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Detection of *Porphyromonas gingivalis* and *Treponema denticola* in chronic and aggressive periodontitis patients: A comparative polymerase chain reaction study

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Abstract

Background: The detection frequency of *Porphyromonas gingivalis* and *Treponema denticola* in chronic periodontitis (CP) and aggressive periodontitis (AgP) is not explored well in Indian population. **Aim:** The study was undertaken to detect *P. gingivalis* and *T. denticola* in CP as well as in AgP patients using polymerase chain reaction (PCR), and to determine the relationship between the frequency of these two microorganisms and the severity of clinical periodontal parameters. **Materials and Methods:** Subgingival plaque samples were collected from ninety participants (thirty CP patients, thirty AgP patients, and thirty healthy participants) and the aforementioned two microorganisms were detected using PCR. **Results:** However, when CP and AgP were compared for the detection frequency of two microorganisms, no statistically significant difference was noted. A statistically significant increase in the number of bacteria-positive sites increased as the score of plaque index (PI), gingival index (GI), and clinical attachment level of CP and AgP patients increased. Coexistence of *P. gingivalis* and *T. denticola* was frequently observed in deep periodontal pockets. **Conclusions:** Study findings suggest that *P. gingivalis* and *T. denticola* are significantly associated with the severity of periodontal tissue destruction. Statistically significant association exists between clinical periodontal parameters such as PI, GI, periodontal pocket depth (PPD), and clinical attachment loss and presence of both the microorganisms.

Keywords: Aggressive periodontitis, chronic periodontitis, polymerase chain reaction, *Porphyromonas gingivalis*, *Treponema denticola*

Introduction

Periodontitis is a significant global health concern and is probably the most common chronic infectious disease of human being. It is defined as "an inflammatory disease of the supporting tissues of teeth caused by specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession, or both."[1] More than 500 bacterial species can be identified within the subgingival plaque.[2] 10–30 bacterial species play a more critical role in the pathogenesis of periodontitis.[3]

A marked qualitative and quantitative difference in microorganisms has been observed between periodontally healthy and periodontitis patients.[4] Gram-negative anaerobic bacteria are found to be increased in the subgingival microflora with active periodontal disease.[5] Among these Gram-negative bacteria, *Porphyromonas gingivalis*, *Tannerella forsythia,* and *Treponema denticola* have been designated as the red complex and these microorganisms are significantly associated with periodontal disease progression.[4]

In the recent consensus report of new classification, *Actinobacillus actinomycetemcomitans* was confirmed as the main causative bacterium in aggressive periodontitis (AgP) and characteristically present in higher proportions. Many other bacteria were also mentioned in the etiology of AgP, but their role is less vital than *A. actinomycetemcomitans*.[6]

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However, several studies in the past have either reported low prevalence or have not identified *A. actinomycetemcomitans* in AgP patients.[7] Assuming that the role of *A. actinomycetemcomitans* is less in AgP patients and other periodontopathic bacteria such as *P. gingivalis* and *T. denticola* play an important role.[8,9]

Detection frequency of major periodontal pathogens such as *P. gingivalis* and *T. denticola* can indicate future risk of the periodontal disease, its aggressiveness, and type of treatment required. Different methods have been developed and applied for identification and isolation of such these bacteria. Microbial culture is frequently considered as the "gold standard." However, culture-based techniques have limitations that they are time-consuming, laborious, require viable microorganisms, and the sensitivity of culture methods is low.[1,6,7] This due to the very specific growth requirements and extremely slow growth of some oral pathogens, several alternative methods have been developed for the detection of such bacteria such as immunoassays, DNA probe assays, and polymerase chain reaction (PCR) assays. PCR technique has higher sensitivity and specificity as compared to microbial culture technique in the detection of such bacteria.[8]

PCR has emerged as a very powerful diagnostic tool. This is an *in vitro* method of synthesis of nucleic acids, wherein a specific DNA segment is amplified rapidly without concomitant replication of the rest of the DNA molecule.[9] In recent years, PCR-based tests having a great interest in detecting bacteria which uses the bacterial small subunit 16S rRNA (16S rDNA) gene for the detection of bacterial pathogens. Molecular analysis based on PCR of 16S rRNA (16S rDNA) gene is revolution in oral microbiology that explores the composition of the subgingival microflora and the true extent of the bacterial communities present, free of the limitations of culture.[10]

