, J., Zhu, L., Shi, W. & Qi, F. (2006). "Cell density- and ComE-dependent Expression of a group of mutacin and mutacin-like genes in *Streptococcus mutans*", *FEMS microbiology letters;* 265**, (1):**11-17.

Kreth, J., Zhu, L., Merritt, J., Shi, W. & Qi, F. (2008). "Role of sucrose in the fitness of *Streptococcus mutans*", *Oral microbiology and immunology; 23***(3)** 213-219.

Kuhnert, W.L., Zheng, G., Faustoferri, R.C. & Quivey, R.G.,Jr. (2004). "The F-ATPase operon promoter of *Streptococcus mutans* is transcriptional regulated in response to external pH", *Journal of Bacteriology,* **186(24):**8524-8528.

Kulkarni GV, Chan KH, Sandham HJ. (1989). An investigation into the use of restriction endonuclease analysis for the study of transmission of mutans streptococci. *J Dent Res;* **68:** 1155-1161.

Kuramitsu HK. (1993). Virulence factors of mutans streptococci: role of molecular genetics. *Crit Rev Oral Biol Med;* **4:**159-76.

Kuramitsu, H. K. (2001). Virulence properties of oral bacteria: impact of molecular biology. *Cur Issues Mol Biol 3*, 35–36.

Kuramitsu, H.K. & Ingersoll, L. (1978). "Interaction of glucosyltransferase with the cell Surface of *Streptococcus mutans*", *Infection and immunity;* 20**(3):** 652-659.

Kuriakose S. Joseph E. (1999). Caries prevalence and its relation to socio-economic status and oral hygiene practice in 600 Pre-school children of Kerala- India. *J. Indian Soc pedod prev Dent;* 17**(3):** 97-100.

Kutsch VK, Kutsch CL. (2006). Disease prevention: Caries risk assessment. *Dent Today; 25*(10**):86,** 88-99.

Kutsch Vk, Milicich G, Domb W, Anderson M, Zinman E. (2007). How to integrate CAMBRA into private practice. *J Calif Dent Assco ;* **35(11);** 778-85.

Lagerlof F, Oliveby A (1994). Caries-protective factors in saliva. *Adv Dent Res* **8:**229-238.

Leclere, R., E. Derlot, J. Duval, and P. Courvalin. (1988). Plasmid-mediated resistance to vancomycin and teicoplannin in *enterococcus faecium. N. Engl.J.Med.* **(319):**157-161.``

Lemos JA, Abranches J, Burne RA. (2005). Responses of cariogenic streptococci to environmental stresses. *Curr Issues Mol Bio;* **7:** 95-107.

Len, A.C., Harty, D.W. & Jacques, N.A. (2004b). "Stress-responsive proteins are upregulated in *Streptococcus mutans* during acid tolerance", *Microbiology (Reading, England);* 150**(5)**:1339-1351.

Lenander-Lumikari,M. V. Loimaranta. (2000). Saliva and Dental Caries, *Adv Dent Res 14* **(12):**40-47.

Lendenman K, Grogan J, Oppenheim FG.(2000). Saliva and dental pellicle-a review. *Adv Dent Res ;* **14:** 22-28.

Li Y, Caufield PW (1995). The fidelity of initial acquisition of mutans streptococci by infants from their mothers. *J Dent Res* 7**4:**681-685.

Liebana, J., Castillo, A., Peis, J. Bala, P., Piedrola. G. (1991). Antimicrobial susceptibility of 1042 strains of *Streptococcus mutans* and *Streptococcus Sobrinus*. Comparison from 1985 to 1984. *Oral Microbiology Immunology,* **(6):** 146-150.

Liljemark WF, Bloomquist C. (1996). Human oral microbial ecology and dental caries and periodontal diseases. *Crit Rev Oral Biol Med;* **7:** 180-198.

Locker D. (1992). Smoking and oral health in older adults. *Can J. Public health***. 83:** 429-432.

Loesche, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiol. Rev*. **50**:353–380.

Loesche, W.J. (1986). "Role of *Streptococcus mutans* in human dental decay", *Microbiological reviews,* **50(4):**353-380.

Lukacs J: (2011). Sex differences in dental caries experience: clinical evidence, complex etiology. *Clin Oral Investig;* 15**(5):**649–656.

