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Research Article

Characterization and Antimicrobial Sensitivity of Periodontopathic Bacteria in Chronic Periodontitis

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Abstract

Objective(s): To evaluate the periodontopathic bacteria through anaerobic culture and 16S rRNA sequencing for its identification and to validate any underlying sub-species present, and to determine anti-bacterial susceptibility in chronic periodontitis.

Method(s): Subgingival plaque was collected from chronic periodontitis patients (n=10) for anaerobic culture and species characterization using conventional analysis. Microbial samples were subjected to 16S rRNA sequencing followed by basic local alignment search tool for species identification. Antibiotic sensitivity tests were performed using disk diffusion method.

Result(s): Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella spp and Peptostreptococcus micros were identified by culture. These microorganisms after validation by molecular sequencing, revealed two species of genus Prevotella namely, P.Intermedia and P.Nigrescens. Antibiotic sensitivity tests showed significant susceptibility to metronidazole, amoxicillin/clavulanic acid, tetracycline and highest resistance to clindamycin.

Conclusion(s): 16S rRNA sequencing may aid in identification of phenotypically similar but molecularly variable bacterial species thereby providing insights on their role in pathogenesis and treatment outcome of chronic periodontitis.

Keywords: Periodontopathic Bacteria; Anaerobic Culture; 16S rRNA Sequencing

Abbreviations

16S rRNA: 16S Ribosomal RNA; BLAST: Bio-Informatics Tools Basic Local Alignment Search Tool; FASTA: Fast-All; P. Gingivalis: Porphyromonas Gingivalis; F. Nucleatum: Fusobacterium Nucleatum; P. Micros: Peptostreptococcus Micros; P. Intermedia: Prevotella *Intermedia*; P. Nigrescens: Prevotella *Nigrescens*

Introduction

Periodontitis is defined as an inflammatory disease of supporting tissues of teeth caused by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with periodontal pocket formation, gingival recession or both [1]. There are approximately 800 species of bacteria identified in the oral cavity [2]. Complex interaction of pathogenic bacterial infection and host response, modified by behavioural factors such as smoking, can result in periodontal disease [3]. Compared with developed countries, developing nations have higher prevalence of calculus and bleeding on probing among adolescents. 14-47% of adult populations in developed countries had calculus deposits compared with 36-63% of adults in developing nations [2]. However, developed countries have higher percentage of individuals with periodontal pockets of 4-5 mm. Greater proportions of older individuals (65-74 years) exhibit periodontal pockets of 6 mm or above compared with adult

populations in both developed and developing countries [4]. The number of bacterial species that can inhabit periodontal lesions has been estimated 4500 of which approximately 50% can be cultured [5]. The number of cultivable species that has been associated with disease progression is limited to less than 10 species and includes Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia and Fusobacterium spp. Conventional anaerobic culture technique has been used to detect and quantify marker bacteria for disease progression bacteria followed by suitable antimicrobial drug [6]. Despite of availability of knowledge of these microorganisms and antimicrobial drug, several patients are showing Refractory periodontitis, most probably due to an unusual pathogenic and virulent bacterium which frequently remains unnoticed, being the reason for increasing tendency for resistance rates worldwide [7]. Hence, DNA based techniques for detection of human microbial pathogens in periodontal microbiology have shown good sensitivity and specificity for detection of periodontal pathogens especially their sub-species [8]. So, accurate identification of periodontopathic bacteria, would prove to provide valuable assistance in effectively eliminating all periodontal pathogens [4]. The present study aims to detect various pathogenic periodontopathic bacteria and assess their antimicrobial susceptibility, which would prove to provide valuable assistance in effectively eliminating all periodontal pathogens. Further aid the clinician towards a specific antimicrobial drug therapy either through local delivery system or systemic administration [9-20].

Materials and Methods

Study design

The study had been intended to be a cross-sectional observational study following institutional review board approval (IRB No: 156/IRB/*****22) and patients were selected from our institute's Department of Periodontology for the subsequent study.

