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PREVALENCE, CHARACTERIZATION AND HETEROGENEITY STUDIES ON *STREPTOCOCCUS MUTANS* ISOLATED FROM BANGALORE URBAN POPULATION

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ABSTRACT

Streptococcus mutans is a well-known causative agent in the development of dental caries. Over the last few decades there has been a remarkable increase in its prevalence rate among children and elders. In the present study, 38 % of *S. mutans* was isolated from the dental plaque samples collected from patients. Antibiotic sensitivity tests revealed the emergence of multi-drug resistance with all the isolates being completely resistant to penicillin, amoxicillin and ampicillin. Also, a decrease in sensitivity to Bacitracin was observed. The isolates were sensitive to the antibiotics erythromycin, clindamycin, ciprofloxacin and azithromycin. Further, Genomic DNA from all the clinical isolates and standard MTCC 497 was obtained and subjected further for PCR-RFLP analysis targeting the *dex* gene. RFLP with various restriction enzymes indicated that *Hae III* enzyme successfully digested the amplicons but the fragments had no heterogeneity among the resistant and the sensitive isolates including the standard.

KEYWORDS: ***Streptococcus mutans*, *dex* gene, RFLP, dental caries, multi-drug resistance.**

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INTRODUCTION

Streptococcus mutans is a Gram-positive, facultative anaerobic bacterium commonly found in the human oral cavity. It is the leading cause of dental caries (tooth decay) worldwide and is considered to be the most cariogenic of all of the oral *streptococci*[1]. Since the discovery of *S. mutans* as a causative agent of dental caries, much attention is focused on this organism for the prevention and management of dental caries.. Dextran is a homoglycan, which is a component of dental plaque, is considered to contribute to the development of dental caries. Dextranase is an enzyme that hydrolyzes glucans in the plaque matrix and is believed to be responsible for the pathogenesis of dental caries[2,3]. Dextranase is widely distributed among oral *Streptococci*, including mutans *Streptococci* and *S. salivarius*. In *S. mutans* dextran is degraded by the dextran hydrolyzing enzyme, dextranase, encoded by the *dex* gene. The dextranase enzyme degrades dextrans to isomaltosaccharides, predominantly isomaltotriose, isomaltotetraose and isomaltopentaose. The extracellular dextranase partially degrade the glucans or modify glucans by altering the ratio of 1, 6-α- to l, 3-α-linked chains and hence decrease solubility in water and therefore affect virulence of *S. mutans.* Thus the involvement of dextranase activity in affecting the composition of dental plaques has been evident[4].The conventional antibiotic therapy is effective in treating bacterial infections. However, with increasing antibiotic resistance, there is a need for newer approaches in the management of these infections. In recent years, the isolates of *S. mutans* have begun to show considerable resistance to commonly used antibiotics[5]. The virulence determinants of *Streptococcus mutans* have been investigated by various molecular and biochemical techniques. The characterization of these drug resistant isolates and their heterogeneity studies may provide more information on their genetic makeup and also be useful in the management and control of drug resistance to at least that region. A number of genotyping techniques like ribotyping, pulse field gel electrophoresis, random amplified polymorphic DNA (RAPD) analysis are being applied worldwide for characterizing the drug resistant isolates. But, these molecular methods used are expensive, time-consuming and difficult to interpret. There is therefore a need for a more reliable, economical and simple method for differentiation. PCR-RFLP has the potential for rapid screening of different types of *Streptococcus mutans.* Polymerase chain reaction (PCR) offers a more highly sensitive and specific detection method for bacteria than conventional culture methods[6]. PCR – RFLP studies on the genetic make -up of virulence factors of *S. mutans* aid in the treatment of dental caries and their prevention. PCR-RFLP can differentiate any resistant strains from contemporaneous background strains of the same serotype. In India, literature regarding genotyping of *S. mutans* isolates is largely lacking. A careful ponderance on scientific literatures pertaining to the *dex* gene and their sequencing reveal, such information are still obscure. Therefore, the present study was aimed to determine the heterogeneity in the *dex* gene among the clinical isolates obtained from Bangalore urban population patients by PCR RFLP methods.