Lundgren M, Emilsson CG, Osterberg T. (1998). Root caries and some related factors in 88-years-old carriers and non-carriers of *Streptococcus sobrinus* in saliva. *Caries Res* **32:**93-99.

Ma, Y., Lassiter, M.O., Banas, J.A., Galperin, M.Y., Taylor, K.G. & Doyle, R.J. (1996). "Multiple glucan-binding proteins of *Streptococcus sobrinus*", *Journal of Bacteriology;* **178(6**): 1572-1577.

Madigan MT, Martinko JM, Parker J. Plasmids. In: Brock biology of microorganisms. Madigan MT, Martinko JM, Parker J, eds. Prentice Hall: Englewood Cliffs, *NJ* (1997a); 328-333.

Madlena M, Hermann P, Jahn M, Fejerdy P. (2008). Caries prevalence and tooth loss in Hungarian adult population: results of a national survey, *BMC Publ Health;* 8**(1):**364.

Mandel ID (1987). The functions of saliva. / *Dent Res* **66(Spec Iss):**623-627.

Mandel, I. D. (1974). Relation of saliva and plaque to dental caries. J. *Dent. Res;* **53:**246-266.

Mansson-Rahemtulla B, Baldone DC, Pruitt KM, Rahemtulla F. (1987). Effects of variations in pH and hypothiocyanite concentrations on *S. mutans* glucose metabolism. / *Dent Res* **66:**486 491.

Marsh PD. (1994). Microbial ecology of dental plaque and its significance in health and disease. *Adv Dent Res***. (8):** 263-271.

Marsh PD. (2006). Dental plaque as a biofilm and microbial community implications for health and disease. *BMC Oral Health;* **6 Suppl 1:** S14.

Marsh, P.D. (2004). "Dental plaque as a microbial biofilm", *Caries research;* 38**(3):** 204-211.

Marshall TA, Eichenberger-Gilmore, Broffitt BA, Warren JJ, Levy SM. (2007) Dental caries and childhood obesity: roles of diet and socioeconomic status. *Community Dent Oral Epidemiol 35*:449-458.

Marthaler Tm, O'Mullane Dm, Vrbic V. (1996). The prevalence of dental caries in Europe 1990-1995. ORCA Saturday afternoon symposium 1995. *Caries Res;* 30**(4):**237-55.

Maslow J, Mulligan ME. (1996). Epidemiologic typing systems. *Infect Control Hosp Epid;* **1**7**:**595-604.

Matsumoto-Nakano, M., Tsuji, M., Amano, A. & Ooshima, T. (2008). "Molecular Interactions of alanine-rich and proline-rich regions of cell surface protein antigen in *Streptococcus mutans* ", *Oral microbiology and immunology;* 23**(4):**265-270.

Mattos-Graner, R.O., Jin, S., King, W.F., Chen, T., Smith, D.J. & Duncan, M.J. (2001). "Cloning of the *Streptococcus mutans* gene encoding glucan binding protein B and analysis of genetic diversity and protein production in clinical isolates", *Infection and immunity ;*69**(11**): 6931-6941.

Mattos-Graner, R.O., Porter, K.A., Smith, D.J., Hosogi, Y. & Duncan, M.J. (2006). "Functional analysis of glucan binding protein B from *Streptococcus mutans*", *Journal of Bacteriology ;*188**(11):**3813-3825.

Mattos-Graner, R.O., Smith, D.J., King, W.F. & Mayer, M.P. (2000). "Water-insoluble glucan synthesis by mutans streptococcal strains correlates with caries incidence in 12- to 30-month-old children", *Journal of dental research;* 79**(6):** 1371-1377.

McDonald R, Avery DR, Dean JA. (2004). Dentistry for the child and adolescent. *8th Edition. Mosby:* 2004.

Mejàre B, Mejàre I, Edwardsson S. (1973). Bacteria beneath composite restorations a culturing and histobacteriological study. *Acta Odontol Scand*; **3**7**:**267-275.

Mejare I, Kallestal C, Stenlund H, Johansson H. (1988). Caries development from 11 to 22 years of age: a prospective Radiographic study. *Caries Res;* 32**(1):**10-6.

Merrit J, Anderson MH, Park NH, Shi W. (2001). Bacterial biofilms and dentistry.J *Calif Dent Assoc;* 29**(5):**255-60.

