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ORIGINAL ARTICLE

Determination of prevalence of genus *Prevotella* from the subgingival plaque samples of chronic periodontitis and in healthy individual through polymerase chain reaction

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Abstract

Background: Members of genus *Prevotella* are Gram-negative anaerobic bacilli mainly residing in the oral cavity. These are pigmented and non-pigmented organisms. Pigmented organisms can be easily identified and isolated through culture, whereas non-pigmented ones are difficult to identify through culture due to their fastidious nature. Studies have been carried out identifying different phylotypes of *Prevotella*, but studies are lacking where only prevalence of genus *Prevotella* is studied. Hence, the present study is aimed to determine the prevalence of genus *Prevotella* from the subgingival plaque samples of chronic periodontitis and in healthy individual through polymerase chain reaction (PCR). **Materials and Methods:** A total of 200 individuals (100 chronic periodontitis and 100 healthy individuals) were considered for the study. Detailed demographic data were obtained from both the groups once they met all the inclusion and exclusion criteria. For chronic periodontitis individuals, detailed clinical examination was done and periodontal index was noted. The subgingival plaque was collected from the individuals and transferred into reduced transport media and sent to a laboratory for DNA extraction. Followed by DNA extraction PCR analysis was carried out for genus Prevotella with conserved region of 16s rDNA as the target. **Result:** Overall 94.5% of cases were positive for genus *Prevotella*. In chronic periodontitis, positivity was marginally high with 96% when compared to healthy individuals which were 93%. When grades of periodontitis were compared, severe periodontitis showed more number of positive cases. However, the difference was statistically insignificant. **Conclusion:** This is the preliminary study which gives an insight for the prevalence of genus *Prevotella* in oral cavity. Higher prevalence was observed in chronic periodontitis individuals when compared to healthy, thus suggesting its role in causing periodontitis. However, further study has to be carried out to identify different species of *Prevotella* and their role in healthy individuals and chronic periodontitis.

Introduction

Anaerobic bacteria constitute members of indigenous microbiota in humans and animals. These organisms do not usually cause disease, but have the capacity to induce mixed or monomicrobial infections if the hosts immune status of the individual is weakened.[1] One such disease that affects the oral cavity due to reduced host defense is periodontal disease, mainly chronic periodontitis. Periodontal disease affects a large proportion of the world's population and is one of the important diseases of the developing countries. The subgingival microbiota involved in the disease progress of periodontium has been the major topic of research in the recent past. Recent researchers based on 16s rRNA gene sequencing, 800–1000 bacterial, and Archaea oral species representing 19,000 pathogens have been identified, and of these, many are uncultivable through culture due to their fastidious nature.[2-7] In spite of the sizeable microbial diversity, only about 50 bacterial species are closely related to periodontal breakdown.[2,8,9] Evidence for bacterial specificity in periodontitis comes from culture studies on microbial occurrence in health

and disease. A significant number of microbial species present in the oral cavity are anaerobic in nature. Among these plethora of organisms, red complex bacteria such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia,* and *Treponema denticola* are proven pathogens.[2,9] Other species such as *Prevotella* may also contribute to the destructions of periodontium. Members of genus *Prevotella* are Gram-negative anaerobic bacilli that comprise of more than 40 different species, both pigmented and non-pigmented organisms, and majority of them reside in oral cavity.[1,2] It was in 1990 Shah and Collins on the bases of phenotypic and phylogenetic evidence, noting that some species initially classified within the genus bacteroides to be categorized into new taxa.[1,10] Based on their phenotypic characteristics and 16SrRNA gene phylogeny, 16 species were transferred into new genus named *Prevotella* in honor of the French microbiologist AR Prevot.[10]

Various methods have been developed for cultivation and identification of genus *Prevotella,* and also different sampling techniques have been employed for the identification of *Prevotella* residence in oral cavity. The common site of locations of these organisms is in the gingival crevice. One of the conventional methods and gold standards is by culture for many years. It has many limitations in respect to detecting non-viable bacteria and inability of some species to grow on selective media. Above all, it is time-consuming and is labor intensive. Moreover, classification and identification based on phenotypic traits do not always provide clear-cut results and are sometimes unreliable.[3,11,12] Followed by this, definitive diagnosis is done based on a biochemical test in combination with gas-liquid chromatography. However, this method is also labor intensive and expensive.[1,13] On the other hand, molecular techniques such as polymerase chain reaction (PCR) using 16S rRNA gene sequence for identification appear to be sensitive and specific for individual genus.[1,3,14] Studies in the literature have focused mainly on the identification of various species of *Prevotella* and studies are lacking where the prevalence of genus *Prevotella* has been identified and compared between disease and health of the oral cavity. Thus, the aim of the study is to detect the prevalence of *Prevotella* genus in chronic periodontitis and in healthy individuals through PCR using subgingival plaque samples.

