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Molecular Analysis on Streptococcous Mutans, In Detecting the Mutacin I and II and GTF Genes in Caries Free and Caries Active Individuals

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Abstract

Streptococcus mutans is a major pathogen implicated in dental caries. Its virulence is enhanced by its ability to produce bacteriocins, called mutacins, which inhibit the growth of other Grampositive bacteria. Sucrosedependent adherence is mediated by glucans, polymers of glucose synthesized from sucrose by glucosyltransferase (Gtf) enzymes. S. mutans makes several proteins that have the property of binding glucans. We hypothesized that three of these glucanbinding proteins (Gbps), Gbps A, C and D, contribute to the carcinogenicity of S. mutans. The availability of suitable in vitro and in vivo systems and a thorough understanding of the genetics, biochemistry and physiology of the dental pathogen Streptococcus mutans have greatly advanced in understanding of important areas in the field of bacteriology such as interspecies biofilms, competence development and stress responses. S. mutans, as an organism had evolved in close association with the human host, as a novel Grampositive model organism. The role of mutacins in vivo is unclear, however the antimicrobial activity of these substances may confer an ecological advantage for the producing strain in bacterial communities such as dental biofilm (56), and they may also be important for the establishment of S. mutans in vivo . Mutacins are peptide or protein antibiotics that are mainly bactericidal for other bacteria of the same or closely related species, as well as for other Grampositive microorganisms, and are likely to confer an ecological advantage in diverse bacterial communities such as dental biofilm. The relationship between caries activity and the higher synthesis of some virulence factors by different genotypes of S. mutans has been related. In this study, of 17 isolates, 10 isolates from cariesactive and 7 isolates from cariesfree were randomly selected for the molecular analysis for the mutacin I and II and for the (GTF) genes studies. It is concluded in the study that Mutacin I and II and (GTF) enzymes detected contribute to the carcinogenicity of S. mutans through a mechanism that may involve. alteration of biofilm architecture. In addition, reports of higher numbers of S. mutans genotypes with increased virulence in cariesactive Subjects suggest the importance of microenvironmental factors in increasing the risk of caries active individuals.

Keywords: S. Mutans; Caries; Biofilm; Glucan; Mutacin I and II; Glycosyltransferase Gene (GTF).

Introduction

Dental caries is a transmissible infectious disease in which mutans streptococci (MS) play the major role. As in many infectious diseases, colonization by pathogens is required before the disease can occur. MS are generally considered to be the principal etiological agent of dental Caries. There is a range of virulence factors important for the establishment of MS in the complex microbial community of dental biofilm. Studies of the

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virulence factors of S. mutans and their correlation with species biodiversity are fundamental to understanding the role played by colonization by different genotypes in the same individual, and the expression of characteristics that may or may not influence their virulence capacity and survival ability under different environmental conditions. In 1924, J. Clarke isolated an organism. from carious lesions and called it *S. mutans*, because he thought the oval-shaped cells observed were mutant forms of streptococci (Clarke, 1924). However, it was only in the late 1950s that *S. mutans* received greater attention from the scientific community and, by the mid 1960s, it was recognized as a major aetiological agent in dental caries (Loesche, 1986). In the subsequent two decades, researchers began to uncover the pathophysiology of *S. mutans*.

The major virulence traits of *S. mutans* were established: (i) the ability to produce large quantities of organic acids (acidogenicity) from metabolized carbohydrates; (ii) the ability to survive at low pH (aciduricity); and (iii) the ability to synthesize extracellular glucan-homopolymers from sucrose, which play a critical role in initial attachment, colonization and accumulation of biofilms on tooth surfaces (Banas & Vickerman, 2003; Bowen & Koo, 2011; Burne, 1998; Loesche, 1986). With the advances in molecular genetics techniques in the 1980s and 90s, scientists began to more rapidly understand how metabolic pathways enabled *S. mutans* to evolve into a specialized dental pathogen.

In this study we have, selected *S. mutans* isolates from both with caries active 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. 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. 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 *gtf*B, production of adhesive substances and percentages of growing bacteria adhering to glass surfaces in the presence of sucrose has been observed. 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.

Materials and Methods

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. We selectedtwodental 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.

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 socioeconomic background. Unstimulated 2–3 ml of saliva sample was collected into a pre-labeled 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*. 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.

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	1000.0ml
Final pH = 7.0 ± 0.2	

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 Himedia Pvt. Ltd., Mumbai and other biochemical tests were prepared and Performed according to the procedures described in the standard microbiology laboratory manuals.