The aim of the present study was to detect and compare the frequency of *P. gingivalis* and *T. denticola* in patients with chronic periodontitis (CP) as well as AgP exhibiting different levels of destruction using PCR assay and correlation with clinical periodontal parameters such as plaque index (PI), gingival index (GI), periodontal pocket depth (PPD), and clinical attachment level (CAL).

Materials and Methods

This study was approved by GDCH, Nagpur and MUHS, Nashik (MH) Ethics Committee. Participants in the present study included thirty healthy participants and sixty periodontitis patients. Periodontitis patients were divided into two groups: CP ($n = 30$) and AgP ($n = 30$).

- Group I: Healthy control group
- Group II: Patients of CP ($n = 30$)
- Group III: Patients of AgP ($n = 30$).

All participants were in good general health, and informed consent was obtained from each participant. In healthy participants, alveolar bone loss was not observed, and local deposits were not seen.

Exclusion criteria

- Systemic diseases such as diabetes mellitus and HIV infection
- Environmental factors such as tobacco habits and smoking in any form and emotional stress
- Patients had periodontal treatment or antimicrobial medication during the last 6 months
- Patients with gingival enlargement were excluded from the study.

The following characteristics are used to diagnose patients of CP.[1]

- CP patients had at least four sites showing ≥3 mm clinical attachment loss (CAL)
- Amount of destruction consistent with local factors
- Horizontal bone loss radiographically
- Subgingival calculus frequently found
- Slow to moderate rate of progression with possible periods of rapid progression.

AgP is recognized as a specific type of periodontitis and following diagnostic criteria are used for this:[1]

- Rapid attachment loss
- AgP patients had at least four sites showing ≥3 mm CAL
- Vertical or saucer-shaped bone destruction seen radiographically specially with central incisors and first molars
- Amount of destruction not consistent with local factors
- Fair oral hygiene
- Familial aggregation.

Clinical evaluation

All clinical parameters were evaluated by the one investigator. Moreover, following parameters were recorded at plaque sampling sites:[11]

a. PI
b. GI
c. Periodontal pocket depth (PPD)
d. CAL.

Microbial sampling

Entire periodontium was evaluated, and sampling sites were isolated with cotton rolls and air-dried after thorough removal of supragingival plaque with sterile cotton pellets. Subgingival plaque samples were collected from the deepest periodontal pocket of any first molar in each patient. Plaque sample was collected from the depth of the periodontal pocket by a single stroke of Gracey curette and immediately suspended in 50 µl of Tris-HCl buffer.[12] Plaque samples

were immediately incubated at 50°C for 10 min and then stored at −20°C (Cryo Scientific) and further processed after collection of all samples (after 6 months).

DNA extraction and nested polymerase chain reaction detection

After thawing, the 50 µl of Tris-HCl buffer containing plaque sample was incubated at 65°C for 15 min. Samples were centrifuged at 10,000 rpm for 5 min and supernatant was discarded. The washed cells were then suspended in 25 µl of 0.5N NaOH solution. Care was taken to prevent the formation of any air bubbles for efficient lysis of cell wall and release of DNA. The suspension was incubated at room temperature for 30 min. This was followed by addition of 25 µl of 1 M Tris buffer (pH 7.5) for neutralization and the solution was mixed. Final volume was made by adding 450 µl of sterile distilled water and an even mixing was brought about by vortex mixing and stored at −20°C (Cryo Scientific) till PCR.