Michalek, S.M., Shiota, T., Ikeda, T., Navia, J.M. & McGhee, J.R. (1975b). "Virulence of *Streptococcus mutans:* biochemical and pathogenic characteristics of mutant isolates", *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.),* 150**(2)**498-502.

Miller, W. D. (1890). The micro-organisms of the human mouth. The S.S. White Manufacturing Co., Philadelphia.

Miravet A, Montiel company JM, Almerich Silla JM. (2007). Evaluation of caries risk in a young adult population. *Med oral patol oral Cir Bucal*. **(5):**412-418.

Mitchell, T.J. (2003). "The pathogenesis of streptococcal infections: from tooth decay to meningitis", *Nature reviews.Microbiology;* **1(3):** 219-230.

Miura H, Isogai E, Hirose K, Wakizaka H, Ueda I, Ito N (1991). Application of sucrose indicator strip to evaluate salivary sucrose clearance. / *Dent* **19:**189-191.

Moss ME, Zero DT. (1995). An Overview of caries risk assessment and its potential utility. *J dent Educ.1;* 59**(10):**932-40.

Moynihan P.J (2002). The role of diet and nutrition in the etiology and prevention of oral disease. *Bull world Health organ***, (83):** 694-699.

Mulvany D (2001).Letters: Soft Drinks and caries. *J Am Dent Assoc 132*: 1083.

Napimoga MH, Jose Francisco Hofling, Marlise Inez Klein, Regianne Umeko Kamiya and Reginaldo Bruno Goncalves (2005). Transmission, diversity and Virulence factors of Streptococcus mutans genotypes. *J Oral Science.,* **47 (2):** 59-64.

Napimoga MH, Kamiya RU, Rosa RT, Rosa EA, Höfling JF, Mattos-Graner RO, Gonçalves RB(2004). Genotypic diversity and virulence traits of *Streptococcus mutans* in caries-free and caries-active individuals. *J Med Microbiol.,* **53:** 697-703.

Narinder, Tewari A, Chawla HS. (1986). Inter comparison of sugar and dental caries relationship in urban and rural children. *J Indian SOC. Pedod Pre Dent;* 17 **(1):** 52-59.

Newbrun E. Cariology. 3rd Edition.quintessence Publishing CO, Inc, Chicago, Illinois (1989).115-21.

Newman HN (1990). Neutrophils and IgG at the host-plaque interface on children's teeth. / *Periodontol.,* **51:**642-651.

Novak J., Caufield P.W., Miller E.J.,(1994). Isolation and biochemical characterization of a novel lantibiotic mutacin from *S.mutans*. *J Bacteriol.,* **176**:4316-4320.

Nyvad, B. & Kilian, M. (1990). "Comparison of the initial streptococcal micro flora on Dental enamel in caries-active and in caries-inactive individuals", *Caries research.,* 24**(4)**: 267-272.

Oho, T., Yamashita, Y., Shimazak, Y., Kushiyama, M. and Koga, T. (2000). Simple and rapid detection of *Streptococcus mutans* and *Streptococcus Sobrinus* in human saliva by polymerase chain reaction. *Oral Microbiol Immunol*. **(15):** 258-262.

Okada M, Soda Y, Di T, Suzuki J, Miura K. (2005). Longitudinal study of dental caries incidence associated with *Streptoccous mutans* and *Streptococcus Sobrinus* in Pre-School children. *J Med Micrbiol.,* **(54):** 661-665.

Olive DM, Bean P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol.,* **3**7**:** 1661-1669.

Ooshima, T., Matsumura, M., Hoshino, T., Kawabata, S., Sobue, S. & Fujiwara, T. (2001). "Contributions of three glycosyltransferases to sucrose-dependent adherence of *Streptococcus mutans*", *Journal of dental research.,* **80(**7**):** 1672-1677.

Ozawa Y, Chiba J, Sakamoto S. (2001). HLA Class II alleles and salivary numbers of mutans streptococci and lactobacilli among young adults in Japan. *Oral Microbiol Immunol.,* **16:**353-357.

Parrot, M., Caufield, P.W. Lovoie, M.C. (1990). Preliminary Characterization of four Bacteriocins from *Streptococcus mutans*. *J.Microbiol***., (36):** 123-130.

