Research Article

# Virulent Gene Screening of Pathogens from Saliva Samples of Caries Patients

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**ABSTRACT**

The aim of the study is to determine the virulence of pathogens from saliva samples of dental caries patients. The virulence of the pathogen is detected from saliva samples collected from dental caries patients by using the techniques of molecular biology. Virulent factors are molecules produced by pathogens that contribute to the pathogenicity of the organism. Estimating this from the saliva of the patients with caries is a highly sensitive method for detection of virulence strain causing caries. If the organisms in the oral cavity are found to be virulent, this may be a factor that induces dental caries. Techniques of Molecular biology can be applied in the field of dentistry and can be used to prevent caries.

**Keywords:** gftB gene, Streptococcus mutans, caries, virulence, pathogenicity.

## INTRODUCTION

Virulence-The degree of pathogenicity within a species of parasites as indicated by case fatality rates or the ability to invade the tissues of the host organism. Molecules produced by pathogens that contribute to the pathogenicity of the organism are called virulent factors.[1] The word "virulence" currently is used to characterize the relative capacity of a microbe to cause disease and has traditionally been used to describe a microbial characteristic[2]. Such organisms have a strong capacity to contribute towards causing diseases. White focusing on the infectious aspects of dental diseases. Herpesvirus species comprise the most prevalent viral family in human saliva and are important periodontopathic agents. Odontopathic bacteria are found in the saliva before colonization of dental surfaces[3]. These can also mix with our blood stream and lymphatic system, being virulent they are very harmful.

Streptococcus mutans is a Gram-positive bacterium that is the agent in the formation of dental cavities. Conditions in the oral cavity are diverse and complex, frequently changing from one extreme to another. Thus, to survive in the oral cavity, S. mutans must tolerate rapidly harsh environmental fluctuations and exposure to various antimicrobial agents to survive[4]. Streptococcus mutans is identified by a chalky white spot on the surface of the tooth indicating an area of demineralization of enamel, which is commonly referred to as a carious lesion. This lesion further leads to tooth decay.

Dental caries is closely associated with the virulence of Streptococcus mutans. Streptococcus mutans, the major etiological agent in dental caries, colonizes the multispecies microbial biofilms that adhere to tooth surfaces. Sophisticated environmental adaptation is critical for the survival and prevalence of S. mutans in the oral cavity, which is a dynamic habitat subject to a wide range of harsh and rapidly changing physiological conditions, including extreme acidity, fluctuation of nutrients, osmotic stress, shifts in temperature and reactive oxygen species, etc. S. mutans has been shown to produce 3 types of GTFs (glucosyltransferases) (GTFB, GTFC, and GTFD), whose cooperative action is essential for cellular adhesion. Environmental conditions encountered by S. mutans in dental biofilms are highly variable, including frequent shifts in pH from above 7.0 to as low as 3.0 during the ingestion of dietary carbohydrates by the host. Thus, pH exerts significant ecological pressure on S. mutans, and its ability to tolerate and grow in low pH environments is crucial for its survival and Pathogenicity. Streptococcus mutans possesses three virulence factors: water insoluble glycans, acid tolerance, and production of lactic acid[5]. The gftB gene is the one which was found to be responsible for virulence of the pathogen by this study.

## MATERIALS AND METHODS

### Subculturing of S. Mutants

Saliva samples were taken from caries patients followed by the subculturing of S Mutans from the sample in to nutrient agar plate. S Mutant colonies where identified and it was isolated from the plate. A broth was prepared so that a single culture was obtained and the virulence of S Mutans can be studied followed by the isolation of DNA.

### Isolation of Genomic DNA

Genomic DNA was isolated from the b oath of S. mutants by cell lysis method. Phenol: chloroform is used to precipitate and separate proteins from DNA. 1.5 ml of bacterial culture was transferred to a micro centrifuge tube and spun at 10000 rpm for 2 mins at 4° C. The supernatant was decanted and tube was drained well onto a tissue paper. The pellet was re-suspended in 467µl of TE buffer by repeated pipetting. 30µl of 10% SDS and 3µl of 20mg/ml of Proteinase K was added to the sample and incubated for 1 hr at 37° C. Equal volumes of Phenol:

Chloroform (24:1) was added and mixed gently by inverting the tubes until the phase was completely mixed. The tubes were spun at 12,000 rpm for 10 mins at 4° C. The upper aqueous layer was transferred to a new tube and an equal volume of Chloroform was added. The samples were mixed by gently inverting the tubes and spun at 12,000 rpm for 10 mins at 4°? The upper aqueous phase was transferred to a new tube and 1/10$^{th}$ volume of 3M sodium acetate was added. Double the volume of 95% ice cold ethanol was added and mixed by inversion until the DNA was precipitated. The tube was spun for 10 min at 12,000 rpm at 4° C and the supernatant was discarded. The pellet was washed with 0.2 ml of 70% ethanol and tube was spun as before. 70% ethanol was discarded and the pellet was air dried. The DNA was then suspended in TE buffer and run on 0.8% Agarose gel.