MATERIALS AND METHODS

Isolation and Identification

Dental plaque samples were collected from patients visiting M. S. Ramaiah Dental College, Bangalore, India. Ethical clearance and prior consent from the patients was obtained before collection of dental plaque samples. A total of 100 dental plaque samples were obtained from the patient's molar teeth using a sterile tooth pick and was immediately transferred to the transport medium and was subjected to isolation and identification studies as described elsewhere[7]. The isolates were identified as *Streptococcus mutans* using routine lab diagnostic tools such as hemolysis on blood agar plates, gram staining and phenol red test.

A standard *S. mutans* strain MTCC 497 received from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India was also included as control. All the isolates along with the standard were then sub-cultured and maintained in Todd Hewitt medium.

Antibiotic sensitivity Tests

All the isolates identified as *S. mutans* and the MTCC standard were tested for their sensitivity pattern with the routinely used antibiotics by Kirby- Bauer disk diffusion technique. The antibiotics tested were amoxicillin (10µg), ampicillin (10µg), azithromycin (30µg), bacitracin (10µg), ciprofloxacin (05µg), clindamycin (02µg), erythromycin (15µg), penicillin (10 units), and streptomycin (10µg). The tests were conducted in triplicates to determine the resistance pattern among the isolates. For each antibiotic the diameter of zone of inhibition was calculated. The antibiotic susceptibility testing results were interpreted according to Clinical and Laboratory Standard Institute guidelines[8].

DNA Isolation and Amplification of the dex gene

Extraction of chromosomal DNA from streptococcal strains was carried out as described elsewhere[9]. The DNA samples were then stored in TE buffer at 4°C till amplification. The amplification of the *dex* gene was performed using a Gradient PalmCycler™ (Corbett Research Labs). The primers were designed based on the homologous sequences coding for the *dex* gene in *Streptococcus mutans*. Amplification of the dextranase sequence was performed using a thermocycler and PCR reaction ReadyMix™ (Sigma-Aldrich) as described previously[9]. Briefly the reaction mixture contained 50 pmol of each primer, 1x PCR buffer, 200 µM of each dATP, dCTP, dGTP and dTTP, 2.5U of Taq DNA polymerase, and 4 µl of template solution, along with 2 µl each of both primers. Amplification was performed using the thermo cycler, programmed for 1 cycle of 1 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 46°C, and 1 min at 72°C, followed by 1 cycle of 2 min at 72°C, and finally 1 cycle of 3 min at 30°C. The PCR products were visualized on Agarose gel after electrophoresis using Tris-acetate-EDTA buffer stained with Ethidium bromide[10].

Restriction Digestion of PCR products

The PCR products were purified to remove excess dNTPs and then were subjected to restriction digestion using different restriction enzymes specific for *dex* gene as described earlier[2]. Digestion was carried out using *HindIII*, *PvuII*, *PstI*, *EcoRI* and *HaeIII* (Sigma-Aldrich), individually. Briefly, 3 µl of purified PCR products were digested with 5 U of each of the restriction enzymes for 60 min at 37°C. After digestion, DNA fragments were analyzed by electrophoresis on 2.0% agarose gels.

RESULTS AND DISCUSSION

Isolation and Identification

Among the 100 plaque samples collected, 38 % were confirmed to be harbouring *S. mutans* by routine lab diagnostic tools. All the 38 GAS isolates were identified as gram positive *cocci* in chains. The colonies grown on Mitis salivarius bacitracin agar plates tested positive for greenish hemolysis on blood agar plates, and positive for sorbitol & mannitol fermentation studies confirming the identification of *S. mutans.* The study revealed a high rate (38%) of *Streptococcus mutans* incidence in Bangalore urban population as described in our previous study[4]. In a similar study conducted to compare the prevalence and pattern of caries in children of urban Bangalore and non-urban Chickaballapur within Karnataka state, India, the results showed caries prevalence of 66.3% in Bangalore city whereas in Chickballapur, the prevalence was 58.4% [11]. Whereas in a recent study, it was concluded that caries prevalence in preschool children of urban Bangalore was 27.5% and showed a decreased trend of caries prevalence in urban Bangalore from 1987 to 2005[12]. But the present study significantly showed that 38 % dental plaque samples harbouring *S. mutans,* the predominant oral

