Resist Gene Screening of Pathogens from Saliva Sample of Caries Patients

Sandhya*, Gayathri R, Vishnupriya V
Saveetha Dental College, 162, P.H. Road, Chennai, Tamilnadu, India.
*Corresponding author’s E-mail: sandhyasan37@gmail.com

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ABSTRACT
The aim of the study is to evaluate the resistance gene of pathogens from saliva samples of dental caries patient. Resistance genes are genes that convey resistance by producing R proteins. Resistance gene of pathogens present in saliva samples collected from the dental caries patient is screened using the technical of molecular biology. The presence of antibiotic resistance gene in the saliva of caries patient might render the infection resistant to common antibiotics. Antibiotic resistance is a major concern of overuse of antibiotics. The aim of this project is to identify selected antibiotic resistance genes in dental caries patient. This research helps us to find pathogens with resistant gene which may ensure the better oral health of the patient. The resistance gene screening in salivary samples of dental caries patients was done.

Keywords: Streptococcus mutans, GTFB, OXA and TEM genes, caries, antibiotic resistance, nucleotide binding domain.

INTRODUCTION
Resistance- Resistance genes (R-genes) are genes that convey the disease resistance against pathogens by producing R proteins. The structures of most are consistent with a role in pathogen recognition and defence response signalling. Resistance genes are very abundant in plant genomes and most belong to tightly linked gene families. The main class of R-genes consist of a nucleotide binding domain (NB) and a leucine rich repeat (LRR) domain(s) and are often referred to as (NB-LRR) R-genes. Once the R protein has detected the presence of a pathogen, it can create a defence against the pathogen. Because the R genes confer resistance against specific pathogens.

The pathogenic species, streptococcus mutans is closely associated with the dental caries. The streptococcus mutans is a Gram-positive bacterium and the Conditions in the oral cavity are diverse and complex, frequently changing from one extreme to another. 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.

Dental caries are caused by microbiological infection which is the most common chronic infectious disease that is prevalent among most of the humans. There are many oral bacteria that is responsible for the caries such are streptococcus mutans, S. salivarius, candida spp, prevotella spp. On the surface of the tooth the streptococcus mutans proliferates as a biofilm from where it gains nutrients and dietary carbohydrate. Streptococcus mutans produce three types of GTFs, Glucosyl transferases (GTFB, GTFC, GTFD) whose coordinated action is essential for cellular adhesion. The S. mutans frequently shift its pH from above 7.0 to as low as 3.0 during the ingestion of carbohydrates by the host. Hence, pH exerts a significant ecological pressure on S. mutans.

Approximately seven hundred microbial species in the biofilm of the oral cavity are nourished with nutrients and provided with a conductive habitat. In addition, the dental plaque provides a cocoon for them, while the dextran they secrete allow them to bind to tooth surfaces. In species like strep. Mitis and strep. sanguis combined there is a good correlation between in vivo binding properties and cariogenicity.

The type and distribution of oral microorganism in the biofilm vary with dietary and cultural habits and the health of individuals. A good understanding of the types and characteristics of the microbes of the oral cavity would be useful in managing infections caused by them. Knowledge of the susceptibility or resistance of these species to specific antibiotics will enhance therapeutic efficacy.

MATERIALS AND METHODS
Subculturing of Microorganism
Reagents and chemicals required was procured from sigma and Himedia.

Subculturing of Microorganism, Saliva of caries patients was collected after getting consent from them. The saliva was streaked on nutrient agar plate and incubated. The streptococcus mutans which is present in the saliva samples of dental caries patient was identified and isolated.

A single colony of the organism was isolated and subcultured in a nutritive broth with constant stirring.
The growth of the organism was monitored and the broth was utilised for the further experiments.

Isolation of Genomic DNA

Genomic DNA of S. mutans was isolated. 1.5ml of bacterial culture was transferred to a micro centrifuge tube and spun at 10000 rpm for 2 minutes 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 hour 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 minutes 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 minutes at 4°C. The upper aqueous phase was transferred to a new tube and 1/10th 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.

Agarose Gel Electrophoresis

The casted agarose 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 2/3rd of the gel. This gel was then viewed under UV Trans-illuminator.