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* isolates 10 form without caries and 7 from with caries individuals for molecular typing of *gtf-B* and mutacin genes by PCR amplification.

PCR Amplification of gtf B Gene

In this study, the 517 bp region off gtf B was amplified using the following primers by simple PCR (Oho *et al.,* 2000):

gftB Forward 5′-ACTACACTTTCGGGTGGCTTGG-3′
gftB Reverse 5′-CAGTATAAGCGCCAGTTTCATC-3′

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

Conditions for Gft B Amplification

Initial denaturation step: at 95˚C for 5 min

For each cycle (toal 30 cycles)

Followed by denaturation: 95˚C for 30 seconds,

Annealing at: 59˚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

PCR Amplification of Mutacin Genes

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′

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}Cfor 2 min

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

Results and Discussion

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 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 individual.

Molecular Studies of S.mutans Isolates

Mutacin I, the focus of this study, is a 24 aa lantibiotic with a molecular mass of 2364 Da. The mutacin I biosynthesis gene locus consists of 14 genes in the order of mutR, mutA, mutA9, mutB, mutC, mutD, mutP, mutT, mutF, mutE, mutG, orfX, orfY, orfZ (Qi *et al.,* 2000). MutR is thought to be the positive regulator for the expression of the mutacin I operon. MutA and MutA9 show strong similarity

to each other. While mutA is the structural gene for prepromutacin I, mutA9 is not required for mutacin I activity. MutB, MutC and MutD constitute the modification apparatus for the premature peptide, and MutT and MutP are the ABC transporter and protease, respectively, for the transporting and processing of premature mutacin. MutF, MutE and MutG are probable immunity proteins for mutacin I. The functions of OrfX, OrfY and OrfZ proteins in mutacin I production are unknown (Qi e*t al.,* 2000) (gtf) enzymes contribute to the ability of *S. mutans* to induce caries in the presence of sucrose. Of the individual Gbp mutants, only the gbpD strain was attenuated relative to the parental. We propose that the combined loss of Gbps A and C has the most dominant effect on S. mutans cariogenicity, though the additive loss of GbpD extends the magnitude and breadth of the attenuation. While the loss of GbpD alone mostly affects caries development on sulcal surfaces, the combined loss of Gbps A and C has its greatest impact on smooth (buccal) Surfaces. Clearly, each Gbp makes a unique contribution to the caries process, but the mechanistic contributions of each cannot be explained by the in vivo results alone. We can only speculate why the *gbpD* mutant is attenuated but the gbpAD and *gbpCD* mutants are not. It would appear that the loss of either *GbpA* or *GbpC* in some way compensates for the loss of *GbpD*, but when paired, the gbpAC mutant is significantly less cariogenic. Evidence supporting regulated expression of *GbpC* was provided by Biswas *et al*.[33], where expression of gbpC mRNA was observed to peak at mid-log phase and was extremely diminished in stationary phase cultures.

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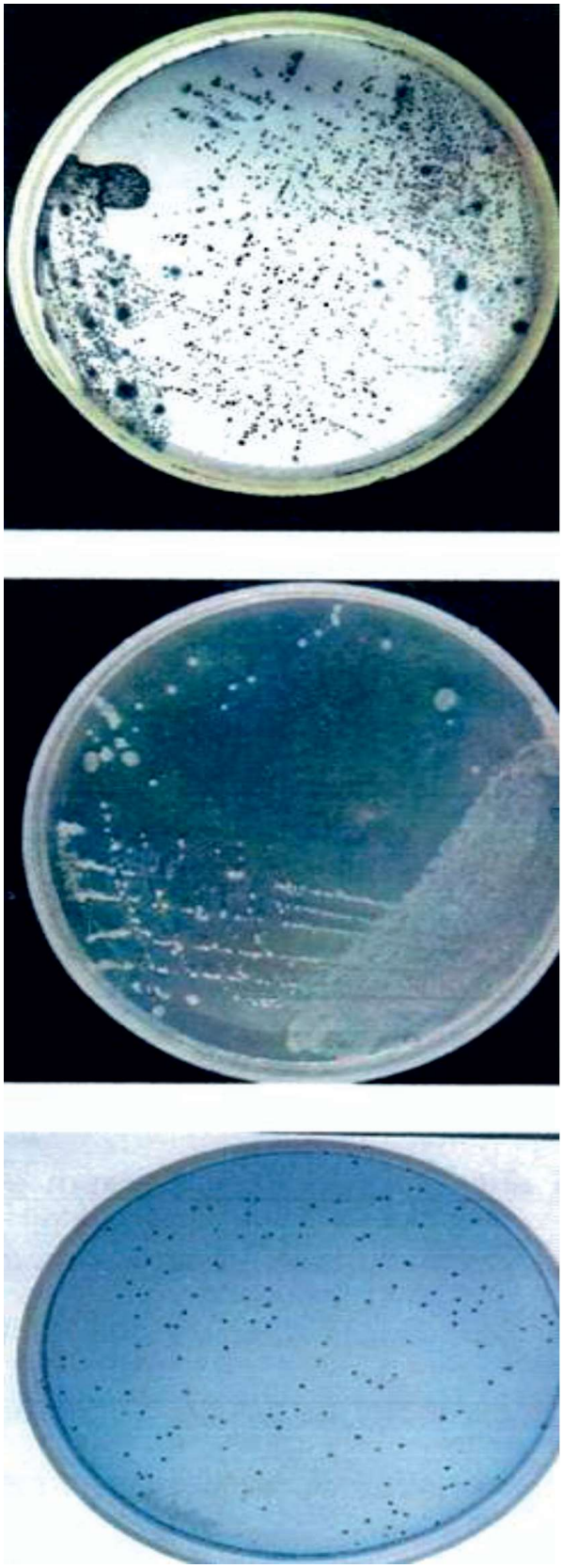

