Prevalence of Periodontopathic Bacteria in the Subgingival Plaque of a South Indian Population with Periodontitis

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ABSTRACT
Background: Periodontitis is an important global public health problem which involves mostly the adult population over 35-40 years of age. Strongly considering that periodontitis is a polymicrobial infection, the screening of the selective microbial population, rather than the isolation of a single member of the subgingival flora, should give a more wide-ranging perception in the aetiology of periodontitis. Different compositions of the bacterial species in the subgingival microflora of the periodontitis patients have been reported in diverse ethnicity. Similar studies on the bacterial aetiology of periodontitis is completely lacking in the Indian population.

Objectives: To detect and compare the prevalence of the eight putative, periodontal bacterial pathogens (Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, Aggregatibacter actinomycetemcomitans, Campylobacter rectus, Eikenella corrodens, Prevotella intermedia and Prevotella nigrescens) among periodontitis patients and healthy subjects.

Materials and Methods: A total of four hundred subgingival plaque samples which were collected from two hundred periodontitis patients (chronic periodontitis patients-ChP, n=128, aggressive periodontitis patients-AgP, n=72) and two hundred healthy subjects was subjected to Polymerase Chain Reaction (PCR) with the help of species specific primers which targeted the 16S rRNA gene of the bacterial species. The statistical analysis was performed by using the Chi-square test.

Results: The prevalence of various microorganisms in chronic periodontitis (n=128), aggressive periodontitis (n=72) and in healthy subjects (n=200) was 80.5%, 73.6% and 11% for P. gingivalis, 73.4%, 59.7% and 10.5% for T. forsythia, 71.1%, 33.3% and 5.5% for T. denticola, 32.0%, 61.1% and 2.5% for A. actinomycetemcomitans, 17.2%, 11.1% and 8.5% for C. rectus, 15.6%, 11.1% and 6% for E. corrodens, 16.4%, 25.0% and 7.5% for P. intermedia and 13.3%, 13.9 and 14% for P. nigrescens respectively.

Conclusion: The present study demonstrated a high prevalence of the red complex group (P. gingivalis, T. forsythia and T. denticola) in the ChP patients. The high odds ratio for P. gingivalis, T. forsythia, T. denticola and A. actinomycetemcomitans suggested a strong association between them and periodontitis. The incidence of A. actinomycetemcomitans along with P. gingivalis and T. forsythia was high in AgP. An appropriate therapeutic strategy can be considered in view of these bacterial consortiums. In addition, two formerly unreported symbiotic relationships were found between the 8 bacterial species which were analyzed.

Key Words: Aggressive periodontitis, Anaerobic bacteria, Chronic periodontitis, PCR, Red complex group, Sub-gingival plaque

INTRODUCTION
Periodontitis is a progressive disease which is widely regarded as the second most common disease worldwide after dental decay. It is caused by microorganisms that adhere to and grow on the tooth surfaces, along with an excessively aggressive immune response against these microorganisms. It involves the progressive loss of the alveolar bone around the teeth and if left untreated, it can lead to the loosening and the subsequent loss of teeth. It had been previously estimated that about 500 bacterial species colonized the human oral cavity [1, 2]. A majority of these organisms were found to be commensals and they existed in complex communities, forming oral biofilms on the tooth surfaces, although only a selective number of the anaerobes had been implicated as periodontal pathogens. The predominating microorganisms which are isolated from the teeth and the gingival sulcus of the individuals with a healthy periodontium include mainly gram-positive, facultative, anaerobic bacteria and rarely, gram-negative, anaerobic bacilli [3]. The gram-negative anaerobic bacteria, on the other hand, have been found to predominate in the subgingival niche with increasing severity of the periodontal disease [4, 5]. Among these gram-negative bacteria, Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola have been designated as the red complex [6].

