Research Article

Resistance Gene Screening of Pathogens from Saliva Sample of Caries Patients

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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 gene 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, GFTB, 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[1]. 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[2].

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[3].

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*[4]. On the surface of the tooth the streptococcus mutans proliferates as a biofilm from where it gains nutrients and dietary carbohydrate[5]. 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[4].

Approximately seven hundred microbial species in the bioflim of the oral cavity are nourished with nutrients and provided with a conductive habitat. In addition, the dental plague 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[6].

The type and distribution of oral microorganism in the bioflim 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[6].

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.mutants 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 electrophoreses 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:

Concentration of ds DNA= A260 * 50µg * dilution factor

Purity of the DNA:

A260: A280ratio = 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

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

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

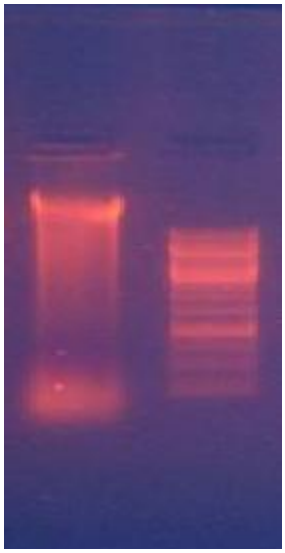

Figure 1

Lane 1: Genomic DNA.

Lane 2: 1Kb Ladder.

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

DNA Quantification by Spectrophotometric Method

Sample	OD at 260 nm	OD at 280nm	Concentration (ng/µl)	Purity
Blank	0.000	0.000	---	---
1	0.335	0.175	16750	1.91

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.mutants 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)

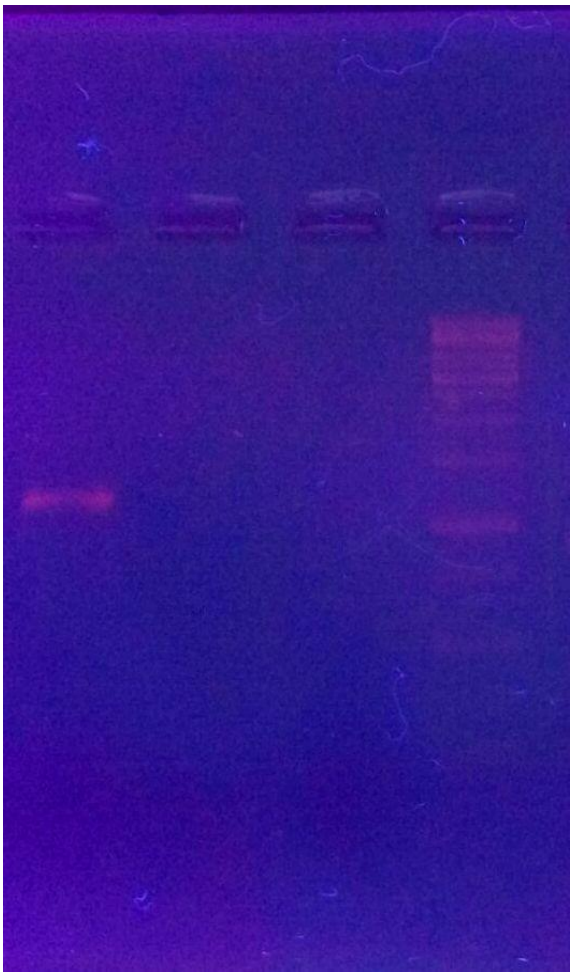

Figure 2

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

1. Knepper, Caleb; Day, Brad. "From Perception to Activation: The Molecular-Genetic and Biochemical Landscape of Disease Resistance Signalling in Plants". The Arabidopsis Book: 1–17, March (2004).
2. Hulbert SH1, Webb CA, Smith SM, Sun Q. Annu Rev Phytopathol. Resistance gene complexes: evolution and utilization. 39, 2001, 285-312.[PMD]
3. Biswas S; Biswas. I (2911). "Role of VItAB, an ABC transporter complex, in viologen tolerance in streptococcus mutans". Antimicrobial agents and

chemotherapy. 55(4), 2011 Apr, 1460-9. doi: 10.1128/AAC.01094-10. Epub. [PMD]