Material And Methods

A total of 200 subjects with an equal number of periodontally healthy individuals and patients with chronic periodontitis were enrolled in the study. Ethical clearance was obtained from the Institutional Ethical Review Board before the commencement of the study. Patients were selected from outpatient department of the institute. After obtaining informed consent from the subject, subjects who fulfilled the following inclusion and exclusion criteria were enrolled for the study. Age of the subject included for the study was between 20 and 70 years. This research is a part of the project funded from Rajiv Gandhi University of Health Sciences.

The inclusion criteria for healthy subjects were probing depth ≤3 mm, no clinical signs of inflammation, no clinical attachment loss, and no tooth mobility.

For chronic periodontitis subjects, inclusion criteria were clinical attachment loss ≥3 mm in at least 4 or more teeth, bleeding on probing and probing depth ≥5 mm in at least 4, or more teeth. Exclusion criteria for both subjects were patients with any systemic diseases/medically compromised patients, patients who have received periodontal therapy, antibiotics/antimicrobials within 3 months before sampling, pregnant and lactating women, smokers, and subjects using smokeless tobacco.

A thorough clinical examination was done, and the following clinical details were noted: Plaque index (Silness and Loe), gingival index,[15] probing pocket depth, and clinical attachment loss for subjects with chronic periodontitis. Clinical grading periodontitis was done based on probing pocket depth and clinical attachment loss, as mild, moderate, and severe.

After obtaining written informed consent from the subjects, subgingival plaque sample was collected. After stripping off the supragingival plaque, the subgingival plaque sample was collected with sterile Gracey Curettes from at least four teeth in healthy subjects and four deepest pockets sites or most diseased sites in the chronic periodontitis patients. The samples collected were transferred to reduced transport fluid and transported to the laboratory at the earliest. The samples were processed at the Central Research Laboratory of the Institute. DNA extraction was done using modified proteinase K method from the plaque sample.

DNA extraction

The obtained sample was centrifuged at 5000 rpm for 3–5 min room temperature and the supernatant was discarded. The sediment was left in the pellet. To the pellet, add 600 µl of extraction solution and 20 µl of proteinase K, and vortex the pellet. Keep the pellet in water bath and incubate for 2 h at 60°C. To this, add 4 µl of RNA-free DNA and incubate it at 37°C for 5–10 min. Add 200 µl of protein precipitation solution and vortex it, followed by centrifuge at 10,000 rpm for 3–5 min at room temperature. Transfer the supernatant to fresh vial without disturbing the precipitate. To this, add 600 µl of isopropanol for the precipitation of DNA and centrifuge for 1 min at high speed at room temperature. Discard supernatant carefully without disturbing the pellet. Wash the pellet with 1 ml of 70% ethanol. Centrifuge again for 1 min at high speed. Remove the supernatant complete and discard, leave the pellet to dry for 5–10 min at room temperature, add 100 µl of distilled water to the pellet, and stored at −20°C until further use.

Following DNA extraction, PCR was performed for the detection of Prevotella genus with conserved region of 16S rDNA as the target. The primers used to target Prevotella conserved region is: Forward primers 5'-CCAGCCAAGTAGCGTGCA-3' and reverse primer as 5'-TGGACCTTCCGTATTACCGC-3'. PCR reaction mixture with a total reaction volume of 25 µl was prepared in a 0.2 ml PCR tube. Taq DNA Polymerase 1.1x Master Mix RED (Ampliqon, Denmark) was used for the preparation of PCR reaction mixture which consists of Tris-HCl pH 8.5, $(NH4)_2S04$, 1.65 mM $MgCl_2$, 0.11% Tween20, 0.22 mM of each dNTP, 0.11 units/µl Ampliqon Taq DNA polymerase, Inert red

dye, and stabilizer. Primer concentration of 10 pmole/μl was used. 3 μl of DNA template was added to the mixture with a concentration of approximately 100 μg/ml. PCR tubes were kept in a Veriti Thermal Cycler (Applied Biosystems, California, USA) to carry out the thermal cycling conditions as follows: Initital denaturation was done at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C, annealing at 54°C, and extension at 72°C for 1 min each. Final extension was done at 72°C for 5 min.