The anaerobic culture methods are arduous and they may fail in identifying all the organisms due to the diverse growth requirements of the bacteria which inhabit the subgingival microflora. The 16S rRNA gene detection by PCR for the organisms that are uncultivable and are difficult to identify can eliminate the ambiguity in the diagnostic microbiology. As the literature on the bacterial aetiology of periodontitis is completely lacking in the Indian scenario, this study was planned, to detect the eight putative periodontal pathogens.

MATERIALS AND METHODS
The Study Population
Two hundred periodontitis patients (chronic periodontitis patients-ChP, n=128, aggressive periodontitis patients-AgP, n=72) were recruited from the Department of Periodontics and Implantology,
Tamil Nadu, Government Dental College and Hospital and the Sree Balaji Dental College and Hospital for the study. The age of the study population ranged between 20-60 years. More than 3 teeth with a pocket probing depth (PPD) of ≥5 mm and bleeding on probing were the inclusion criteria. Patients who were on periodontal therapy and dental hygiene procedures in the past year, those who were on antibiotic therapy during the past six months, pregnant women and those with a history of diabetes, other systemic conditions and smoking were the exclusion criteria. 200 healthy subjects with a PPD of ≤3 mm and ≤3.6% of sites which exhibited gingival bleeding were included as the control group. A written informed consent was obtained from the patients and the healthy subjects. This study was reviewed and approved by the human ethical committee of the Dr A.L.M Post Graduate Institute of Basic Medical Sciences, University of Madras, Chennai.

Sample collection and DNA isolation
The PPD and the clinical attachment level CAL (mm) was recorded. The PPD was measured with a graduated Williams periodontal probe. The subgingival plaque was collected from the periodontitis patients and the healthy subjects, from three different sites, by using a sterile Gracy curette after the careful removal of the supragingival plaque with a sterile cotton roll. The samples from each patient were pooled into 500µl of phosphate buffered saline (PBS, pH 8.0), transported in ice and stored at -20°C till they were assayed. DNA was extracted from them as per the method of Wu et al. [7]

PCR Assays
All the PCR analyses were performed in duplicate. Positive and negative controls were incorporated in all the batches of the samples which were examined. The positive controls consisted of DNA which was extracted from clinical samples (subgingival plaque), which was previously tested and was found to be positive for each of the bacterial species, as was confirmed by PCR and sequencing. Sterile millipore water was included as a negative control. One negative control was included for a batch of ten samples which were analyzed. The PCR reactions were run in a thermal cycler (Eppendorf Mastercycler Gradient; Eppendorf AG, Hamburg, Germany). The PCR reaction mixture (50µl) for the detection of the 16S rRNA gene of P. gingivalis, T. forsythia, T. denticola, E. corrodens, C. rectus, A. actinomycetemcomitans, P. intermedia and P. nigrescens contained 5µl of 10X PCR buffer (20 mM/L Tris-HCl, 50 mM/L KCl, pH 8-4), 1.25 U Taq DNA polymerase (Bangalore Genei, India.), 0.25 mM/L of each dNTP (Medox Biotech India Pvt Ltd), 1.5mM MgCl2(Sigma-Aldrich Pvt Ltd, India), 0.5 µM of each primer (Sigma-Aldrich Pvt Ltd, India) and 5 µl of the template. The PCR thermocycling conditions for the detection of the 16S rRNA of P. gingivalis, T. forsythia, T. denticola, E. corrodens and C. rectus included an initial denaturation at 94°C for 2 min, followed by 36 cycles of denaturation at 95°C for 30 sec, primer annealing at 60°C for 1 min and extension at 72°C for 1 min, and subsequently, a final extension step at 72°C for 2 min. The temperature profile for the detection of the 16S rRNA of A. actinomycetemcomitans, P. intermedia and P. nigrescens included an initial denaturation at 95°C for 2 min, followed by 36 cycles at 94°C for 30 sec, at 55°C for 1 min and at 72°C for 2 min, and a final extension step at 72°C for 10 min.