**Analysis of DNA Using Agarose Gel Electrophoresis**

For the majority of DNA samples, electrophoretic separation is carried out in agarose gels. Separation on agarose gels is achieved because of resistance to their movement caused by the gel matrix. Thus the largest molecules will have difficulty moving, whereas the smallest molecules will be relatively unhindered. Consequently the mobility of DNA molecules during gel electrophoresis will depend on size.

Agarose was weighed and transferred to a conical flask. 50 ml of 1X TAE was added and Agarose was melted to a clear solution by heating. It was allowed to cool until it reached bearable temperature. 2.5µl of ethidium bromide stock solution was added. A gel casting tray was placed in a table and the melted agarose was poured. After the gel solidified, the comb was taken out carefully. The casted gel was placed in an electrophoresis tank and 1X TAE buffer was added until the gel was completely submerged. DNA sample was mixed with the gel loading buffer and loaded into the well. The samples were then electrophoresed at 50V until the gel loading buffer reached 2/3$^{rd}$ of the gel. This gel was then viewed under UV Trans-illuminator.

**Qualitative and Quantitative Determination of DNA by Spectrophotomeric Method**

Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength[7]. A solution of nucleic acids strongly absorbs UV with an absorbance maximum of 260nm and proteins at 280nm which is linearly related with the concentration of DNA and RNA and the amount of contamination in the solution. The intense absorption is primarily due to the presence of aromatic rings in the purine, pyrimidine. The concentration of nucleic acid in a solution can be calculated with the value of $A_{260}$ of the sample. Proteins are usually the major contaminants in nucleic acids extract and these have absorption maximum at 280nm. The ratio of absorbance at 260 and 280nm hence provides a clear idea about the extent of contamination in the preparation. A ratio between 1.7 and 1.9 is indicative of fairly pure DNA preparation. But values less than 1.8 signify the presence of proteins as impurities. The values greater than 1.8 signify the presence of organic solvent in the DNA preparations. A ratio of 1.8 determines the pure DNA preparation.

The concentration of DNA in the sample was calculated using the given formula:

**Concentration of dsDNA**

$A_{260}$ * 50µg * dilution factor

**Purity of the DNA**

$A_{260:}$ $A_{280}$ratio = A260/ A280

= 1.8: pure DNA

= 1.7 – 1.9; fairly pure DNA (acceptable ratio for PCR)

= less than 1.8; presence of proteins.

= greater than 1.8; presence of organic solvent

**Polymerase Chain Reaction (PCR)**

PCR is an *in vitro* method of enzymatic synthesis of specific DNA sequence developed by Karymuller in 1983[8]. It is a very simple and inexpensive technology for characterizing, analyzing, synthesizing, a specific DNA or RNA from virtually, any living organism, plant, animal, virus or bacteria. It exploits the natural function of polymerase present in all living things to copy genetic material or to perform molecular photocopy. PCR consists of three steps:

**Denaturation**

During this step, the two strands melts, open to form single stranded DNA and all enzymatic reaction stoops. This is generally carried out at 92 to 96C.

**Annealing**

Annealing of primer to each original strand for new strand synthesis is carried out between 40–60C.

**Extension**

The polymerase adds dNTPs complementary to the template strand at the 3'end of the primers. Since both the stands are copied in the PCR there is an exponential increase in the number of copies of the required gene.[9]

These 3 steps are repeated for about 20 to 30 times in an automated thermal cycler, which heat and colds the reaction mixture in the tube in a very short time.

This result in exponential increase accumulation of the specific DNA fragments.

100ng of DNA is used for molecular identification of respective sample.

