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RESEARCH ARTICLE

GENETIC DIVERSITY OF POLYMORPHIC GINGIVITIS IN DENTAL PLAQUES

***[1]Raghadsharqi Luaibi Alrazn and [2]Senthil Kumar, R.**

[1]Department of Microbiology, Indian Academy Degree College, India
[2]Department of Biotechnology, Indian Academy Degree College, India

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ABSTRACT

Gingivitis is caused by substances derived from microbial plaque accumulating at or near the gingival sulcus; all other suspected local and systemic etiologic factors either enhance plaque accumulation or retention, or enhance the susceptibility of the gingival tissue to microbial attack. *Porphyromonasgingivalis* (formerly Bacteroides gingivalis) is an anaerobic, asaccharolytic, gram-negative coccobacillus that is frequently a component of the flora of subgingival lesions of adult patients with periodontitis. The organism is often present in the oral cavities of periodontally healthy children and adolescents, but, because it occurs in densities below the detectable limit of anaerobic culturing (<1% of the total flora) it is not routinely recovered from such individuals. Hence sensitive and sophisticated methods of detection from the biological samples need to be employed. The genetic diversity of bacteria were analyzed based on RAPD analysis and ARDRA based fingerprinting. Based on the RAPD pattern all the bacteria are belonged to the different species or strains. PCR analysis of the 16S rDNA region also revealed that the strains are from the *Gingavilis* species. We carried out DNA fingerprinting profile using ARDRA, for the identification and classification of *Gingavilis* isolates. Traditional PCR was initially used for the detection of bacteria.

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INTRODUCTION

Gingivitis is a common and mild form of gum disease (periodontal disease) that causes irritation, redness and swelling (inflammation) of gums. Because gingivitis can be mild, you may not be aware that you have the condition. But it's important to take gingivitis seriously and treat it promptly. Gingivitis can lead to much more serious gum disease (periodontitis) and eventual tooth loss (Allaker, 2009). Gingivitis is caused by substances derived from microbial plaque accumulating at or near the gingival sulcus; all other suspected local and systemic etiologic factors either enhance plaque accumulation or retention, or enhance the susceptibility of the gingival tissue to microbial attack (Armitage,1982). If ignored, the bacteria and inflammation causing gingivitis will destroy the periodontal attachment fibers between the gums and teeth, allowing bacteria to invade and destroy the underlying bone. When the attachment fibers have been lost, the patient is diagnosed. Several of the studies on oral bacteria have demonstrated a tremendous richness of species within the microbial community associated with periodontitis, a very common oral disease.

Corresponding author: Raghadsharqi Luaibi Alrazn
Department of Microbiology, Indian Academy Degree College, India

No single species could be identified as the decisive pathogen in periodontitis; instead the disease is induced by the activity of a mixed bacterial biofilm growing under anaerobic conditions. Several studies have shown an association between painful exacerbation of periapical lesions and the presence in the root canal of specific bacteria. Black-pigmented bacteria belonging to the genera Porphyromonas, Prevotella, and Bacteroides have been cultured from root canals in a significant proportion of cases in those studies (Griffee, 1980; Sundqvist 1989, Yoshida, 1987) and are frequently present in the same canals as members of the generaPeptostreptococcus and Fusobacterium (Gomes, 1994, Gomes, 1996, Haapasalo, 1989). However, the findings of different studies based on culturing of canal contents vary significantly.

This may be due, at least in part, to the reduced reliability and sensitivity of culturing techniques. The persistence or further expansion of a periapical lesion, despite seemingly adequate endodontic treatment and timely restoration of the tooth, is usually attributed to the persistence of pathogenic microorganisms in the root canal system. Recent investigations have documented that the presence of cultivable bacteria from canals at the time of obturation was critical in predicting failure of treatment (Sjogren, 1997). However, the microorganisms most commonly associated with failed endodontic cases are

different from those cultured from canals with pulp necrosis. Studies reveal that most of these failed cases have gram-positive strains such as enterococci, streptococci, and eubacteria, with occasional Candida, peptostreptococci, and fusobacteria (Molander, 1998). Although enterococci were the most prevalent microorganisms in the last three studies, being present in 54, 70, and 38% of the cases, respectively, the percentages of different strains identified again vary significantly among the studies, and in a considerable number of cases there were no cultivable microorganisms. Therefore, sensitive and accurate molecular techniques are necessary to accurately characterize the root canal microbial irritants in order to determine their association with clinical symptoms and the prognosis of treatment.