Perch, B., E. Kjems, and T. Ravn. (1974). Biochemical and serological properties of *Streptococcus mutans* from various human and animal sources. *Acta Pathol Microbiol Scand*., **82:**357-370.

Petersen, P.E., Bourgeois, D., Ogawa, H., Estupinan-Day, S. & Ndiaye C. (2005). The global burden of oral diseases and risks to oral health. *Bulletin of the World Health Organization*; 83**(9):** 661-9.

Petersen, PE. (2003). The World Oral Health Report 2003: continuous improvement of oral health in the 21st century-the approach of the Who Global Oral Health Programme. *Community Dent Oral Epidemiology*, **31:** 3-23.

Peterson PE, Yamamoto T. (2005). Improving the oral health of older people: the approach of the WHO Global Oral Health Programme. *Community Dent Oral Epidemiol ;* 33**(2):**81-92.

Petersson GH, Brathall D. (1996). The caries decline: A review of reviews. *Euro J Oral Sci;* 104**(42):**436-43.

Pitts NB. (1998). Risk assessment and caries prediction. *J Dent Educ;* 62**(10):**762-70.

Powell LV. (1998). Caries prediction: A review of the literature. *Community Dent Oral Epidemiol ; 26* **(6):**361-71.

Preza D, olsen I, Aas JA, Willumsen T, Grinde B, Paster BJ. (2008). Bacterial profiles of root caries in elderly patients. *J Clin Microbiol 46* **(6):** 2015-2021.

Preza D, olsen I, Aas JA, Willumsen T, Grinde B, Paster BJ. (2008). Bacterial profiles of root caries in elderly patients. *J Clin Microbiol 46* **(6):** 2015-2021.

Preza D, Olsen I, Aas JA, Willumsen T, Grinde B, Paster BJ.(2008). Bacterial profiles of root caries in elderly patients. *J Clin Microbiol* ; **46:** 2015-2021.

Proctor GB, Pramanik R, Carpenter GH, Rees GD. (2005). Salivary proteins interact with dietary constituents to modulate tooth staining. *J Dent Res;* **84:** 73-78.

Qi, F., Chen, P. Caufield, P.W. (1990). Functional analyses of the promoters in the lantibiotic mutacin II biosynthetic locus in *Streptococcus mutans*. *Appl Environ Microbiol*. **(65):**652-658.

Qi, F., Chen, P. Caufield, P.W. (2001) The group I strain of *Streptococcus mutans.* VA 140 produces both the lantibiotic mutacin- I and a non-lantibiotic Bacterocin, Mutacin- 4. *Appl Environ Micrbiol***. (6**7**):** 15-21.

Quivey, R.G., Kuhnert, W.L. & Hahn, K. (2001). "Genetics of acid adaptation in oral streptococci", Critical reviews in oral biology and medicine: an official publication of the American Association of Oral Biologists; 12 **(4):** 301-314.

Ravald N, Birkhed D. (1992). Prediction of root caries in periodontally treated patients maintained with different fluoride programmes. *Caries Res;* 26**(6):**450-8.

Reich E, Lussi A, Newbrun E, (1999). Caries –risk assessment. *Int Dent J;* 49**(1):**15-26.

Rose G. (2001). Sick individuals and sick populations. *Int J Epidemiol;* 30**(3):**427-32.

Rudney JD (1995). Does variability in salivary protein concentrations influence oral microbial ecology and oral health? *Crit Rev Oral Biol Med* ***6*:**343-367.

Russell JI, MacFarlane TW, Aitchison TC, Stephen KW, Burchell CK (1990). Caries prevalence and microbiological and salivary caries activity tests in Scottish adolescents. *Community Dent Oral Epidemiol* **18:**120-125.

Russell, R.R. (1979). "Glucan-binding proteins of *Streptococcus mutans* serotype c", *Journal of general microbiology;* 112**(1):** 197-201.

Russell, R.R. (2009). "Changing concepts in caries microbiology ", *American Journal of Dentistry;* 22**(5**): 304-310.

Russell, R.R., Aduse-Opoku, J., Sutcliffe, I.C., Tao, L. & Ferretti, J.J. (1992). "A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolisms", *The Journal of biological chemistry;* 267**(7):** 4631-4637.

Saarela M, Alaluusua S, Takei T, Asikainen S. (1993). Genetic diversity within isolates of mutans streptococci recognized by an rRNA gene probe. *J Clin Microbiol;* **31:** 584-587.