Patient sample

The study population included 10 patients with chronic periodontitis having periodontal pocket ≥ 5mm, to meet this criterion every patient was subjected to complete periodontal and radiographic examination, including probing depth, clinical attachment loss and bleeding on probing. According to inclusion criteria, none of the periodontal patients had received antibiotics for three months prior to sample collection. Informed and written consent of all subjects was taken for the study.

Samples were collected with complete aseptic precautions from the deepest site. Initially, the site of sample collection was, carefully cleaned with sterile gauze, and isolated with cotton rolls to prevent saliva contamination. For single sites, two sterile paper points were inserted to the bottom of the pocket for a period of 15s. Immediately, the paper points were immediately transferred to anaerobic medium. The samples were processed on the same day and were plated on Neomycin blood agar plates. The plates were incubated in Anaerobic jar at 37ºC under anaerobic conditions. After 14 days of anaerobic incubation, suspected colonies were further identified by microscopy, gram-staining biochemical and enzyme activity included catalase, oxidase, indole hydrolysis, esculin hydrolysis, gelatin hydrolysis, urea hydrolysis and fermentation of sugars. DNA extraction was carried out by QIAMP DNA(Qiagen) extraction kit as per manufacturers guidelines. The quality of DNA was evaluated by taking absorbance ratios at 260nm and 280nm using spectrophotometry. The qualified samples were sent for bacterial 16S ribosomal RNA sequencing and the results of the sequencing were analyzed using bio-informatics tools Basic Local Alignment Search Tool (BLASTn).

Organisms identified were evaluated using anti-bacterial susceptibility test. Evaluation was done using disk diffusion method. The following antimicrobials obtained commercially were evaluated: ciprofloxacin 5 µg (CIP), azithromycin 15 µg (AZM), clindamycin 2 µg (CD), tetracycline 30 µg (TE), amoxicillin 10 µg (AML), amoxicillin + clavulanic acid 30 µg (20/10) (AUG), metronidazole 5 µg (MTZ). Susceptibility breakpoints were interpreted according to Clinical and Laboratory Standards Institute (CLSI) breakpoint criteria.

Results Periodontopathic organisms isolated after anaerobic culture were Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella spp and Peptostreptococcus micros (Table 1, Figure 1). These organisms isolated using anerobic culture were subjected for 16S rRNA sequencing for validation and identify any underlying sub-species (Table 2). Confirmation on the molecular platform, revealed two species of genus Prevotella namely, Prevotella Intermedia and Prevotella Nigrescens (Figure 2). Antibiotic sensitivity test showed significant susceptibility to Amoxicillin, metronidazole, amoxicillin/clavulanic acid, tetracycline and highest resistance to clindamycin (Table 3).

Sr. No.	Species isolated	Out of 10 samples in %
1	Fusobacterium nucleatum	70%
2	Porphyromonas gingivalis	60%
3	Prevotella spp.	30%
4	Peptostreptococcus micros	10%

Table 1: Shows organisms isolated after anaerobic culture out of 10 samples.