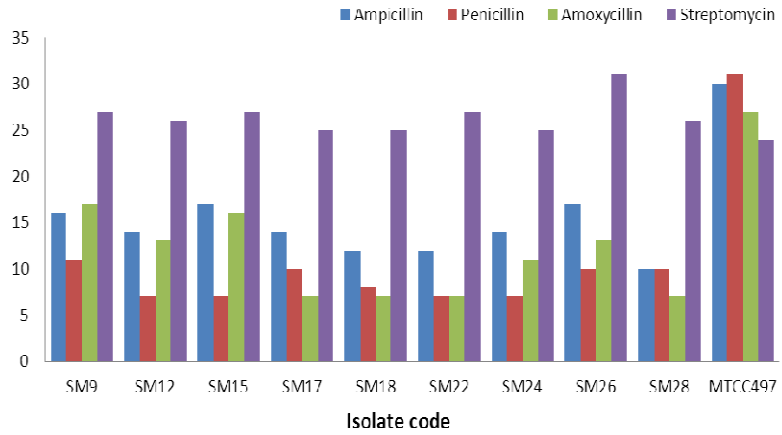
microflora causing dental caries. In another study conducted among the school children of Urban Delhi, the prevalence of dental caries was found to be 52.3%. Although it can be seen that the prevalence of caries in urban Bangalore population is comparatively lower than Delhi, but it is alarming that the rate has significantly increased in Bangalore population too which emphasizes the need for regular surveillance programs to screen for *S. mutans* and its role in causing dental caries.

Antibiotic sensitivity Tests

In order to understand the antibiotic susceptibility pattern among the clinical isolates of *Streptococcus mutans*, a battery of routinely used antibiotics were tested for its sensitivity. The Kirby-Bauer disk diffusion technique was performed on Mueller-Hinton agar as recommended by the Clinical and Laboratory Standards Institute (CLSI). Zone diameters were determined and interpreted according to CLSI standard guidelines. Sensitivity was observed for the antibiotics Ciprofloxacin, erythromycin, clindamycin and azithromycin. The isolates showed decreasing sensitivity towards Bacitracin. While 30% of the isolates showed intermediate sensitivity to Amoxicillin, 70% showed complete resistance. The isolates showed complete resistance to penicillin and ampicillin (Graph 1). Ciprofloxacin, erythromycin, clindamycin and azithromycin proved to be the most effective antibiotics against *S. mutans*. The standard MTCC 497 was sensitive to all the antibiotics tested.

Graph 1

Antibiotic resistance pattern of S. mutans to Ampicillin, Penicillin, Amoxicillin and Streptomycin



Viridans group *Streptococci* resistant to antibiotics have increasingly been reported over the past decade, while studies on antibiotic resistance of *Streptococcus mutans* group are few. *Streptococcus mutans,* being gram-positive cocci, is expected to be certainly susceptible to cell wall synthesis inhibiting antibiotics like penicillin, cephalosporin and protein synthesis inhibiting antibiotics like chloramphenicol and tetracycline. But recent studies indicate the emergence of penicillin resistance in *Streptococcus mutans*, which are generally considered uniformly susceptible to penicillin. In a recent study from the isolates obtained from a hospital, the *Streptococcus mutans* was found to be uniformly resistant to the β – lactam antibiotic, penicillin[13]. In another recent study, *S. mutans* isolates were multi drug resistant with resistance to amoxicillin, ceftriaxone, chloramphenicol, erythromycin, and tetracycline [14]. The results of disk diffusion revealed that most acidogenic bacteria were found resistant to antibiotics tested. “Out of seven acidogenic bacteria”, four were resistant to vancomycin, chloramphenicol, and erythromycin. In other study it was similarly reported that dental caries bacteria were resistant against vancomycin, chloramphenicol, penicillin, bacitracin and

streptomycin[15]. Thus the present study which revealed the emergence of multi drug resistance in *Streptococcus mutans* in the urban Bangalore region has actually not only made general prophylaxis a very cumbersome affair but also has further intricate the antibiotic resistance pattern of this region.

PCR – RFLP analysis of the dex gene

Genomic DNA obtained from each isolate showed no visible RNA contamination. The amplified PCR products of all the clinical isolates and standard were visualized on 1.5% agarose gel. The products showed successful amplification of the *dex* gene. The gel images were analyzed by Digital Gel Documentation and Analysis Software (DGelDAS, Biotek, Yercaud), to determine their molecular size. The molecular size of the PCR products were compared with molecular weight DNA ladder. The amplicons were about 520 to 570 bp, confirming successful amplification of the *dex* gene (Fig 1). The PCR products were purified to remove excess dNTPs and then were subjected to restriction digestion using different restriction enzymes specific for *dex* gene. It was found that *Hind III, Pvu II, Pst I, EcoR I* showed no visible digestion, as they all produced only single bands. Alternatively *Hae III* was successful in digesting the PCR amplicons. Every sample showed a set of bands that were uniform in band length, expressing no heterogeneity between the strains (Fig 2).

