

Identification and Detection of Streptococcus MUTANs in Plaque Samples

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Abstract: Streptococcus mutans have been proposed as the main causative agent of dental cavities. Dental caries is among the most common diseases in the world, this study was done to determine whether PCR analysis is useful to identify and even classify oral and other streptococci. 20 dental plaque samples were collected from teeth, 10 normal sample and 10 from persons with oral problems. The samples were diluted and plated in Mitis-Salivary media, Bacterial DNA was extracted from all specimens, then analysed by PCR with 16s universal primer and S. mutans serotype-specific primer sets. Then it was sequenced, The strains reported in this paper were identified as S. mutans on the basis of the fact that the sequence generated was Streptococcus mutans.

Keywords: Streptococcus Mutans, Dental Caries, Dental Plaque, PCR.

I. INTRODUCTION

Dental caries is a multifactorial, chronic bacterial disease, that causes demineralization and destruction of the hard tissues, usually by production of acid by bacterial fermentation of the food debris accumulated on the tooth surface (1). Dental caries is one of the most common chronic infectious diseases in the world (2,3). It is considered to be a major public health problem (4). the most cariogenic bacteria in dental plaque is streptococcus mutans (5,6). This is due to their ability of rapid lactic acid formation from dietary carbohydrates, mainly sucrose and glucose(7). S.mutans is a facultative anaerobic, Gram-positive cocci bacterium which appears in chains on Gram stain (8). S. mutans is classified into four serotypes (c/e/f/k) based on the chemical composition of its cell surface serotype specific rhamnoseglucose polymers (RGPs), which form a backbone of rhamnose polymers with side chains of glucose polymers (9,10). Serotype c is reported to be the most prevalent in oral isolates at approximately 70–80 %, followed by e, f and k (11, 12). Species identification of mutans streptococci has been based on conventional methods including complicated biochemical tests, but is time-consuming and sometimes unsatisfactory (13). Molecular biology methods have been developed to overcome culture problems, polymerase chain reaction (PCR) is now used in bacterial identification in environmental and clinical specimens. PCR methods are more sensitive and specific, and faster than conventional methods in bacterial determination. They allow the detection of viable and nonviable microorganisms, and consume less time and effort than conventional methods (14).

II. MATERIALS AND METHODS

A. Samples Collection

Twenty plaque samples were collected from Oxford Dental College (Bangalore, India) in sterile tubes containing 2ml normal saline and 1% yeast extract. Samples were stored at least 12hrs in a cold place, 10 normal sample and 10 from persons with oral problems.

B. Isolation of Bacteria

Samples were diluted into different serial dilutions. The dilutions of each sample were spread by sterile spreader on glass on MS -agar plates. The cultures were incubated for 24-48 hrs.

III. CHROMOSOMAL DNA ISOLATION

The method used to isolate chromosomal DNA from S. mutans was as follows. Cultures of S. mutans were initiated from pure streaked for single colonies, and dissolved in DNA extraction buffer and 20% SDS. Kept for incubation for 2 hours at 65°C. It was vortexed to mix the components. The whole mixture was centrifuged at 10,000rpm for 10 mins, and the clear solution was taken. To it equal volume of chloroform:isoamyl alcohol(24:1) was added and centrifuged at 10,000rpm for 10 mins. The upper clear layer was taken and to it 1/6th volume of Isopropyl alcohol was added and kept in -20°C for 20 mins. The final mixture was centrifuged at 10,000rpm for 10 mins. The pellet was rinsed with 70% ethanol and centrifuged again at 10,000rpm for 10 mins. The pellet was finally dried and Millipore water was added. The concentration of chromosomal DNA was determined spectrophotometrically (260nm) by standard procedures and its quality was assessed by the 260:280 nm absorbance ratio and by electrophoresis on agarose gel, the PCR products were analysed by electrophoresis on an agarose gel. Amplification and sequencing of the 16S rRNA for the amplified sample was performed to confirm bacterial identification. Universal primers for 16S rRNA were used.