4. Maripandi A, Arun Kumar T, and Ali A. AI Salamah. Prevalence of dental caries bacterial and evaluation of inhibitory concentration effect of different tooth pastes against streptococcus spp. A African journal of microbiology research Vol. 5(14), 18 July, 2011, 1778-1783, DOI: 10.5897/AJMR11.40 ISSN 1996-0808.
5. Smith EG, Spatafora GA J Dent Res. Gene regulation in S. mutans: complex control in a complex environment. 91(2), 2012 Feb, 133-41.[PMD]
6. Philip K, Teoh W. Y, Muniandy S and Yaakob H. Pathogenic Bacteria Predominate in the Oral Cavity of Malaysian Subjects. Journal of Biological Sciences, 9, 2009, 438-444.
7. Twetman L, Twetman S. Comparison of two chair-side tests for enumeration of Mutans Streptococci in saliva. Oral Health Dent Manag. 13(3), 2014 Sep, 580-3. [PMD]
8. Darshana Bennadi, Veeara Reddy, Nandita Kshetrimayum. Influence of genetic factor on dental caries. J Int Soc Prev Community Dent. 4(3), 2014 Sep-Dec, 154–158. [PMD]
9. Hong X, Hu DY. Salivary Streptococcus mutans level: Value in caries prediction for 11-12-year-old children. Community Dent Health. 27, 2010, 248–52. [PMD]
10. Pradopo S. The colony number of Streptococcus mutans and Lactobacillus in saliva of dental caries and free caries children. Dent J. 41, 2008, 53–5. [PMD]
11. Pannu P, Gambhir R, Sujlana A. Correlation between the salivary Streptococcus mutans levels and dental caries experience in adult population of Chandigarh, India. Eur J Dent. 7, 2013, 191–5. [PMC free article] [PMD]
12. Farsi N. Dental caries in relation to salivary factors in Saudi population groups. J Contemp Dent Pract. 9, 2008, 16–23. [PMD]
13. J Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE. Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol. 46, 2008, 1407–17. [PMC free article] [PMD]
14. Hegde PP, Ashok Kumar BR, Ankola VA. Dental caries experience and salivary levels of Streptococcus mutans and Lactobacilli in 13-15 years old children of Belgaum city, Karnataka. J Indian Soc Pedod Prev Dent. 23, 2005, 23–6. [PubMed].
15. Ogaya Y, Nomura R, Watanabe Y, Nakano K. Detection of Helicobacter pylori DNA in inflamed dental pulp specimens from Japanese children and adolescents. Med Microbiol. 64(Pt 1), 2015 Jan, 117-23. [PMD]
16. Yergeau E, Masson L, Elias M, Xiang S, Madey E, Huang H, Brooks B, Beaudette LA. Comparison of Methods to Identify Pathogens and Associated Virulence Functional Genesin Biosolids from Two Different Wastewater Treatment Facilities in Canada. 11(4), 2016 Apr 18, e0153554. [PMD]
17. Lee PY, Costumbrado J, Hsu CY, Kim YH. Agarose gel electrophoresis for the separation of DNA fragments. J Vis Exp. (62), 2012 Apr 20, pii: 3923. [PMD]
18. Holmes DL, Stellwagen NC. Electrophoresis of DNA in oriented agarose gels. J Biomol Struct Dyn. 7(2), 1989 Oct, 311-27. [PMD]
19. Moore D, Chory J, Ribaudo RK. Isolation and purification of large DNA restriction fragments from agarose gels. Curr Protoc Immunol. 2001 May, Chapter 10, Unit 10.5.[PMD]
20. Trinh KT, Zhang H, Kang DJ, Kahng SH, Tall BD, Lee NY..Fabrication of Polymerase Chain Reaction Plastic Lab-on-a-Chip Device for Rapid Molecular Diagnoses. Int Neurourol J. Epub, 20(Suppl 1), 2016 May,26, S38-48. [PMD]
21. Levin AE, Williamson PC, Bloch EM, Clifford J, Cyrus S, Shaz BH, Kessler D, Gorlin J, Erwin JL, Krueger NX, Williams GV, Penezina O, Telford SR, Branda JA, Krause PJ, Wormser GP, Schotthoefer AM, Fritsche TR, Busch MP.Serologic screening of United States blood donors for Babesia microti using an investigational enzyme immunoassay. Transfusion. 2016 May 25. [Epub ahead of print] [PMD]
22. Carvalho YB, Da Silva Santos JJ, Raibenberg FC, Poersch LH, Romano LA. Use of Polymerase Chain Reaction for Bivalve Pathogen Surveillance in the Yellow Clam Mesodesma mactroides. J Aquat Anim Health. 28(2), 2016 Jun, 114-7. [PMD]
23. Trumbo TA, Schultz E, Borland MG, Pugh ME. Applied spectrophotometry: analysis of a biochemical mixture. Biochem Mol Biol Educ. 41(4), 2013 Jul-Aug, 242-50. [PMD]
24. Wimardhani YS, Sasanti H, Wardhany II, Sarsito AS, Pradono SA, Subita GP, Soegyanto AI, Rahmayanti F, Chamusri N, Iamaroon A. Saliva-Based Screening of High-Risk Human Papillomavirus Strains: Detection in Female Indonesian and Thai Dental Students. Asian Pac J Cancer Prev. 16(13), 2015, 5525-9. [PMD]
25. Flake C, Arafa J, Hall A, Ence E, Howard K, Kingsley K. Screening and detection of human papillomavirus (HPV) high-risk strains HPV16 and HPV18 in saliva samples from subjects under 18 years old in Nevada: a pilot study. BMC Oral Health. 12, 2012 Oct 22, 43. [PMD]
26. Walsh DJ, Corey AC, Cotton RW, Forman L, Herrin GL Jr, Word CJ, Garner DD. Isolation of deoxyribonucleic acid (DNA) from saliva and forensic science samples containing saliva. J Forensic Sci. 37(2), 1992 Mar, 387-95. [PMD]

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