Fig. 1: Streptococcus mutans growth on themitis salivarius bacitracin (MSB) agar

Table 1: Over all incidence of Streptococcus mutans in saliva samples

		No. of Samples male	female	Total	male	Incidence of *S. Mutans* female	Total
With Caries	Habitual	58	4	56	33 (63.46%)	01 (25.00%)	34 (60.71%)
	Non-habitual	38	48	86	20 (52.63%)	27 (56.25%)	47 (54.65%)
Total		90	52	142	53 (58.88%)	28 (53.84%)	81 (57.04%)
Without Caries	Habitual	43	06	49	03 (06.97%)	01 (16.66%)	04 (08.16%)
	Non-habitual	24	39	63	14 (54.16%)	21 (53.84%)	35 (34.82%)
Total		67	45	112	17 (25.37%)	22 (48.88%)	39 (34.24%)
Grand Total		157	97	254	70 (44.58%)	50 (51.54%)	120 (47.24%)

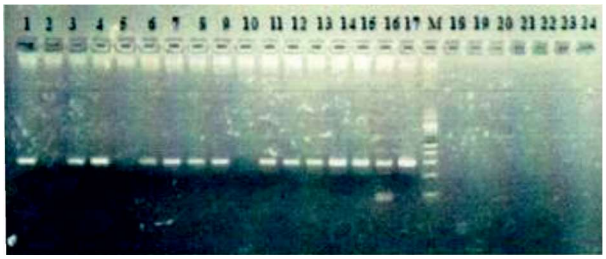

Fig. 2: Mutacin genes mut I and II in S. mutans.

Lane M-250 bp DNA ladder
Lane 1 to 10: mut I gene amplicons of S. mutans isolates from without caries individuals
Lane 11 to 17: mut I gene amplicons of S. mutans isolates from with caries individuals
Lane 18 to 24: mut II gene amplicons of S. mutans isolates from with caries individuals shows no amplicons.

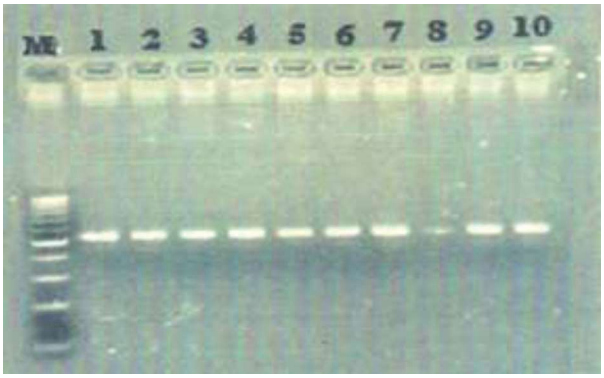

Fig. 3: GTF B gene amplicons of S. mutans isolates.
Lane M-100 bp DNA ladder
Lane 1 to 7: S. mutans isolates from with caries and
Lane 8 to 10: S. mutans isolates from without caries individuals

Conclusion

1. 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.
2. 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. 3. 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.
3. 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 and mut genes in the cariogenesis of S. mutans.
4. 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.
5. 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.

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