In the first amplification, the bacterial 16S rRNA gene was amplified by PCR using universal primers 27F and 1492R and Taq DNA polymerase according to the manufacturer's instructions. The primer sequences were 27F, 5'-AGA GTT TGA TCC TGG CTC AG-3'; and 1492R, 5'-TAC GGG TAC CTT GTT ACG ACT T-3'. The PCR mixtures were 5 µl of genomic DNA, and 95 µl of reaction mixture amplified in a PCR thermal cycler (BIO-RAD, USA) and the predicted PCR product was obtained at 1505 bp in length with universal primers.[13]

These two microorganisms were identified by amplification of the first PCR amplification products using species-specific primers based on 16S rRNA gene sequences. The reaction mixture for each *P. gingivalis/T. denticola* contained 5.0 µl 10X PCR buffer (10 mMol), 2.5 µl dNTPs (200 mMol), 2 µl primer of each (25 pmol), 1 µl Taq DNA polymerase (Cinna Gen Co, Iran) (1 U), 3 µl $MgCl_2$ (3 mM), 5.0 µl DNA template of each microorganism, and water (milli-Q) 31.5 µl. Species-specific primer sequences were used:[13]

P. gingivalis P1 (forward)-5'GCG TAT GCA ACT TGC CTT AC 3'

P2 (backward)-5'GTT TCA ACG GCA GGC TGA AC 3'

T. denticola P1 (forward)-5'TAA TAC CGA ATG TGC TCA TTT ACA T 3'

P2 (Backward)-5'TCA AAG AAG CAT TCC CTC TTC TTC TTA 3'

The PCR amplification was performed in a DNA thermal cycler (BIO-RAD, USA). The temperature profile for *P. gingivalis* and *T. denticola* included an initial denaturation step at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min and extension at 72°C for 1 min, and then final extension step at 72°C for 10 min in thermal cycler.[14] The expected amplicon 518 bp for *P. gingivalis* and 316 bp for *T. denticola* was analyzed on comparison with the standard 1 kb ladder (Invitrogen Corp., Carlsbad, CA, USA) on a 1.5% agarose gel (Merck, Germany) electrophoresis performed at 4V/cm in Tris-acetate ethylenediaminetetraacetic acid buffer. The gel was stained with 0.01 µg/ml ethidium bromide and visualized under 300 nm UV light on Biorad XR (California, USA) gel documentation unit.

Statistical analysis

Statistical software STATA Version 10.0 was used for data analysis. All the variables were presented as a mean ± standard deviation. The detection frequency of *P. gingivalis* and *T. denticola* was expressed in actual numbers and percentages. PI, GI, PPD, and CAL were compared in healthy, chronic, and aggressive using one-way analysis of variance. Multiple comparisons were performed using Bonferroni test. Odds ratio and 95% confidence interval were calculated to find association between CP and AgP patients and presence of *P. gingivalis* and *T. denticola*. The Chi-square test or linear trends were used for association between PI, GI, and CAL and detection frequencies of *P. gingivalis and T. denticola* in CP and AgP. $P < 0.05$ was considered statistically significant.

Results

In the present study, all the AgP patients (Group III) were younger than patients of CP (Group II) and healthy participants were in comparable age group. No statistically significant difference was observed in gender-wise distribution in all study groups [Table 1]. The mean values of PI, GI, PPD, and CAL were lower in healthy participants than those of CP or AgP patients. However, the degree of gingival inflammation and plaque accumulation was less in AgP patients than CP patients [Table 2].

P. gingivalis and *T. denticola* were detected with more frequency at sites that showed deep periodontal pockets and severe attachment loss and increased severity of gingival inflammation in both CP and AgP patients. The Chi-square test for linear trends proved a statistically significant association between PI, GI, and CAL with the presence of *P. gingivalis* and *T. denticola* in CP and AgP patients.

Table 1: Age and gender distribution in healthy, chronic periodontitis, and aggressive periodontitis patients group

	Healthy (30)	CP (30)	AgP (30)
Gender			
Male	16	19	13
Female	14	11	17
Age (mean age±SD)	31.13±5.97	37.5±6.39	21.26±3.27

CP: Chronic periodontitis, AgP: Aggressive periodontitis, SD: Standard deviation

Table 2: Comparing mean level of clinical periodontal parameters (plaque index, gingival index, periodontal pocket depth, and clinical attachment loss) in chronic and aggressive periodontitis patients[7]