The PCR products were fractionated in a 1.5% agarose gel electrophoresis in Tris-Borate EDTA buffer. The gel was stained with 0.5µg/ml ethidium bromide and it was photographed by using a BioRad UV gel documentation system. A 100 bp ladder (Medox Biotech India Pvt Ltd) served as the molecular weight marker.

Primer Specificity
The species-specific primers for P. gingivalis, T. forsythia, T. denticola, E. corrodens, C. rectus, P. intermedia and P. nigrescens were as per Ashimoto et al’s protocol [9]. The primer for A. actinomy cetemcomitans was as per Slots et al’s protocol [9]. The species specificity was confirmed by sequencing one PCR product from a clinical sample for each primer in an Applied Biosystem (ABI) 3130 Genetic Analyser, ABI PRISM Big Dye Terminators Version 3.1. The sequences which were generated were compared with those which were available in the GenBank database.

Nucleotide sequence analysis
All the sequenced products unveiled nucleotide identities of ≥99% with the corresponding taxa when they were blasted in the Genbank database, thus ensuring reliability in the detection protocol. The sequences for the 16S rRNA gene of P. gingivalis, T. forsythia, T. denticola, A. actinomycetemcomitans, C. rectus, E. corrodens, P. intermedia and P. nigrescens were submitted to GenBank under the accession nos, HQ112349, HQ112350, HQ202265, HQ188689, HQ269826, HQ269827, HQ202263 and HQ202264 respectively.

Statistical analysis
All clinical measurements such as PPD and CAL were computed for each subject and they were then averaged across the subjects within the groups. Any difference of \( P < 0.05 \) was considered as statistically significant. The prevalence of the eight bacterial species among the periodontitis patients and the healthy subjects was compared with the Pearson’s Chi-square test. A \( P \) value of < 0.05 was considered as statistically significant. The odds ratio was calculated by using the Chi-square test with a 95% confidence interval, to analyze the association between two different bacterial species among the periodontitis patients and the eight bacterial species with disease and health. A \( P \) value of <0.0001 was considered as statistically significant.

Results
The periodontal clinical parameters of the two subject groups are shown in [Table/Fig-1]. A significant difference was observed between the groups with respect to age \( (p < 0.0001) \). No significant differences were observed between the groups with respect to the gender (male/female ratio) \( (p = 0.5) \). The subjects with periodontitis presented significantly higher values of the mean PPD \( (p < 0.0001) \) and CAL \( (p < 0.0001) \), than the periodontally healthy individuals. [Table/Fig-1]

The species specific primers for all the eight bacterial species successfully detected a single band of the expected size. [Table/ Fig-2] shows the prevalence of the eight bacterial species in ChP, AgP and in healthy subjects. A co-occurrence of P. gingivalis / T. forsythia was observed in (71) 55.5% of the ChP patients. Correspondingly, a co-occurrence of P. gingivalis / T. denticola was observed in (72) 56.3% of the ChP patients. Among the AgP patients, the co-occurrence of P. gingivalis / T. forsythia was found to be (32) 44.4%. Conversely, a co-occurrence of P. gingivalis / T. denticola was observed only in (13) 18% of the AgP patients.

The co-occurrence of T. forsythia / T. denticola was (66)51.5% and (13)18% in the ChP and the AgP patients respectively. A co-occurrence of the red complex group (P. gingivalis, T. forsythia and T. denticola) was observed in (47) 36.7% of the ChP and in (5) 6.9%
The odds ratio was calculated to assess the association between the bacterial species. Twenty eight bacterial combinations were tested for the periodontitis group (both ChP and AgP) by using the Chi-square test [Table/Fig-3]. A statistically significant odds ratio ($p<0.01$) was obtained for 19 of the 28 bacterial combinations. A low odds ratio occurred for C. rectus/ P. intermedia and E. corrodens / P. nigrescens at 0.72 and 0.67 respectively.