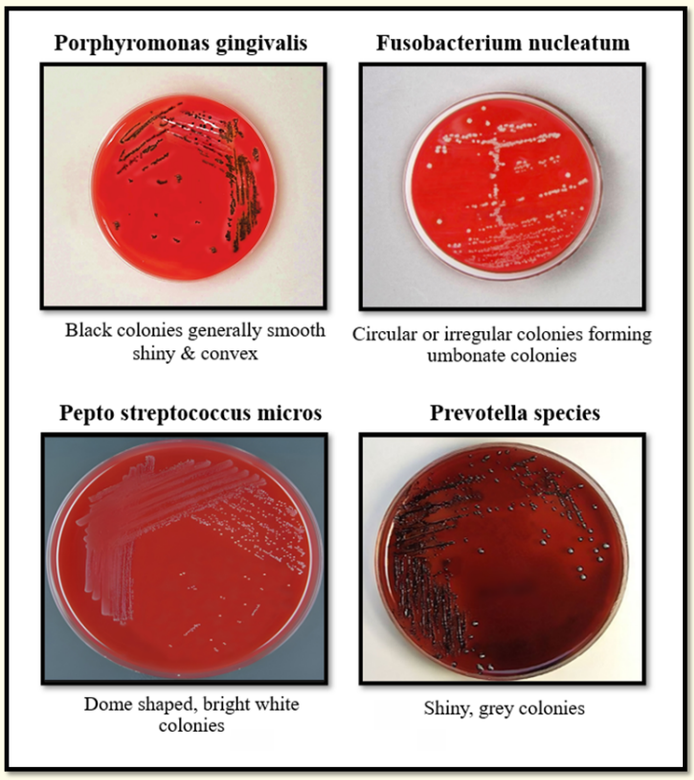
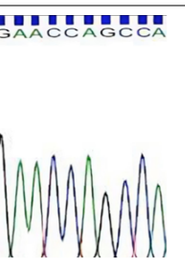
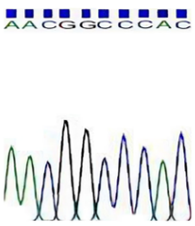
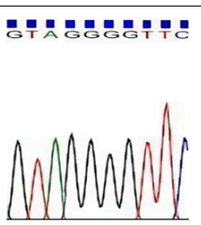


Figure 1: Shows the picture of isolated organisms on culture plates.

Species	FASTA sequence	Chromarogram	Confirmed organism by 'BLAST'
1	AACAGAAGAAGTGACGGCTAAATACGTGCCAG CAGCCGCGGTAATACGTATGTCACNAGCGTTATC CGGATTTATTGGGCGTAAAGCGCGTCTAGGTGGT TATGTAAGTCTGATGTGAAAATGCAGGGCTCAAC TCTGTATTGCGTTGGAAACTGTATAACTAGAGTA CTGGAGAGGTAAGCGGAACTACAAGTGTAGAGG TGAAATTCGTAGATATTTGTAGGAATGCCGATGG GGAAGCCAGCTTACTGGACAGATACTGACGCTA AAGCGCGAAAGCGTGGGTAGCAAACAGGATTAG ATACCCTGGTAGTCCACGCCGTAAACGATGATTA CTAGGTGTTGGGGGTCGAACCTCAGCGCCCAAG CTAACGCGATAAGTAATCCGCCTGGGGAGTACGT ACGCAAGTATGAAACTCAAAGGAATTGACGGGG ACCGCAC		Fusobacterium nucleatum
2	AAAGACGGACTAAAACCGCATACACTTGTATTATTGCA TGATATTACAAGGAAATATTTATAGCTGTAAGATAGGCA TGCGTCCCATTAGCTAGTTGGTGAGGTAACGGCTCACC AAGGCGACGATGGGTAGGGGAACTGAGAGGTTTATCC CCCACACTGGTACTGAGACACGGACCAGACTCCTACG GGAGGCAGCAGTGAGGAATATTGGTCAATGGGCGAGA GCCTGAACCAGCCAAGTCGCGTGAAGGAAGACTGTC CTAAGGATTGTAAACTTCTTTTATACGGGAATAACGGG CGATACGAGTATTGCATTGAATGTACCGTAAGAATAAG CATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG AGGATGCGAGCGTTATCCGGATTTATTGGGTTTAAAGG GTGCGTAGGTTGTTCGGTAAGTCAGCGGTGAAACCTG AGCGCTCAACGTTCAGCCTGCCGTTGAAACTGCCGGG CTTGAGTTCAGCGGCGGCAGGCGGAATTCGTGGTGTA GCGGTGAAATGCATAGATATCACGAGGAACTCCGATTG CGAAGGCAGCTTGCCATA		Porphyromonas gingivalis
3	AACGGCCCACCAAGGCTACGATCAGTAGGGGT TCTGAGAGGAAGGTCCCCCACATTGGAACTGAG ACACGGTCCAAACTCCTACGGGAGGCAGCAGT GAGGAATATTGGTCAATGGACGTAAGTCTGAAC CAGCCAAGTAGCGTGCAGGATTGACGGCCCTAT GGGTTGTAAACTGCTTTTGTTGGGGAGTAAAGC GGGGCACGTGTGCCCYTTTGCATTTACCCTTCGA ATAAGGACCGGCTAATTCCGTGCCAGCAGCCGC GGTAATACGGAAGGTCCAGGCGTTATCCGGATTT ATTGGGTTTAAAGGGAGTGTAGGCGGTCTGTTA AGCGTGTTGTGAAATTTAGGTGCTCAACATCTAC CTTGCAGCGCGAACTGGCGGACTTGAGTGCACG CAACGTATGCGGAATTCATGGTGTAGCGGTGAA		Prevotella Intermedia
4	GTAGGGGTTCTGAGAGGAAGGTCCCCCACA TTGGAACTGAGACACGGTCCAAACTCCTACGGG AGGCAGCAGTGAGGAATATTGGTCAATGGACGY AAGTCTGAACCAGCCAAGTAGCGTGCAGGATGA CGGCCCTATGGGTTGTAAACTGCTTTTATGTGGG AATAAAGTGGCGCACGTGTGCGCCATTGCATGT ACCTCATGAATAAGGACCGGCTAATTCCGTGCCA GCAGCCGCGGTAATACGGAAGGTCCAGGCGTTA TCCGGATTTATTGGGTTTAAAGGGAGTGTAGGCG GTCTGTTAAGCGTGTTGTGAAATTTAGGTGCTCA ACATTTAACTTGCAGCGCGAACTGTCAGACTTG AGTACACGCAGCGCAGGCGGAATTCATGGTGTA GCGGTGAAATGCTTAGATATCATGAGGAACTCCG ATCGCGAAGGCAGCCTGCGGGAGTGTTACTGAC		Prevotella Nigrescens