Variables	Group	Mean±SD	*F*	Multiple comparison (Bonferroni correction)		
				Healthy versus CP patients	Healthy versus AgP patients	AgP versus CP
PI	Healthy	0.48±0.24	115.47	*P*<0.01*	*P*<0.01*	1.21 *P*>0.05
	CP	2.45±0.43				
	AgP	1.24±0.72				
GI	Healthy	0.14±0.21	204.93	*P*<0.01*	*P*<0.01*	1.09 *P*>0.05
	CP	2.55±0.43				
	AgP	1.46±0.63				
PPD	Healthy	1.96±0.76	96.72	*P*<0.01*	*P*<0.01*	0.36 *P*>0.05
	CP	7.93±2.13				
	AgP	8.30±2.52				
CAL	Healthy	1.02±0.36	93.56	*P*<0.01*	*P*<0.01*	0.41 *P*>0.05
	CP	7.93±2.14				
	AgP	8.13±2.41				

**P*<0.05 then it was considered significant (S). PI: Plaque index, GI: Gingival index, PPD: Periodontal pocket depth, CAL: Clinical attachment loss, CP: Chronic periodontitis, AgP: Aggressive periodontitis, SD: Standard deviation

Thus, it was observed that while comparing the detection frequency of *P. gingivalis* and *T. denticola* between healthy individuals and CP or AgP, a significant difference was observed. Whereas comparing the detection frequency of *P. gingivalis* between CP and AgP, a nonsignificant difference was observed [Table 3].

The odds ratio was more than two in any combination of bacteria and plaque samples. In particular, *P. gingivalis* and *T. denticola* were frequently detected in plaque samples, and this combination showed the highest odds ratio in AgP patients with *P. gingivalis* [Table 4].

In particular, *T. denticola* was frequently detected together with *P. gingivalis* in plaque samples [Table 5].

Discussion

The periodontal disease is significantly associated with a multiple number of periodontal pathogens such as *P. gingivalis*, *T. denticola,* and *T. forsythensis*. It is well established that periodontal disease occurs as a result of subgingival plaque with specific bacteria, particularly Gram-negative anaerobes.[15]

These red complex species appears later in biofilm development, and these are considered periodontal pathogens, namely, *P. gingivalis*, *T. denticola,* and *T. forsythia*.[3] Out of the three members of red complex, *P. gingivalis* and *T. denticola* have been strongly associated in the pathogenesis of periodontitis.[4] In the present study, PCR technique was employed for detecting the presence of *P. gingivalis* and

Table 3: Bacterial detection frequencies in all three groups[7]

Group (%)	*Porphyromonas gingivalis* (%)	*Treponema denticola* (%)
Healthy	3 (10)	4 (13.3)
CP	22 (73.30)	23 (76.7)
AgP	24 (80)	24 (80)

**P*<0.001 (found significant at 5% level of significance, when compared CP and AgP with Healthy). CP: Chronic periodontitis, AgP: Aggressive periodontitis

Table 4: Odds ratios of association among *Porphyromonas gingivalis* and *Treponema denticola* in plaque samples of chronic and aggressive periodontitis patients (compared with healthy)

Organism	AgP	CP
Porphyromonas gingivalis	20.0** (4.64–92.49)	13.75** (3.42–59.77)
Treponema denticola	16.0** (3.91–69.26)	13.14** (3.34–54.50)

**OR (95% CI). CI: Confidence interval, CP: Chronic periodontitis, AgP: Aggressive periodontitis, OR: Odds ratio

Table 5: Coinfection of *Porphyromonas gingivalis* and *Treponema denticola* in study groups

Porphyromonas gingivalis and *Treponema denticola*	Number of cases	Percentage
Healthy	2	6.66
CP	24	80
AgP	21	70

CP: Chronic periodontitis, AgP: Aggressive periodontitis

T. denticola and association of the detection frequency of *P. gingivalis* and *T. denticola* with that of clinical periodontal parameters, i.e. PI, GI, PPD, and CAL were also assessed.

In the present study, detection frequency of both microorganisms differed in various groups; in healthy control participants, *P. gingivalis* was detected in only 10% and *T. denticola* was detected in only 13.33% patients.