The odds ratio which was calculated by using the Chi-square test for the bacterial species for periodontitis (ChP and AgP) and health revealed a statistically significant positive association for P. gingivalis, T. forsythia, T. denticola and A. actinomycetemcomitans towards both the groups of periodontitis. A high odds ratio occurred for P. gingivalis and A. actinomycetemcomitans The lowest odds ratio occurred for P. nigrescens [Table/Fig-4]. No negative association (odds ratio <0.5) was observed.

The odds ratio which was calculated to assess the association of the eight bacterial species only for ChP or AgP [Table/Fig-5] revealed a positive association for T. denticola towards ChP and for A. actinomycetemcomitans towards AgP ($P<0.0001$). None of the other bacterial species showed a significant odds ratio solely for ChP or AgP.

**DISCUSSION**

Owing to the low sensitivity and the ambiguity in the identification of anaerobes to the species level by cultivation, the present study used the 16S rRNA- based PCR method of detection.

Among the eight putative periodontal pathogens which were screened, the red complex group (P. gingivalis, T. forsythia and T. denticola) showed a higher prevalence in the periodontitis group as compared to other gram-negative anaerobes, which is well in agreement with several other existing data, which suggested that this group was associated with the severity of periodontal tissue destruction [6,10,11]. On the other hand, the percentage prevalence of the red complex in the healthy periodontium was low. Our study showed an association of only the red complex group and A. actinomycetemcomitans to adult periodontitis as against Ashimoto et al’s findings [8], who reported this association for all the eight bacterial species. The present study supported the fact that the red complex group of bacteria were putative periodontopathogenic bacteria, which was in concurrence with the findings of existing studies in a diverse population. In the present study, the members of the red complex were frequently detected together, as was also reported by previous studies [12, 13, 2, 14] and this exhibited a very strong correlation with the pocket depth, which is in accordance with the earlier findings of Socransky et al [6]. The presence of the red complex consortium was observed in a majority of the ChP patients. A striking colonization of the red complex group was completely absent among the healthy subjects. A majority of the ChP patients who harboured the red complex group showed a PPD of ≥6mm and a CAL of ≥8mm. Conversely, AgP patients who were positive for the red complex group revealed a PPD and a CAL of ≥8mm and ≥10mm respectively.

About (9) 4.5% ChP patients colonized A. actinomycetemcomitans along with the presence of the red complex group. Such an association was totally absent among the AgP patients. Two ChP patients harboured all the seven organisms except E. corrodens. Four ChP patients colonized 5 species (P. gingivalis, T. forsythia, T. denticola A. actinomycetemcomitans and P. intermedia). Two healthy subjects harboured four organisms, a combination of T. forsythia, A. actinomycetemcomitans, P. intermedia and P. nigrescens/ P. gingivalis T.forsythia, C.rectus and P. nigrescens. P. gingivalis and T. forsythia co-occurred in (5) 2.5% of the healthy subjects. P. gingivalis, T.forsythia and C.rectus was colonized in 2 healthy subjects. Overall, the prevalence of P. gingivalis was high in both the ChP and the AgP patients, followed by T. forsythia and T. denticola in the ChP patients and A. actinomycetemcomitans and T. forsythia in the AgP patients respectively.
A. actinomycetemcomitans was a predominant pathogen in the aetiology of AgP. The occurrence of A. actinomycetemcomitans in ChP and AgP was associated with the severity of the disease with respect to PPD and CAL. A. actinomycetemcomitans was most frequently detected in sites with a pocket depth of ≥5 mm and a clinical attachment loss of ≥7 mm. A statistical significance (p<0.0001) was observed for the prevalence of A. actinomycetemcomitans among the AgP patients. The association of A. actinomycetemcomitans with the red complex group was completely absent among the AgP patients, thus suggesting its pronounced role in AgP.