Saarela M, Hannula J, Mättö J, Asikainen S, Alaluusua S.(1996). Typing of mutans streptococci by arbitrarily primed polymerase chain reaction. *Arch Oral Biol;* **41:** 821-826.

Saarela, Caufield Pw. (1998). Arbitrarily primed polymerase chain reaction finger printing for the genotype identification of mutans-streptococccci from humans. *Oral Microbiol Immunol*, **(13):**17-22.

Sajith, D.W. Banting. Ellen, R.P., (2006). *Streptococcus mutans* and *Lactobacillus* detection in the assessment of dental root surface caries risk. *J.Dent.Res.* **64:**1245-1249

Samaranayake L. (2002)*:* Essential microbiology for Dental students.- *3rd edition, livingstone Elsevier Harcourt Publishes* Limited**:**268-*69.*

Satcher D (2000). Oral health in America: A report of the surgeon general. http://www.surgeongeneral.gov/library/oralhealth/.Accessed march 2007.

Sato, Y., Yamamoto, Y. & Kizaki, H. (1997b). "Cloning and sequence analysis of the gbpC gene encoding a novel glucan-binding protein of *Streptococcus mutans", Infection and immunity;* 65**(2):** 668-675.

Scheinin A, Pienihakkinen K, Tiesko J, Holmberg S. (1992). Multifactorial modeling for root caries prediction. *Community Dent Oral Epidemiol;* 20**(1):** 35-7.

Schilling, K.M. & Bowen, W.H. (1988). "The activity of glucosyltransferase adsorbed onto saliva-coated hydroxyapatite", *Journal of dental research; 67***(1**): 2-8.

Scully C (1980). Comparative opsonic activity for *Streptococcus mutans* in oral fluids, and phagocytic activity of blood, crevicular fluid and salivary polymorphonuclear leukocytes in Rhesus monkeys. *Immunology; **39:**101*-107.

Selwitz RH, Ismail AI, Pitts NB: Dental caries. *Lancet* (2007); **369:**51-9.

Sgan-Cohen, H.D., J. Katz, T. Horev, A. Dinte, A. Eldad, (2000). Trends in caries and associated variablesamong young Israeli adults over 5 decades. *Community Dent Oral Epidemiol.* 28**(3):** 234-240.

Shah H and Hardie. (1982). Proposal for reclassification of *Bacteroides asaccharolyticus, Bacteroides gingivalis*, and *Bacteroides endodontalis* in a new genus, Porphyromonas. *International Journal of Systematic Bacteriology* **38:**128-131.

Shah, D.S. & Russell, R.R. (2004). "A novel glucan-binding protein with lipase activity from the oral pathogen *Streptococcus mutans*", *Microbiology (Reading, England;* 150**(6):** 1947-1956.

Shanmuk S. Jamshetty, (2004). Epidemiology of etiological agents of dental disorders in Gulbarga division. Thesis, Gulbarga University, Karnataka.

Shklair IL, Keene HJ. (1976). Biochemical characterization and distribution of *Streptococcus mutan*s in three diverse populations. In: *Microbial aspects of dental caries. Stiles HM, Loesche WJ, O'Brien TC, eds. Information Retrieval Inc: Washington,* DC; 201-210.

Shourie KC, (1941). Dental caries in children in madras city in relation to economic and nutritional status. *Ind. J. Med.Res,* **30:**561-572.

Socransky S. (1970). Microbiology of periodontal disease-present status and future considerations. *Journal of Periodontology;* **48:**497-502.

Sogi G and Baskar D, J. (2001). Dental caries and oral hygiene status of 13-14 year old school children of Davangere. *J Indian Soc Pedo Prev Dent;* 19**(3):**113-117.

Sohn W, Burt BA, Sawers MR (2006). Carbonated soft drinks and dental caries in the primary dentition. *J Dent Res 85***(3):**262-266.

Sonbul H, Al-Otaibi M, Birkhed D. (2008). Risk profile of adults with several dental restorations using the Cariogram model. *Acta tOdontol Scand;* 66**(6):**351-7.

Steinberg, D. (2000). Studying plaque biofilms on various dental surfaces. In Handbook of Bacterial Adhesion: *Principles, Methods, and Applications, Edited by Y. H. An & R. J. Friedman. Totowa, NJ: Humana Press:* 353-370.