Amplification of dex gene of S. mutans

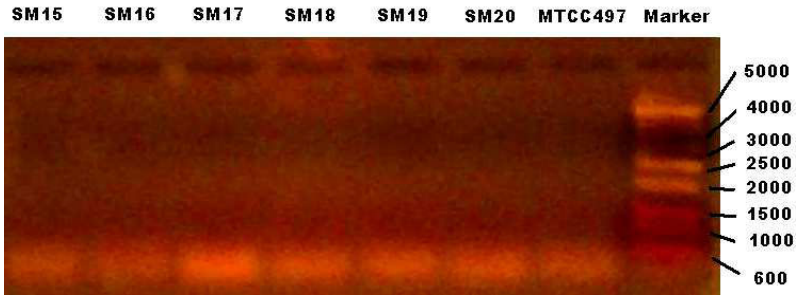


Figure 1

The PCR products of the *dex* gene of the standard and clinical isolates of *S. mutans* visualized in agarose gel electrophoresis were about 520 to 570 bp, confirming successful amplification.

Restriction Digestion of dex gene of S. mutans

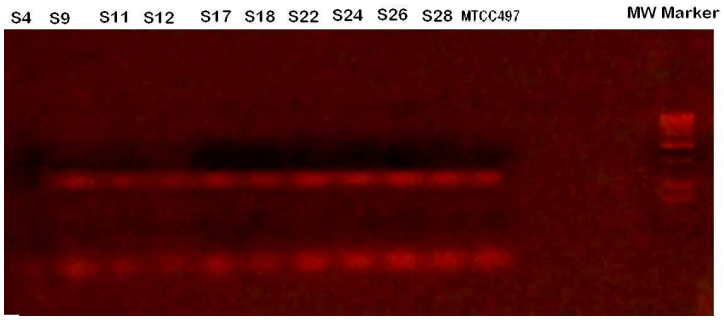


Figure 2

The PCR – RFLP pattern of the dex gene of *S. mutans* visualized on agarose gel electrophoresis. The *Hae* III digestion showed a set of bands that were uniform in band length, expressing no heterogeneity among the isolates and standard.

In a similar study[6], a pair of polymerase chain reaction primers were designed on the basis of the nucleotide sequence homology of dextranase genes of *Streptococcus mutans*, *S. sobrinus* and *S. downei*. The primer pair amplified a 530-bp DNA fragment on the *dex* genes of mutans streptococcal species: *S. mutans*, *S. sobrinus*, *S. downei*, *S. rattus* and *S. cricetus*. *Hae* III digestion of the 530-bp fragments generated species-specific sub fragments, which were easily distinguishable from each other by agarose gel electrophoresis. In another study the mutans streptococci was screened using PCR-RFLP targeting dextranase gene[16]. Their study revealed that PCR-RFLP profile resulting from the *Hae* III-digested 530 bp DNA fragments clearly differentiated each clinical isolate of mutans streptococci at the species level. The size of the PCR products and their *Hae* III-fragments were all matched to those of the type strains. Hence the results of the present study are in agreement with the earlier studies that *dex* gene amplicons are about 520 to 570 bp. Also this study suggests that *Hae* III could be used for restriction digestion of *dex* gene than other restriction enzymes. Additionally the heterogeneity study also revealed that the *dex* gene RFLP pattern was unaltered in the antibiotic resistant clinical isolates. Also there was no heterogeneity seen within the standard, sensitive and resistant isolates.

CONCLUSION

In the present study, the emergence of multi drug resistance in *Streptococcus mutans* was observed. This suggests the need for a regular surveillance program to control and manage the antibiotic resistance in these bacteria. It was concluded that, at least in this region of India, *S. mutans* has become an important matter of concern because of their higher preponderance of isolation and emergence of drug resistance in them. The study also revealed that there was no heterogeneity found in the RFLP pattern of the *dex* gene which is believed to be involved in the pathogenesis of dental caries.

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