The PCR technique can be sensitive enough to detect a few DNA strands of the microorganisms present if adequate primers are used and the PCR conditions are sufficiently optimized. We have recently shown that, after inoculation of three endodontopathogenic bacteria in mouse pulp exposures, PCR was much more accurate than culturing in detecting the inoculated anaerobic bacteria (Fouad, , 2002). Several uncultivable species have been identified from dentoalveolar abscesses by PCR (Wade, 1997). The present study is mainly focused on to trace out the genetic diversity among the individuals which cause the disease. The samples are isolated from the plaques and cultured on selective medium. The genomic DNA is isolated and further used for the amplification of the POG gene. The samples are amplified for the 16s rDNA and the amplified fragments are used for the ARDRA studies.

MATERIAL AND METHODS

Sample collection: Gingivitis Sample has been collected from Dental plaques from Vaidheihi Dental College Bangalore. And the samples were cultured in Brucella Agar Base media.

Bacterial culture isolation: The cultures on the plate were further subcultured to obtain pure isolated bacterial colonies. The isolated colonies were further used for gram staining.

Isolation of Genomic DNA from Bacteria: Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, N-trimethyl-ammonium bromide (CTAB) method. Briefly 1 ml bacterial culture was centrifuged at 10000rpm 2 min. at 4 °C and the pellet obtained was added with 675µl of extraction buffer (buffer (150mM NaCl, 4 mg/ml SDS, 10mM EDTA, 10mM Tris-HCl, pH7.5) and incubated at 37°C for 30 min. To the contents 75µl of SDS (20%) was added and incubated at 65°C for 2 hours. The contents were mixed thoroughly and centrifuged at 10000rpm for 10min at 4°C. The supernatant obtained was collected in a sterile microcentrifuge tube and added with equal volumes of Chloroform: Isoamyl alcohol (24:1). The tubes were then centrifuged at 10000 rpm for 10 min at 4°C.

The aqueous phase was collected in a fresh tube and added with 0.6volumes of isopropyl alcohol and incubated at room temperature for 1hour. The precipitated DNA was further pelleted and washed with 70% ethanol and stored in TE buffer and stored at −20°C until further use. The quantity of the isolated DNA was checked in UV-VIS spectrophotomer (Vivaspec Biophotometer, Germany).

PCR Amplification: The gene selected for the primer designing was POG gene with an amplicon size of 200bp. The specific primers were designed using primer 3 plus software, and the designed oligonucleotides were synthesized in sigma corporation USA. The PCR mixture consisted of 10 × reaction buffer with MgCl2 (1.5mM), 2µL of dNTP mix (2.5mM), 2µl each of forward and reverse primers (10 picomoles/µl each primer), 0.3µL of Taq DNA polymerase (5U/µL), and 50ng/µL of template DNA in a total volume of 20µL. The PCR was performed with the following cycling profile: Initial denaturation at 94°C for 2 minutes, followed by 30 cycles of 50 seconds denaturation at 94°C, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minutes. The time for the final extension step was increased to 6 minutes. The PCR products amplified were then qualitatively analyzed on 1% agarose gel. The PCR product was recovered using the QIA quick gel extraction kit, and the amplified product was then purified and used for cloning purpose.

Primer details: The POG gene was amplified by PCR using purified genomic DNA as a template. Oligonucleotide primers were synthesized to amplify the intact region of POG gene. The forward primer, 5' GTA AGT CAG CGG TGA AAC CTG 3' and the reverse primer, 5' TCA GTG TCA GTC GCA GTA TGG 3', were purchased from Eurofins, Bangalore. These primers correspond to the gene POG and thus the final PCR product was 200bp (Table 1).