PCR product was detected by running the amplified samples on 2% agarose gel electrophoresis. The amplified samples were loaded on the agarose gel matrix and allowed to run at 80V for 2 h. The gel was stained with 0.5 μg/ml of ethidium bromide for 20 min, and then, gel was visualized under UV transilluminator in gel doc system (Major science, USA). The bands were compared with NEX-GEN 100 bp DNA ladder (Genetix Asia Pvt. Ltd., New delhi, INDIA) which was run simultaneously with each run [Figure 1].

Results were analyzed, and statistical analysis was carried out using SPSS software version 17.0. $P < 0.05$ was considered as statistically significant and $P > 0.05$ was considered as statistically non-significant.

Results

Of 200 cases, 116 were females and 84 were males. In healthy subjects, 54 were females and 46 were males. In chronic periodontitis, 62 were females and 38 were males. The age of the mean average age of the subject ranged from 22 years to 67 years [Tables 1 and 2]. Clinically when periodontitis was classified, 50 cases were of severe grade, 29 cases were moderate, and 21 cases were of mild periodontitis [Table 3].

Overall positivity for genus *Prevotella* through PCR was 189 (94.5%) of 200 subjects. When positivity was compared between healthy and chronic periodontitis, the percentage of positivity was slightly high in chronic periodontitis (96.0%) compared to healthy (93.0%). The result was statistically insignificant [Table 4]. Comparison of positivity of genus *Prevotella* between males and females showed higher number of positive cases for females compared to males in both groups, and the result was statistically insignificant [Tables 1 and 2]. Comparison of the mean value of plaque index and gingival index with positive cases of *Prevotella* showed higher mean value for both plaque index (3.4625) and gingival index (3.4458); however, the observation was statistically insignificant [Table 5]. When positivity of genus *Prevotella* was compared between different grades of periodontitis, there was no significant difference observed between the grades. However, it was higher in moderate and severe periodontitis compared to mild [Table 1].

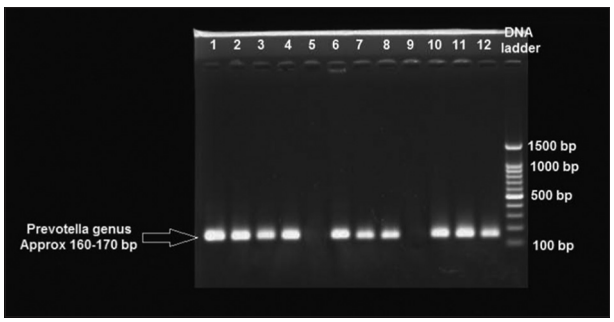

Figure 1: Photograph showing positive bands for *Prevotella* genus at 160–170 bp, samples 5 and 9 are negative

Discussion

The present study aimed to detect the prevalence rate of genus *Prevotella* in subgingival plaque of patients with chronic periodontitis and healthy individuals through PCR. In the present study, we considered patient who has chronic periodontitis and healthy individuals without any tobacco habit or any other deleterious habits. The reason for this is environmental factors such as smoking/alcohol drinking which might have an impact on host response, and the result may vary.[16]

The study showed an overall 94.5% of the positivity of *Prevotella,* and the incidence was slightly higher in chronic periodontitis group (96%) when compared to healthy (93%). On literature search, we found that no studies were done only to detect the prevalence of *Prevotella* genera. This appears to be the first study. Studies have concentrated on *Prevotella* species identification and their associations with endodontic infection, periodontitis, odontogenic infections, and some opportunistic infections of the oral cavity.[1,17] Studies have shown a higher prevalence of *Prevotella intermedia* up to 96.9% and *Prevotella nigrescens* up to 56.2%.[18] Which can be co-related to our study results for genus *Prevotell*a which showed high positivity. Another study by Lie *et al.* showed 73% of the positive samples for *P. intermedia* and *P. nigrescens* and stressed the need of isolation of these organisms in the oral cavity and their role in disease.[19] Studies have also shown variation in species according to their geographic location. *P. intermedia* was more predominated in Brazil and the USA, whereas in Chile, *Prevotella melaninogenica* was in higher proportion. In Sweden, *Prevotella* species formed only a minor component,[20] thus making it essential for us

Table 1: Comparison between males and females for PCR positivity within healthy group using Fisher's exact test

Group	PCR		Total	Fisher's exact test
	Negative for *Prevotella* by PCR *n* (%)	Positive for *Prevotella* by PCR *n* (%)		
Female	3 (5.6)	51 (94.4)	54 (100.0)	*P*-value 0.700, Not significant
Male	4 (8.7)	42 (91.3)	46 (100.0)	
Total	7 (7.0)	93 (93.0)	100 (100.0)	

PCR: Polymerase chain reaction

Table 2: Comparison between males and females for PCR positivity within chronic periodontitis group by Fisher's exact test