For a bacterial species to be considered as a periodontal pathogen, it is expected to exist infrequently in participants with a healthy periodontium. Several reasons have been hypothesized for the presence of periodontal pathogens in healthy participants. One explanation for the presence of such species in healthy participants might be related to the difference in the virulence of such bacterial strains. The organisms harbored by individuals with healthy periodontium are probably strains of low virulence.[16]

The study data showed an increase in the detection frequency of *P. gingivalis* and *T. denticola* with increasing periodontal pocket depth. Socransky and Haffajee[3] compared the microflora of periodontal pockets and found a higher prevalence of *P. gingivalis* and *T. denticola* in deep pockets than in shallow pockets.[3]

Similarly, in the present investigation, we found an increase in the detection frequency of both the microorganisms with increasing periodontal pocket depth in AgP patients also. Takeuchi *et al*.[14] found the detection frequency of both microorganisms in deep periodontal pockets. Thus, it can be inferred from all the studies done including this study that there is a definite increase in the levels of *P. gingivalis* and *T. denticola* with increasing periodontal pocket depth indicating the increasing severity of the disease. However, the exact reason for the increase in frequency of such pathogenic bacteria in AgP and CP is not known.[17] The reasons suggested for such findings are the higher levels of anaerobiosis at deeper sites, i.e., reduced oxygen tension, differences in subgingival temperature, requirement for hemin or other substances, and more easy supply of nutritional materials from gingival crevicular fluid. Thereby providing a more conducive environment for growth of fastidious, anaerobic microorganisms.

The above findings revealed that the detection frequency of *P. gingivalis* and *T. denticola* in both CP and AgP was more or less similar. Similar prevalence of periodontopathic bacteria was also found in the Japanese population.[14]

The present study observed that a statistically significant association exists between clinical periodontal parameters such as PI, GI, PPD, and CAL and presence of both the organisms. This association showed statistically significant increase with an increase in pocket depth in both CP and AgP patients. Colombo *et al*.[16] found that *P. gingivalis* and *T. denticola* play a significant positive role in the more severe forms of AgP.

In the present study, *P. gingivalis* and *T. denticola* were frequently detected together in the same plaque samples. This coexistence is frequently seen in deep periodontal pockets (PPD >5 mm) in both CP as well as AgP patients. This coexistence of *P. gingivalis* and *T. denticola* might be because of some factors produced by *P. gingivalis* which are responsible for the growth of *T. denticola* in the deep periodontal pockets.

Simonson *et al*.[18] had reported that *P. gingivalis* provides one or more factors necessary for the colonization and/or proliferation of *T. denticola*. Nilius *et al*.[19] had identified heat-labile growth factor activity in culture filtrates of *P. gingivalis* and these growth factors produced by *P. gingivalis* may be important for the growth of *T. denticola* in the microenvironment of the periodontal pocket.

Thus, it seems that the presence of these two microorganisms alone is not sufficient to cause disease. Other factors necessary for disease progression should also be considered such as high levels of one or more specific pathogens, low levels of beneficial species, and environmental and genetic factors associated with susceptible host.

The present study highlighted the association of *P. gingivalis* and *T. denticola* with CP as well as AgP. However, more extensive studies are advocated to correlate the periodontopathogens as well as to evaluate the virulence factors such as fimbriae, capsular polysaccharides, and hemagglutinin with *P. gingivalis* and hemolysins, esterases, collagenase, and phospholipase with *T. denticola*, which all contribute in pathogenicity of periodontal disease.

The development of analysis of genomic data makes it possible to explore the patterns of gene expression of these bacteria and thus better define the pathogenesis of the disease. Integration of this information provides the basis for proactive approaches for prevention, diagnosis, and treatment of periodontal disease.

Conclusion

P. gingivalis and *T. denticola* were frequently detected in periodontitis patients by PCR. The prevalence of these two microorganisms was correlated with various clinical parameters. Our data suggest that their presence is associated with the severity of periodontal tissue destruction.

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Conflicts of interest
There are no conflicts of interest.

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