A. PCR Amplification

PCR amplification of 16s region was done in 20 µl of reaction mixture containing PCR buffer, 1X (Kappa, SA); MgCl₂, 3 mM; dNTP mix, 0.25 mM; Taq DNA polymerase, 0.05 U; primer, 1 picomol and template DNA, 50 ng. Sterile nuclease free water is used as negative control. The PCR product was purified and subsequently sequenced in the forward and reverse directions and ultimately compared to the sequences reported in GeneBank (NCBI). PCR was performed to characterize the isolates based on their

serotype. For this, amplification was done in a reaction mixture (25 µl) consisting of PCR master mix that contained an enzyme, buffer, red dye and the required reagents, 25 pmol of each primer and 20–50 ng of the template DNA solution in a thermal cycler. The primer details are given below:

TABLE I: Primers

Primers	Sequences (5'-3')	GC %	Tm Value	Length	Product Size
16S FP	AGA GTT TGA TCC TGG CTC AG	50	51.0 °C	20	1500 bp
16S RP	CGG TTA CCT TGT TAC GAC TT	45	49.7 °C	20	

TABLE II: Primers

Primer	Sequence (5 – 3)	Size	Serotype
SC F	CGGAGTGCTTTTACAAAGTGCTGG	727	C
SC R	AACCACGGCCAGCAACCCCTTAT		
SE F	CCTGCTTTTCAAGTACCTTTCGCC	517	E
SE R	CTGCTTGCCAAGCCCTACTAGAAA		
SF F	CCCACAATTGGCTTCAAGAGGAGA	316	F
SF R	TGCGAAACCATAAGCATAGCGAGG		
SK F	ATTCCCGCCGTTGGACCAATCC	294	K
SK R	CCAATGTGATTCATCCATCAC		

Sequencing: The eluted amplicons were sequenced at Scigenome, Cochin using ABI PRISM Big Dye Terminators v1.1 cycle sequencing kit (Applied Biosystems, USA) according to the manufacturer's instructions.

B. Sequence Analysis And Phylogenetic Analysis

The comparison of the nucleotide sequences of the unique fragment with the sequences available in the GenBank database was carried out using the NCBI BLAST program (<http://www.ncbi.nlm.nih.gov/blast>). Phylogenetic tree were generated on the basis of sequences of Myeloperoxidase gene sequences using Clustal W 1.8 (15). A rooted phylogenetic tree was constructed using the sequences obtained from the samples.

IV. RESULT

The growth of specific colony on Mitis Salivaris media appears in 13 samples among 20. only 3 samples of healthy people have shown growth .

A. Chromosomal DNA Isolation

The DNA extracted from all the bacterial isolates were of good quality and could be used in all the downstream reactions.

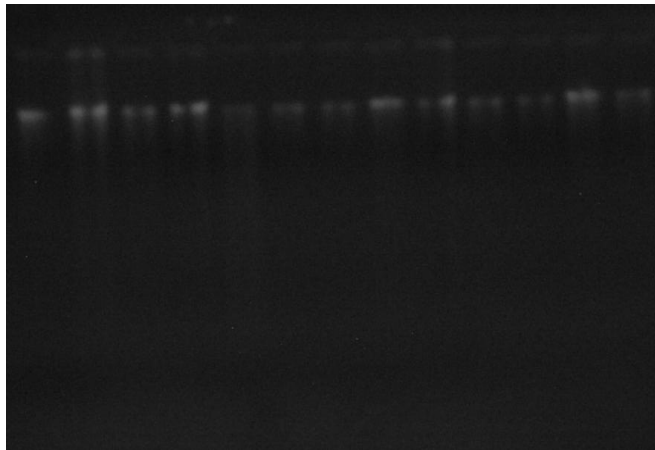


Fig.1.

B. Sequencing

16s rRNA sequencing was done and the electrophoregram shows the sequences are of good base quality. The sequence derived from the electrophoregram was BLAST searched for similarity sequences at the NCBI (National Centre For Bioinformatics) gene database.



Fig.2.