Group	PCR		Total	Fisher's exact test
	Negative for *Prevotella* by PCR *n* (%)	Positive for *Prevotella* by PCR *n* (%)		
Female	2 (3.2)	60 (96.8)	62 (100.0)	*P*-value 0.633, Not significant
Male	2 (5.3)	36 (94.7)	38 (100.0)	
Total	4 (4.0)	96 (96.0)	100 (100.0)	

PCR: Polymerase chain reaction

Table 3: Comparison between mild, moderate, and severe periodontitis groups for PCR positivity using Chi-square test

Group	PCR		Total	Chi-square test
	Negative for *Prevotella* by PCR *n* (%)	Positive for *Prevotella* by PCR *n* (%)		
Mild periodontitis	1 (4.8)	20 (95.2)	21 (100.0)	Pearson Chi-Square value 055.
Moderate periodontitis	1 (3.4)	28 (96.6)	29 (100.0)	Df-2
Severe periodontitis	2 (4.0)	48 (96.0)	50 (100.0)	*P*-value-0.973, Not significant
Total	4 (4.0)	96 (96.0)	100 (100.0)	

PCR: Polymerase chain reaction

Table 4: Comparison between healthy and chronic periodontitis groups for PCR positivity by Fisher's exact test

Group	PCR		Total	Fisher's exact test
	Negative for *Prevotella* by PCR *n* (%)	Positive for *Prevotella* by PCR *n* (%)		
Healthy	7 (7.0)	93 (93.0)	100 (100.0)	*P*-value 0.537, Not significant
CP	4 (4.0)	96 (96.0)	100 (100.0)	
Total	11 (5.5)	189 (94.5)	200 (100.0)	

PCR: Polymerase chain reaction

Table 5: Comparison of plaque and gingival index scores between PCR-positive and negative groups by unpaired t-test

Measurement	PCR	*n*	Mean±SD	Test of significance - Unpaired t-test
Plaque index score	Negative for *Prevotella* by PCR	4	2.5250±0.60759	*P*-value- 0.147, not significant
	Positive for *Prevotella* by PCR	96	3.4625±1.27339	
Gingival index score	Negative for *Prevotella* by PCR	4	3.2250±2.06942	*P*-value- 0.718, not significant
	Positive for *Prevotella* by PCR	96	3.4458±1.15494	

PCR: Polymerase chain reaction, SD: Standard deviation

to know the prevalence of genus *Prevotella* as a whole before speciation among small subgroup of Indian population.

In the present study, the prevalence of *Prevotella* was seen to be higher in chronic periodontitis compared to healthy suggesting its role in disease progression. Although *Prevotella* species such as *P. intermedia, P. nigrescens, P. melaninogenica, Prevotella tannerae,* and *Prevotella denticola* have been well established, its role in periodontitis needs further evaluation.[21,22] A study by Xie *et al*. observed higher frequencies of *Prevotella* species from periodontal abscess patients.[23]

When the severity of chronic periodontitis was compared with the prevalence of *Prevotella,* higher positivity was seen in moderate and severe periodontitis. Furthermore, when mean gingival index and plaque index score was compared between positivity of *Prevotella* between the groups, the mean score was high in chronic periodontitis and was also correlated to the increased number of positive cases for *P. intermedia* in chronic periodontitis. In oral infections, especially periodontal infections such as adult and advanced periodontitis, *P. intermedia* and play an important role as pathogens in early periodontal disease without deep pocket. *Prevotella* species are more oxygen tolerant and can provide environmental conditions suitable for supporting other anaerobes. Several virulence factors in *P. intermedia* such as enzyme production including aminopeptidase, chymotrypsin-elastase, and trypsin-like dipeptidyl peptidase and alkaline phosphatase activities have been reported, and these can be harmful to the host. Oral species of *P. intermedia* show ability to adhere and invade epithelial cells, and this is due to the different types of fimbriae present on *P. inermedia* strains. *Prevotella* species have a significant range of virulence factors that allow them to invade the host tissue, destroy cellular structure and matrix, and stimulate a powerful and persistent inflammatory process.[1,3]

Conclusion

In this study, we made an attempt to study the prevalence of genus *Prevotella* in health and disease. Our finding showed very

high prevalence of genus *Prevotella* using sensitive and scientific tool PCR. This is a preliminary study and gives an insight for the presence of this organism both in healthy and disease. Further study to categorize the species and elucidate their role in chronic periodontitis and healthy individuals is required since more than 90% species of this genus reside in oral cavity. Furthermore, determining their antibiotic resistance to each species would help us in understanding the pathophysiology of this taxa.

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