Elution of DNA from gel: Briefly about 300mg Agarose gel fragment was excised with scalpel and was transferred to 1.5ml or 2 ml tube. 650µl of gel solubilizer was added to the gel piece and incubated for 10min at 50°C. The contents were vortexed and added with 50µl Binding Optimizer. 750µl of sample was transferred to Spin Column and centrifuged at 10000g for 1min. The filtrate obtained was discarded and the column was washed with 700µl Wash Buffer. The process was repeated 2-3 times and the DNA was extracted with 30-50µl Elution Buffer and stored at 4°C, until further use.

16S rDNA PCR Amplification: PCR amplification for 16SrDNA gene was done in 25µl of reaction mixture containing PCR buffer, 1X (Kappa,SA); MgCl2, 2.5mM; dNTP mix, 2.5mM; Taq DNA polymerase, 5U; primer, 10pmol (pA-5' AGA GTT TGA TCC TGG CTC AG 3', pH- 5' AAG GAG GTG ATC CAG CCG CA 3') and template DNA 100ng. The total volume of the mixture being 20µl.

Table 1. Primer Details

Oligonucleotide	Sequences (5'- 3')	GC %	Tm Value	Length	Product Size
POG FW	GTA AGT CAG CGG TGA AAC CTG	52	54.4 ^{0}C	21	200 bp
POG RV	TCA GTG TCA GTC GCA GTA TGG	52	54.4 ^{0}C	21	

Amplified Ribosomal DNA Restriction Analysis (ARDRA):

Amplified 16S rDNA obtained was analyzed by restriction digestion with two restriction endonucleases (EcoR1, Taq1). Restriction was carried out in 10µl volume containing 5µl PCR product, 1µl of 10X buffer, 0.1µl of BSA, 0.2µl of each restriction enzyme(2U). All the reaction mixtures were incubated at 37ºC in water bath for 16 hours except Taq I, which was incubated at 65ºC for 3 hours. The digested samples were run on 3% agarose. Interpretation of ARDRA patterns was based on manual visual scoring the positions of the fragments of molecular size ≥100 bp. Data analysis was done by unweighted pair group method using arithmetic averages (UPGMA) analysis of Dice similarity coefficient using the computer program NTSYSpc 2.0.

RESULTS AND DISCUSSION

Isolation of pure culture: Gingivitis Sample has collected from Dental plaques from Vaidheihi Dental College, Bangalore, and cultured in Brucella Agar Base media. The isolated bacteria are found to be Gram negative. Genomic DNA isolation and quantification: The Gram negative bacteria were cultured in the Brain heart infusion broth media and genomic DNA was isolated by modified CTAB method. The isolated DNA was electrophorized in 1% Agarose gel (Fig.1). The quantity and quality of DNA was analyzed by UV visible spectrophotometer (Data not shown).

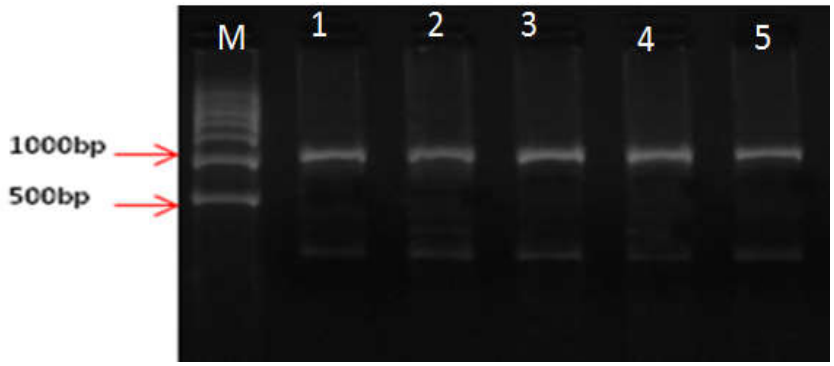

Fig. 1. PCR amplification of partial *Porphyromonas* gene from isolated bacteria. Lane 1-5 DNA samples with the gene amplified. The band length was found to be approximately 1000bp