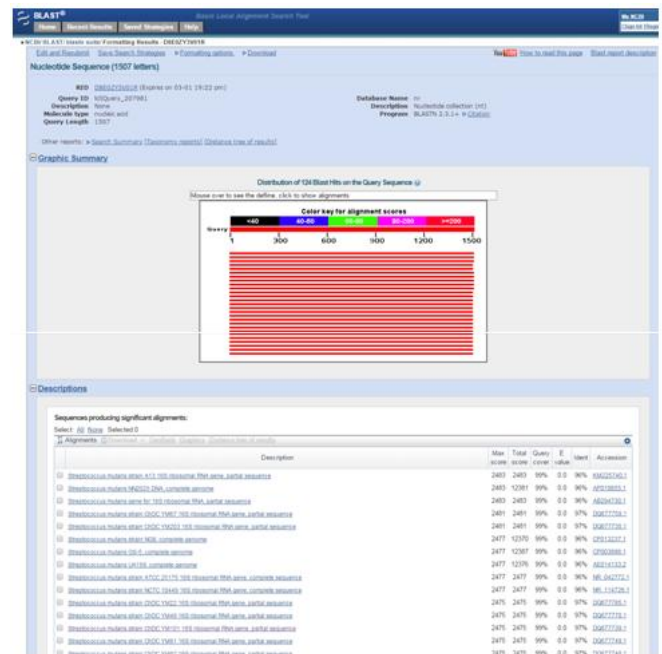


Fig.3.

The similarity search revealed maximum similarity of 96% with Streptococcus mutans.

C. Serotyping

PCR was done according to the above mentioned protocol. The bands were visualized under UV transilluminator which shows bands around 750bp. So from this we can confirm that it is serotype C.

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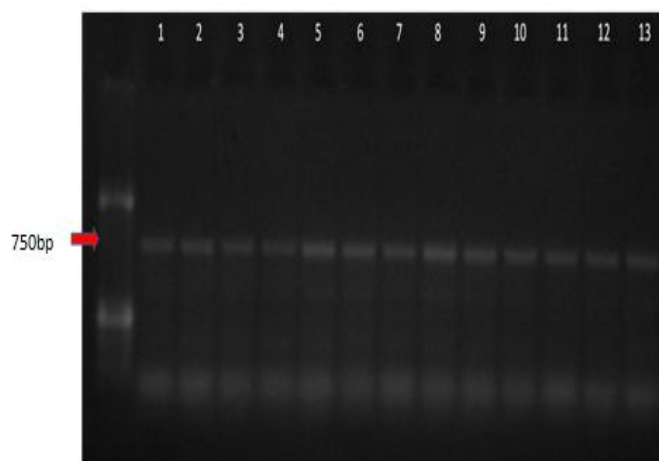


Fig.4. Gel image showing the serotype amplification.

V. DISCUSSION

Firstly 10 diseased samples were collected from dental college and 10 healthy samples. The diseased samples were collected from persons with oral problems. The samples were diluted and plated in MS media and colonies were observed next day. 3 of the normal healthy samples showed colony and all 10 diseased samples showed colony. Being plated on specific medium the colonies were confirmed to be *S. mutans*. But to re-confirm it by sequencing the DNA was isolated and PCR was done with 16s universal primer. As expected band was seen at 1500bp. Then it was purified from the gel and sequenced. The strains reported in this paper were identified as *S. mutans* on the basis of the fact that the sequence generated was *Streptococcus mutans*. To subdivide the type of *Streptococcus mutans* serotyping was done with the known sets of serotype specific primer sequences. From the gel picture it was confirmed that all the diseased patients have serotype C *Streptococcus mutans* infection.

VI. CONCLUSION

Polymerase chain reaction molecular approach was very sensitive, and it was fast and specific in identifying and detecting the presences of *Streptococcus mutans* in plaque samples. If compared to normal culture approach. Finally, the results of the present study suggest that the PCR method is suitable for investigation of the intra-oral distribution of *S. mutans*. PCR results of the present study showed that *S. mutans* had a significantly higher prevalence of caries. Longitudinal studies are required to evaluate the acquisition and colonising combination of *mutans streptococci* in the oral cavity, and to compare the results to the incidence of dental caries.

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