The prevalence of A. actinomycetemcomitans among the ChP group in the present study was consistent with few other reports [8, 29, 30], although its prevalence among ChP and the healthy subjects was inconsistent with the findings of Kumar et al. [18] and Junior et al [31]. The prevalence of A. actinomycetemcomitans in the present study was high among the periodontitis group (both ChP and AgP) as compared to previous reports from different ethnicity [32, 33, 34, 24, 15, 35]. Conversely, few studies have reported a higher prevalence among the ChP and the AgP groups for A. actinomycetemcomitans [20, 7, 23, 31]. The prevalence A. actinomycetemcomitans in AgP was very high as compared to that in healthy subjects, which was similar to the findings of Faveri et al [36].

In the present study, there was a strong association of T. forsythia with periodontitis and a good statistical significance was observed between the periodontitis patients and the healthy subjects (p<0.0001). These findings were consistent with several previous reports [8, 16, 22, 18, 37, 38]. They were not in agreement with Conrads et al's [39] and Lee et al's reports [23].

Among the different species of spirochetes, T. denticola has been extensively characterized in terms of its pathogenicity and involvement in the development of periodontitis. T. denticola has been shown to be associated with severe periodontal disease in adult humans and it can serve as a prognostic marker for the disease recurrence [40]. A good statistical significance was observed between the patients and the healthy group (p<0.0001). These findings were consistent with several previous reports [8, 16, 22, 18]. A high prevalence among the periodontitis patients suggested that this spirochete was associated with alveolar bone loss, particularly in the ChP group. Our study reported a moderately high prevalence of T. denticola among the ChP patients as compared to the findings of Ashimoto et al [8] and Mooter et al [41]. The prevalence of T. denticola was in concurrence to the findings of a previous study that had used the real-time PCR assay [42]. The prevalence of this species was relatively low in comparison to that in earlier studies [18, 23, 15]. The data of the healthy population corroborated the findings of Takeuchi et al [15].

A statistical significance was observed between the patients and the healthy group for C. rectus (p=0.044) and E. corrodens (p=0.008). The present study reported a low prevalence of C. rectus and E. corrodens in the adult periodontitis group as compared to Ashimoto et al's [8] and Mooter et al's findings [16].

A statistical significance was observed between the patients and the healthy group for P. nigrescens (p=0.885). In few previous studies, a high prevalence of P. intermedia and P. nigrescens, which ranged between 52-85% was reported [8, 16], Colombo et al [43] reported a 37 % prevalence of P. intermedia. A very low prevalence of 2.6% was reported by Conrads et al [39].

patients. They were totally absent among the healthy subjects. Our findings showed such a consortium only in five AgP patients. A pathogen may more readily colonize the subgingival sites which are already occupied by other organisms, due to gingival inflammation or the growth factors which are produced by other organisms. However, some organisms may occur together in the periodontitis lesions, merely because they both induce destructive disease without interacting with each other.

P. gingivalis can also be isolated from healthy individuals, but in a very low frequency and it is therefore recognized as a part of the commensal oral microbiome. A good statistical significance was observed between the healthy and the periodontitis group with respect to the P. gingivalis prevalence (p<0.0001). The prevalence of P. gingivalis (11%) in the present study among the healthy subjects was very well in accordance with Takeuchi et al. [15] and Botero et al's findings [16]. As compared to the present study, few previous studies reported a higher prevalence of P. gingivalis among the healthy subjects in diverse ethnic groups [17, 18, 19].

The percentage prevalence of P. gingivalis among the ChP group in the present study was consistent with quite a number of the earlier reports [8, 16, 20, 17, 21]. It was found to be moderately low as compared to a number of previous reports [22, 18, 23, 15, 7]. The prevalence of P. gingivalis among the AgP patients in the present study was high as compared to the finding of Mullally et al. [24] who reported a 16.7% prevalence. Conversely, Botero et al. [16] reported a very high prevalence of 91.6% amongst the AgP group.