Stopplaar JD, de, Konig, KG, Plasschaert AJM, Hoeven, Vander JS. (1971). Decreased Carcinogenicity of a mutant of *streptococcus mutans. Arch Oral Biol ;* **16:** 871-75.

Stull TL, LiPuma JJ, Edlind TD. (1988). A broad-spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. *J Infect Dis;* **15**7**:** 280-286.

Takahashi, N. & Nyvad, B. (2008). "Caries ecology revisited: microbial dynamics and the caries process ", *Caries research;* 42**(6):**409-418.

Takahashi, N., Horiuchi, M. & Yamada, T. (1997). "Effects of acidification on growth and glycolysis of *Streptococcus sanguis* and *Streptococcus mutans*", *Oral microbiology and immunology;* 12**(2):**72-76.

Tanzer JM, Freedman ML, Fitzgerald RJ, Larson RH. (1974). Dimished virulence of glucan synthesis. Defective mutants of *streptococcus mutans*. *Infect Immun;* **10:** 197-203.

Tanzer JM, Livingston J, Thompson AM. (2001). The microbiology of primary dental caries in humans. *J Dent Educ;* **65:** 1028-1037.

Tappuni AR, Challacombe SJ. (1994). A comparison of salivary immunoglobulin A (IgA) and IgA subclass concentrations in predentate and dentate children and adults. *Oral Microbiol Immunol* **9:**142-145.

Tellez M, Sohn W, Burt BA, and Ismail AI. (2006). Assessment of the relationship between neighborhood characteristics and dental caries severity among low-income African-Americans: a multilevel approach. *J Public Health Dent;* 66**(1):**30–36.

Tenovuo J, Grahn E, Lehtonen O-P, Hyyppa T, Karhuvaara L, Vilja P. (1987). Antimicrobial factors in saliva: ontogeny and relation to oral health. / *Dent Res* **66:**475-479.

Tenovuo J, Larjava H (1984). The protective effect of peroxidase and thiocyanate against hydrogen peroxide toxicity assessed by the uptake of [3H]-thymidine by human gingival fibroblasts cultured *in vitro. Arch Oral Biol* **29:**445-451.

Tenovuo J, Lumikari M, Soukka T. (1991). Salivary lysozyme, lactoferrin and peroxidases: antibacterial effects against cariogenic bacteria and potential clinical applications in Preventive dentistry. *Proc Finn Dent Soc* **8**7**:**197-208.

Tenovuo J, Lumikari M. (1991). Organic factors in human saliva in relation to dental caries. In: Dental caries. Markers of high and low risk groups and individuals. *Johnson NW, editor. Cambridge, UK: Cambridge University Press, pp.* 382-399.

Tewari A, Chawla HS, Narinder. (1986). Inter comparison of sugar and dental caries relationship in urban and rural children. *J Indian Soc Pedod Prev Dent,* **1**7**(1):**52-59.

Theilade, E. And theilade, J. (1985). Formation and ecology of plaque at different locations in the mouth. *Scandinavian J Dental Res;* **93:** 90- 95.

Thenisch NL, Bachmann LM, Imfeld T, Leisebach Minder T, Steurer J. (2006). Are mutans streptococci detected in preschool children a reliable predictive factor for dental caries risk? A systematic review. *Caries Res;* **40:** 366-374.

Tinanoff N. (1995). Critique of evolving methods for caries risk assessment. *J Dent Educ;* 59**(10):**980-5.

Tinanoff N. (1995). Dental caries risk assessment and prevention. *Dent Clin North Am;* 39 **(4):** 709-19.

Tsumori, H. & Kuramitsu, H. (1997). "The role of the *Streptococcus mutans* glucosyltransferases in the sucrose-dependent attachment to smooth surfaces: Essential role of the GtfC enzyme", *Oral microbiology and immunology;* 12**(5):** 274-280.

Ullman R, Miller S, Strampeer M, Cunha B. (1998). *Streptococcus mut*ans endocarditis; Report of three cases and review of the literature. *Heart Lung*, **1**7**:**209-212.

Vacca-Smith, A.M. & Bowen, W.H. (1998). "Binding properties of streptococcal glucosyltransferases for hydroxyapatite, saliva-coated hydroxyapatite, and bacterial surfaces", *Archives of Oral Biology;* 43**(2):**103-110.