The PCR reaction is performed for 20µl. PCR reaction was performed for gene. The PCR tubes were placed in thermo-cycler and the reaction was carried inside the thermo-cycle.[9]

**Primers**

gftB –F: AGCCATGCGCAATCAACAGGTT, gftB - R: CGCAACGCGAACATCTTGATCAG

**Reaction Set Up for gftB Gene Amplification**

| Components | Stock Concentration | Final Concentration | Volume for 20 µl Setup |
|---|---|---|---|
| Milli Q water | | | 10.8µl |
| dNTP mix | 2mM | 0.2mM | 2 µl |
| Taq buffer | 10X | 1X | 2 µl |
| Forward Primer | 3µm | 0.3µm | 2 µl |
| Reverse Primer | 3µM | 0.3µM | 2 µl |
| DNA Template | 100ng/µl | 100ng | 1 µl |
| Taq DNA polymerase | 5U/µl | 1U | 0.2 µl |

**DNA Quantification by Spectrophotometric Method**

| Sample | OD at 260nm | OD at 280nm | Concentration (ng/µl) | Purity |
|---|---|---|---|---|
| Blank | 0.000 | 0.000 | -- | -- |
| 1 | 0.328 | 0.189 | 16400 | 1.73 |

**PCR Reaction Conditions for gftB**

Initial denaturation : 94°C – 3min

Denaturation : 94°C – 1min

Annealing : 60°C – 1min

Extension : 72°C – 1min 20sec

Final extension : 72°C – 7min

Hold : 4°C

Total number of cycles 32

**Concentration of DNA**

$A_{260}$ X 50µg/ml X dilution factor

Dilution Factor = 3ml/3µl = 1000

The concentration of DNA was found to be 16,400ng/µL It was found to be 1.73 percent pure.

## RESULTS AND DISCUSSION

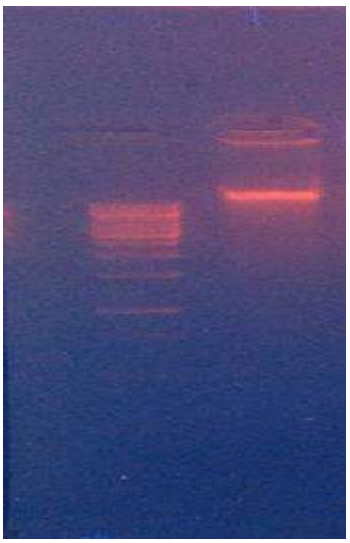
Isolation of Genomic DNA.

DNA was isolated by cell lysis method

**Polymerase Chain Reaction**

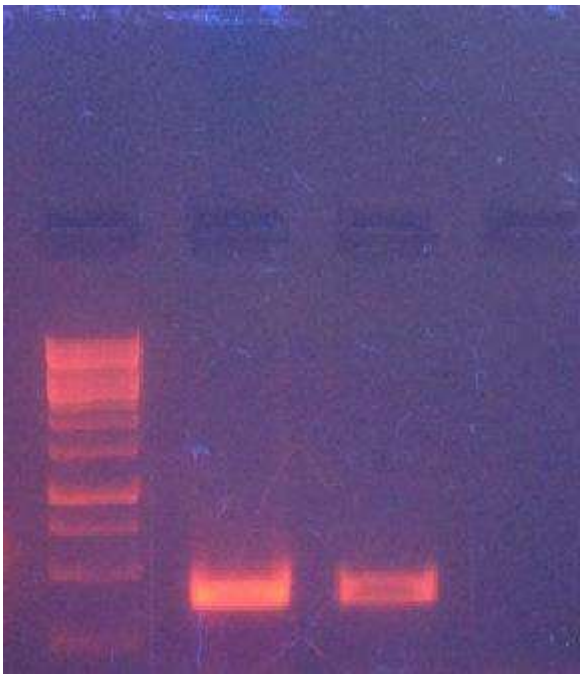
Amplification of gftB Gene (60°C)

Many people have S Mutans in their oral cavity but are found to be resistant to caries. This is because the only factor responsible is virulence, thus the S Mutans if not virulent doesn't cause any caries.[12] But if virulence is observed the patient is diagnosed with caries. Thus the gene responsible for virulence being gftB gene causes extensive attack. Virulent factors determine whether infection occurs and how severe the resulting disease symptoms are. For causing a diseases the bacteria first binds to the host cell surface. Adhesion occurs in short. Colonization of virulent bacteria occurs and finally ends in invasion. The bacterias present actually release a variety of enzymes which cause damage to the host tissues.



Lane 1: 1Kb Ladder

Lane 2: Genomic DNA



Lane 1: 1Kb Ladder

Lane 2: PCR Amplicon gftB

Size: ~420bp

The result shows that gftB gene of S Mutans was amplified.

## CONCLUSION

Thus the virulence of pathogens where studied by taking saliva samples from caries patients and the genes responsible for their virulence where found. The results concluded that gftB genes where the ones responsible for the virulence of the organisms. This study concluded that Techniques of Molecular biology can be applied in the field of dentistry and can be used to prevent caries.

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