In agreement with the findings of previous studies [25, 26, 11, 23, 27, 28], the data of the present study supported the notion that

<table>
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<th>Bacterial species</th>
<th>Odds Ratio</th>
<th>95% confidence interval</th>
<th>p-value</th>
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<tr>
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<td>28.68</td>
<td>16.46-49.97</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>T. forsythia</td>
<td>18.53</td>
<td>10.78-31.86</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>T. denticola</td>
<td>23.24</td>
<td>11.90-45.40</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>A. actinomyctemcomitans</td>
<td>28.82</td>
<td>11.36-73.12</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>C. rectus</td>
<td>1.89</td>
<td>1.01-3.56</td>
<td>0.062491</td>
</tr>
<tr>
<td>E. corrodens</td>
<td>2.55</td>
<td>1.25-5.17</td>
<td>0.012419</td>
</tr>
<tr>
<td>P. intermedia</td>
<td>2.98</td>
<td>1.58-5.6</td>
<td>0.000763</td>
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<tr>
<td>P. nigrescens</td>
<td>0.95</td>
<td>0.54-1.69</td>
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** Table/FIG-4: The odds ratio of bacterial species tested with periodontitis and health

## Significant p value

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<th>Odds Ratio</th>
<th>95% confidence interval</th>
<th>p-value</th>
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<td>T. forsythia</td>
<td>1.86</td>
<td>1.01-3.44</td>
<td>0.064802</td>
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<td>T. denticola</td>
<td>4.91</td>
<td>2.64-9.16</td>
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<td>A. actinomyctemcomitans</td>
<td>0.29</td>
<td>0.16-0.55</td>
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<td>C. rectus</td>
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<td>0.69-3.95</td>
<td>0.342782</td>
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<tr>
<td>E. corrodens</td>
<td>1.48</td>
<td>0.62-3.55</td>
<td>0.502335</td>
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<tr>
<td>P. intermedia</td>
<td>0.58</td>
<td>0.29-1.19</td>
<td>0.197603</td>
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<tr>
<td>P. nigrescens</td>
<td>0.94</td>
<td>0.41-2.20</td>
<td>0.920344</td>
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** Table/FIG-5: The odds ratio of bacterial species tested with ChP and AgP

= Significant p value.
The odds ratio which was calculated to assess the association between the bacterial species among the periodontitis patients in this study revealed a positive association for 19 of the 28 bacterial combinations. Previous positive associations between periodontopathic bacteria have been reported for T. forsythia/ Crectus, P. gingivalis/P. intermedia, P. intermedia/C. rectus, E. corrodens/C. rectus [6] and T.denticola/P. gingivalis [44, 45] and amongst P. gingivalis, P. intermedia and T. forsythia [46, 47]. Earlier, in a similar study, a positive association was reported for 17 of the 28 bacterial combinations [8]. The present study reported an additional positive association for P. gingivalis/E. corrodens and T. denticola/E. corrodens. The high odds ratio between the bacterial species perhaps showed a symbiotic association in the periodontal pockets. The low odds ratios for C. rectus/ Pintermedia and E.corrodens /Pintermedia suggested their negative association.

CONCLUSION

The odds of detecting P. gingivalis, T.forsythia, T.denticola and A.actinomyetemcomitans were 25.68, 15.53, 20.24 and 25.82 times high in individuals with periodontitis as compared to those in the healthy subjects. The high odds ratio for P. gingivalis, T.forsythia, T.denticola and A.actinomyetemcomitans suggested a strong association between them and periodontitis (ChP and AgP). In aggressive periodontitis, in addition to A.actinomyetemcomitans, the prevalence of P.gingivalis and T.forsythia was also high. The significant odds ratio for T.denticola and A.actinomyetemcomitans showed a positive association exclusively with ChP and AgP respectively. A significant difference in the prevalence of P.nigrescens was not observed among periodontitis and the healthy group, thus suggesting their symbiotic role in the periodontal pockets. A large cohort study may help in further substantiating the aetiology of this polymicrobial infection.

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