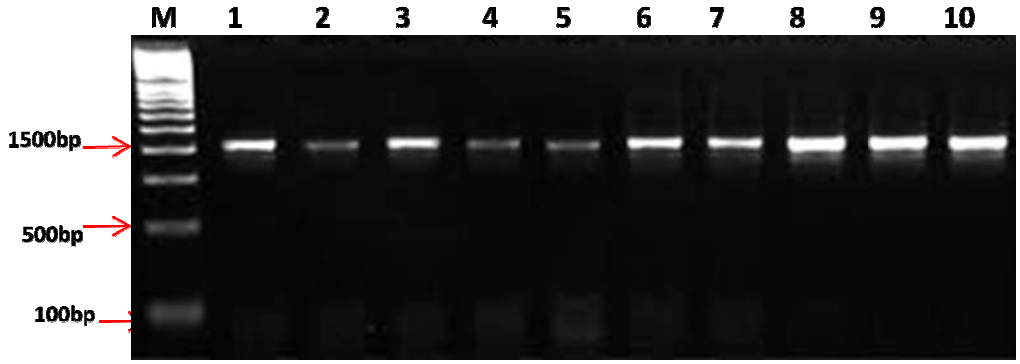

Fig. 2. 16S rDNA PCR amplification of the isolated Periodontal bacteria. The band amplified was found to be approximately 1500bp. Lane 1-10: Amplified samples

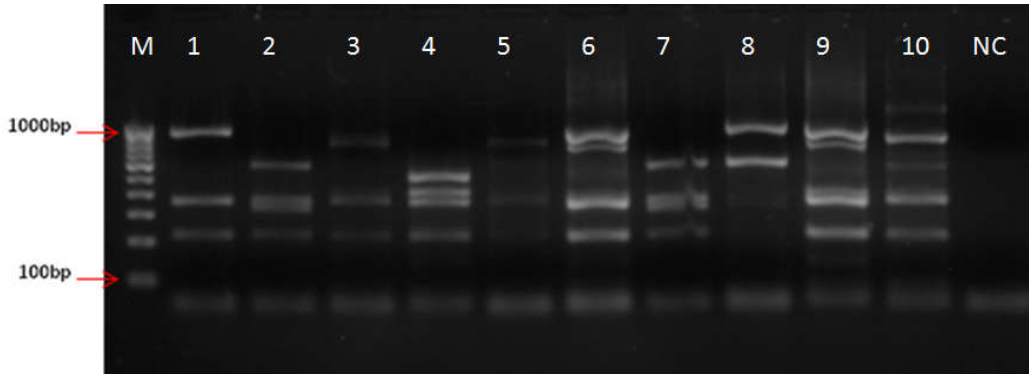

Fig. 3. Restriction patterns obtained after digestion with *Taq*I for amplified 16S rDNA of *bacteria* after running in 2% Agarose gel. Lane M: Molecular Marker. Lane 1-10: amplified samples of the isolated bacterial 16S rDNA