Qualitative and Quantitative Determination of DNA by Spectrophotometric Method

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 spectrophotometer and the UV lamp was switched on. The wavelength was set at 260nm and 280nm. The instrument is set at zero absorbance with T.E buffer or sterile water as blank.5 or 7μl of the sample is taken in a quartz cuvette and made up to 3ml with TE buffer or sterile water. Absorbance of the solution with the sample was read. The concentration of DNA in the sample was calculated using the given formula:

\[ \text{Concentration of ds DNA} = \frac{A260 \times 50\mu g \times \text{dilution factor}}{} \]

Purity of the DNA:

\[ A260: A280 \text{ratio} = A260/A280 \]

= 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

Identification of Resistance Gene by Polymerase Chain Reaction (PCR)

PCR is an in-vitro method of enzymatic synthesis of specific DNA sequence developed by Karymuller in 1988. It is a very simple and inexpensive technology for characterising, analysing, synthesising, 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. 100ng of DNA is used for molecular identification of respective gene. PCR reaction was performed for TEM and OXA gene. The PCR tubes were placed in thermo-cycler and the reaction was carried out inside the thermo-cycler.

Reaction Setup for TEM and OXA Gene Amplification

<table>
<thead>
<tr>
<th>Components</th>
<th>Stock Concentration</th>
<th>Final Concentration</th>
<th>Volume for 20 μl Setup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli Q water</td>
<td></td>
<td></td>
<td>10.8</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2 mM</td>
<td>0.2 mM</td>
<td>2 μl</td>
</tr>
<tr>
<td>Taq buffer</td>
<td>10X</td>
<td>1X</td>
<td>2 μl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>3 μm</td>
<td>0.3 μm</td>
<td>2 μl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>3 μM</td>
<td>0.3 μM</td>
<td>2 μl</td>
</tr>
<tr>
<td>DNA Template</td>
<td>100 ng/ μl</td>
<td>100 ng</td>
<td>1 μl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5U/ μl</td>
<td>1U</td>
<td>0.2 μl</td>
</tr>
</tbody>
</table>

PCR Reaction Conditions for TEM

Initial denaturation : 94°C – 3min

Denaturation : 94°C – 1min

Annealing : 50°C – 1min

Extension : 72°C – 1min 20sec

Final extension : 72°C – 7min

Hold : 4°C

Total number of cycles 32

PCR Reaction Conditions for OXA

Initial denaturation : 94°C – 3min

Denaturation : 94°C – 1min

Annealing : 50°C – 1min

Extension : 72°C – 1min 20sec
Final extension : 72°C – 7min
Hold : 4°C
Total number of cycles 32

RESULTS
Isolation of Genomic DNA

Figure 1
Lane 1: Genomic DNA.
Lane 2: 1Kb Ladder.

The gel profile shows the presence of genomic DNA isolated from S.mutans. (Fig: 1).

DNA Quantification by Spectrophotometric Method

<table>
<thead>
<tr>
<th>Sample</th>
<th>OD at 260 nm</th>
<th>OD at 280nm</th>
<th>Concentration (ng/µl)</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.000</td>
<td>0.000</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1</td>
<td>0.335</td>
<td>0.175</td>
<td>16750</td>
<td>1.91</td>
</tr>
</tbody>
</table>

Concentration of DNA
A260 X 50µg/ml X dilution factor
Dilution Factor = 3ml/3µl = 1000

The concentration of DNA was found to be 16750(ng/µl). Isolated DNA was found to be 1.91 percent pure.

DISCUSSION
TEM and OXA gene is responsible for the antibiotic resistance. In this study genomic DNA of S.mutans was isolated from dental caries patients. Using the technique of PCR the presence of TEM and OXA gene was identified. The presence of TEM and OXA gene suggests that the organism isolated is resistant to antibiotics. This study shows the presence of resistance genes to antimicrobial agents in saliva of dental caries patient. Resistant microbe are increasingly difficult to treat, which requires alternative medication or higher dose of antibiotic, which may be more toxic, a few infection are now completely untreatable due to resistance. Therefore in future research should be focused to improve the development of new antibiotics, other therapeutic and vaccines should betaken to slow the emergence of resistant bacteria and prevent the spread of resistant infection.

Resistance Gene Screening of Polymerase Chain Reaction

Amplification of TEM and OXA Gene (50°C)

Figure 2
Lane 1: PCR Amplicon TEM
Lane 2: PCR Amplicon OXA
Lane 4: 1Kb Ladder

The result shows that TEM and OXA gene is amplified. (Fig: 2)

REFERENCES
3. Biswas S; Biswas. I (2911). “Role of VltAB, an ABC transporter complex, in viologen tolerance in streptococcus mutans”. Antimicrobial agents and