5	AGTTTGATCCCGGCTCAGGACGAA CGCTGGCGGTGTACTTAACACATGC AAGTCGAACGTGATTTNTATGGAA AGCCCTGCGGGGACGGAAATGAAA TGAAAGTGGCGAACGGGTGAATAA CACGTCACCACCCTACCTTACACA GTGGGATAGCCGCTGGAAACGACG ATTAATACCGCATCAGCACCACTAA GTACACATGTTGGGAGGTAAAAGA TTTATCGGTGTAAGCATGGGCTCGC CTCTGATTAGCTAGNTGGAAGGGTA AAGGCCTACCAAGGCGCACGATCA GTTGCCGGTCTGAGAGGATG		Peptostreptococcus micros

Table 2: Validation of the identified bacteria by 16S rRNA Bioinformatic sequencing.

Antimicrobial	F. nucleatum		P. gingivalis		P. micros		P. Intermedia		P. nigrescens	
	S	R	S	R	S	R	S	R	S	R
Amoxicillin	100%	0%	100%	0%	100%	0%	90%	10%	90%	10%
Metronidazole	90%	10%	100%	0%	100%	0%	100%	0%	80%	20%
Ciprofloxacin	100%	0%	90%	10%	80%	20%	90%	10%	90%	10%
Clindamycin	60%	40%	80%	20%	90%	10%	80%	20%	60%	40%
Amox-clav	90%	10%	100%	0%	90%	10%	100%	0%	100%	0%
Tetracycline	90%	10%	100%	0%	90%	10%	100%	0%	100%	0%

Table 3: Susceptibility of selected bacterial species isolated to various antibacterial agents.