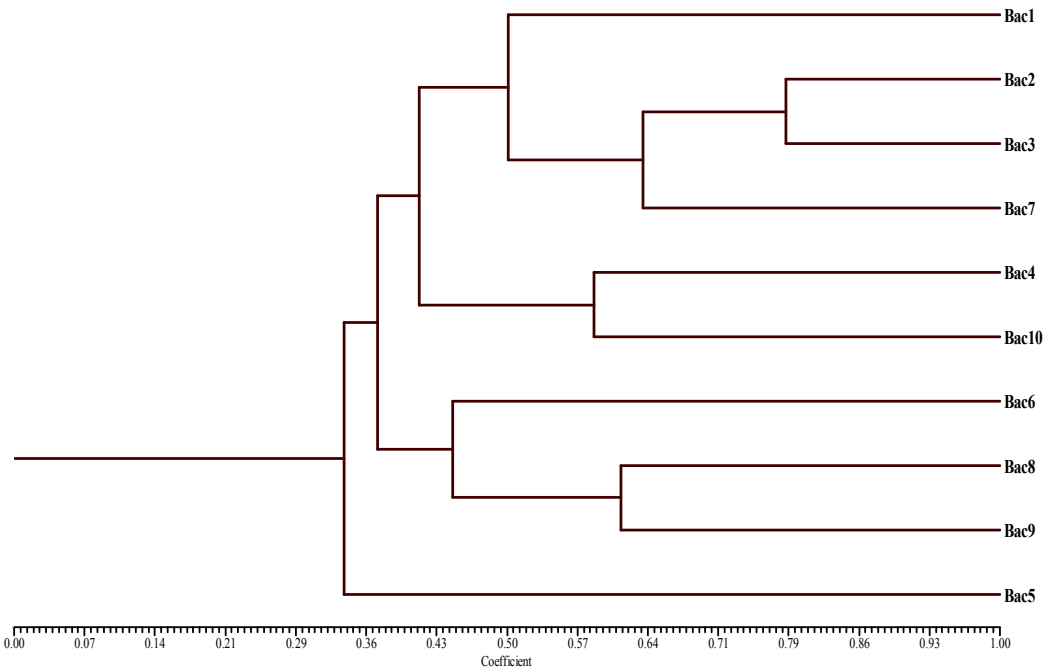

Fig. 4. Unweighted pair group method with arithmetic average cluster analysis using Dice similarity coefficient of the *Taq*I, restriction analysis of 16S rDNA of the isolated bacterial strains. The scale at the bottom represents similarity coefficient

Genetic diversity analysis: Genetic diversity analysis was done using PCR RFLP (Amplified Ribosomal DNA Restriction Analysis) and RAPD method. PCR amplification of POG gene: Porphyromonas specific primers were designed for the using the sequences of gene available in NCBI GenBank using Primer 3 plus Software. The predicted primers were validated initially in silico and subsequently in wet lab. The primers could yield an amplicon of the expected size of ~1000bp. The PCR product was electrophoresed and visualized by 1% Agarose gel. The primers were found to produce ~1000bp amplicon which shown.

16S rDNA PCR: The PCR amplification using the universal primer with the genomic DNA extracted from the sample resulted in a PCR product of approximately 1500bp.

Amplified Ribosomal DNA Restriction Analysis (ARDRA): The PCR product of the 16S rDNA was compared with the fragment pattern by treatment with restriction enzyme (Taq1). ARDRA is a rapid, accurate and reliable technique to assess the microbial diversity. The band pattern obtained indicates the structure of the community present in the environmental system. Our results clearly indicated differences in the microbial community. Significant differences have been observed between the restriction patterns of the isolated periodontal bacteria.

Conclusion

Thus, the application of more reliable and discriminative method was necessary. ARDRA fingerprinting technique was shown to be most suitable method for differentiating indicating high percentage discriminatory power for the method.

In this study, evaluation of genomic ARDRA fingerprinting methods performed by computerized comparison of digitized fingerprinting patterns gives an accurate analysis. Several conventional typing methods, including biotyping, serotyping and antibiogram typing, have been described for P.gingivalis but these methods recognize insufficient heterogeneity between strains to study transmission routes of this suspected periodontal pathogen. New molecular typing methods for bacteria have recently been applied to P. gingivalis. Typing of P. gingivaliis highly sensitive and reveals considerable heterogeneity between strains, but produces large numbers of DNA fragments, resulting in patterns which can be difficult to interpret when large numbers of isolates have to be evaluated.

In contrast, ribotyping of 10 results usually in only four to eight bands. This method has been applied to P. gingivalis isolates from six" and nine" unrelated subjects, all of which were found to be distinct. Random amplification of DNA by PCR with an arbitrary primer (AP-PCR) can also be used for typing bacteria. "Random amplification of genomic DNA, directed by a single oligonucleotide of arbitrary sequence, produces a set of varying short DNA products. The genetic diversity of bacteria were analyzed based on RAPD analysis and ARDRA based fingerprinting. Based on the RAPD pattern all the bacteria are belonged to the different species or strains. PCR analysis of the 16S rDNA region also revealed that the strains are from the Gingavilis species. We carried out DNA fingerprinting profile using RAPD markers and ARDRA, for the identification and classification of Gingavilis isolates. Traditional PCR was initially used for the detection of bacteria. Moreover, besides the inaccurate enumeration, the enrichment step for samples' bacteria did not yield better sensitivity for the detection of bacteria.

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