Vadeboncoeur, C. & Pelletier, M. (1997). "The phosphoenolpyruvate: sugar phosphotransferase system of oral streptococci and its role in the control of sugar metabolism", *FEMS microbiology reviews;* 19 **(3):** 187-207.

Van der Reijden WA, van der Kwaak JS, Veerman ECI, Nieuw Amerongen AV.(1996). Analysis of the concentration and output of whole salivary constituents in patients with Sjogren's syndrome. *Eur J Oral Sci* 104:335-340.

Van Houte J. (1993). Microbial predictors of caries risk. *Adv Dent Res;* 7**:**87–96.

Van Houte, J. (1994). "Role of micro-organisms in caries etiology ", *Journal of dental research;* 7**3(3):**672-681.

Van Palenstein Helderman, W.H., Mafees.I. Van der Hoeven, J.S. Mikx, F.H (1996). Cariogencity depends more on diet than the prevailing mutans streptococcal species. J Dent Res**. (**7**5):** 535-545

Vitorino Rui, Lobo M, Duarte JR, Ferrer-Correia A, Domínguez P, Amado FM.(2005). The role of salivary peptides in dental caries. *Biomed Chomatogr;* **19:** 214-222.

Vitorino Rui, Lobo M, Duarte JR, Ferrer-Correia A, Tomer KB, Dubin JR, Domínguez P, Amado FM.(2004). In vitro hydrox-yapatite adsorbed salivary proteins. *Bioch Bioph Res Commun* ; **320:** *342-346.*

Wan AK, Seow WK, Purdoe DM, Bird PS, Walsh LJ, Tudehope DI. (2001). Oral colonization of *Streptococcus. mutans* in six-month-old predentata infants. *J Dent Res;* **80(b):**2060–5.

Wan AK, Seow WK, Walsh LJ, Bird PS, Tudehope DI, Purdie DM. (2001). Association of *streptococcus mutans* infection and oral developmental nodules in pre-dentate infants. *J Dent Res;* **80(a):**1945–8.

Welin, J., Wilkins, J.C., Beighton, D., Wrzesinski, K., Fey, S.J., Mose-Larsen, P., Hamilton, I.R. & Svensater, G. (2003). "Effect of acid shock on protein expression by biofilm cells of *Streptococcus mutans*", *FEMS microbiology letters;* 227**(2**): 287-293.

Welsh J, McClelland M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res*; **18:**17213-7218.

Whiley RA, Beighton D. (1998). Current classification of the oral streptococci. *Oral Microbiol Immunol* ; **13:** 195-216.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res;* **18:** 6531-6535.

Williams RL, Williams DF (1988). Albumin adsorption on metal surfaces. *Biomaterials* 9:206–212.

Woodward M, Walker ARP. (1994). Sugar consumption and dental caries: evidence from 90 countries. *Br Dent J **176*:**297-302.

Wright JT, Cutter GR, Dasanayake AP, Stiles HM, Caufield PW.(1992). Effect of conventional dental restorative treatment on bacteria in saliva. *Community Dent Oral Epidemiol;* 20**(3):**138-43.

Yaghi MM (2001). Letters: Soda pop and caries. *J Am Dent Assoc 13*2:576-577.

Yamashita, Y., Bowen, W.H., Burne, R.A. & Kuramitsu, H.K. (1993). "Role of the *Streptococcus mutans* gtf genes in caries induction in the specific-pathogen-free rat model", *Infection and immunity ;* 61**(9)** 3811-3817.

Young DA, Featherstone JDB, Roth JR. (2007). Curing the silent epidemic; caries management in the 21 st century and beyond. *J Calif Dent Assoc;* 35**(10):**681-85.

Zero D. (2004). Sugars: the arch criminal*? Caries Res;* 38**(3);** 277-85.

Zhu, L., Kreth, J., Cross, S.E., Gimzewski, J.K., Shi, W. & Qi, F. (2006). "Functional characterization of cell-wall-associated protein WapA in *Streptococcus mutans*", *Microbiology (Reading, England);* 152 **(8**): 2395-2404.

Zickert I, Emilson CG, Krasse B. (1982). *Streptococcus mutans*, lactobacilli and dental health in 13-14 years old Swedish children. *Community Dent Oral Epidemiol;* 10(**2):**77-81.