Discussion

Periodontal disease generally refers to inflammatory pathologic state of the gingiva and the supporting structures of the periodontium which include gingival, alveolar bone, periodontal ligament, and cementum. They are commonly found in most human populations and result in significant morbidity, with exfoliation of the teeth in severe condition.[9] Fusobacterium nucleatum belongs to the family bacteroidaceae. Gram-negative, an anaerobic, spindle-shaped rod dominant microorganism in periodontal tissues, and is associated with periodontitis etiology.[10] Previous research using culture to detect the bacterium in subgingival plaque produced varying results 74%,[11] 45.8%,[12] 0%[13] while our study showed 70%. In the process of dental plaque formation, F. nucleatum in the process of dental plaque formation has the ability to co-aggregate with early and late plaque colonizers through related proteins and receptors in its outer membrane, thereby promoting the development of periodontal diseases.[10] Porphyromonas gingivalis form the classic 'red complex' described by Socransky et al. [14] It is also the bacteria most frequently found in patients with periodontal disease. This bacterium was found in 85.75%,[9] 10%,[13] 8.33%[12] of subgingival plaque samples from patients with chronic periodontitis.[9] Present study, showed (60%) of cases which goes in accordance to above findings. Presence of P. gingivalis in oral habitat relies on the fermentation of amino acids for energy production, a property required for its survival in deep periodontal pocket, where sugar availability is low[15] and capable of eventually pollute soft tissues and flee the surgical debridement of periodontal lesions.[16] F. nucleatum and P. gingivalis noted to play a prime role in pathogenesis of chronic periodontitis. Further, species were confirmed with the aid of 16S rRNA gene sequencing. No significant sub-species was spotted, this might be due to dissimilarity in populations, number of sample and collection site which may influence the results.[17] P. micros may be one of the few gram-positive organisms that acts as a major periodontitis-producing organism. This study found 10% of P. micros among the identified organisms. Other studies significantly showed higher proportions of P. micros in active than in inactive deep periodontitis lesions[17,18] while few remain unidentified in culture.[13]

Among the cultured organisms, an additional anaerobic, gram-negative, rod-shaped bacterium identified as prevotella spp. Prevotella intermedia was the most commonly identified species in the earlier literature. Metabolic activity of P. intermedia often results in an accelerated development of oral biofilm-mediated diseases. These metabolites are known to initiate and promote periodontal disease, both directly and indirectly.[19] On validation using 16S rRNa sequencing revealed, two species of genus prevotella, P. intermedia and P. nigrescens. These two species close resembled each other, they were difficult to identify just merely relying on the colony morphology and biochemical test. According to study by Boutuga K et al they show 6% difference in their 16S rRNA genes, which can aid in identification of the two.[17] P. nigrescens is a very rarely identified it, this organism was noted in a periodontal study by[17] using Real time-PCR and other study by[20] similarly as our study by using 16S rRNA sequencing in a case of cellulitis from extraoral site.

Antiobiotic sensitivity test was done for the above microorganism's amoxicillin and ciprofloxacin proved to be highly effective for F. Nucleatum. For P. Gingivalis along with Amoxicillin, amoxy-clav and tetracycline showed high susceptibility while amoxicillin and metronidazole for P. micros. For the two species of Prevotella, metrodinazole was found highly effective for P. intermedia and less for P. nigrescens. Hence, distinction by PCR-based methods such as 16S rRNA gene sequencing for detecting genotypic differences can be helpful in the management of such anaerobic conditions where sub species remain unidentified and regular treatment line up proves to be less effective.[20] Especially cases of refractory periodontitis this validation process along with antibiotic sensitivity test would could be of a great help to the clinician.

There are certain drawbacks, wherein statistically significant sample size is mandatory to identify the unrevealed subspecies of various periodontal organisms. The 16S rRNA method is expensive and unaffordable; however, it might become significantly less expensive in the long run if the therapeutic protocol were incorporated into the standard therapy scenario of severe recurring patients. Antibiotic sensitivity test for application in patients, proper sample size along with minimal inhibitory concentration for sample individually is mandatory.

Conclusion

Implication of 16S rRNA sequencing can aid in confirmation of identified species on culture yield and identify organisms which are phenotypically similar but molecularly variable along with few unknown species. It may also provide insights on their role in pathogenesis and treatment outcome of chronic periodontitis.

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Conflict of Interest

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