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ORIGINAL ARTICLE

Detection of red complex organisms in chronic periodontitis by multiplex polymerase chain reaction

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Abstract

Background: Periodontitis is widely regarded as the second most common disease worldwide after dental decay. The trigger for the initiation of the disease is the presence of complex microbial biofilm in the subgingival area. The Gram-negative anaerobic bacteria have been found to predominate in the subgingival niche. The red complex, which includes *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* encompasses the most important pathogens in chronic periodontitis. The data of these microorganisms in periodontal disease in our country is sparse, hence, the aim of the study.
Materials and Methods: A total of 170 subjects from both the sexes and within an age group of 21–85 years with chronic periodontitis were selected. Sub-gingival pooled plaque sample was collected by sterile Gracey curette from two deep periodontal pockets (probing depth [PD] ≥5 mm) DNA was isolated using in-house laboratory and multiplex polymerase chain reaction was carried out.
Results: The mean gingival index, PD, and clinical attachment level were calculated, and the prevalence of *P. gingivalis*, *T. denticola,* and *T. forsythia* in individual and combination was also detected. The statistical analysis for the above-mentioned parameters was done using the software.
Conclusion: It was observed that there was a statistically significant increase in red complex bacteria in the age group >41 years, although no gender bias was seen. Out of 170 subjects, 106 showed positive for red complex organisms, either singly or in varied combinations.

Introduction

Periodontitis is regarded as the second most common dental disease worldwide after dental decay. It is caused by microorganisms that adhere to and grow on the tooth surfaces, along with an 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.[1] Periodontal diseases are multifactorial infections elicited by the interaction between various bacterial species and the host immune response toward them. The trigger for the initiation of the disease is the presence of complex microbial biofilm in the subgingival area that houses over 700 bacterial species and phylotypes.[2] 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, and 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 red complex encompasses the most important pathogens in adult periodontal disease.[6] These bacteria are not usually found alone, but in combination in the periodontal pockets, suggesting that some bacteria may cause the destruction of the periodontal tissue in a cooperative manner.[7]

These organisms express a number of potential virulence factors and induce host inflammatory mediators, eventually leading to connective tissue breakdown and alveolar bone resorption.[8]

The data of these microorganisms in periodontal disease in our country is sparse, hence, the aim of the study.

Materials and Methods

This study is a part of the research grant funded by the Indian Council of Medical Research (ICMR) entitled "identification of virulence factors of *T. forsythia* in patients with chronic periodontitis using *in vivo* induced antigen technology."

The ethical clearance from the Institutional Review Board was obtained before the start of the study.

From the subjects who visited the Dental OPD at Maratha Mandal's NGH Institute of Dental Sciences, Belgaum, 170 patients with chronic periodontitis were included in this study. These comprised patients from both the sexes and within an age group of 21–85 years. The other parameters considered were periodontal pocket depth ≥5 mm, clinical attachment loss ≥3 mm and bleeding on probing. The subjects with any systemic diseases/medically compromised or who have received periodontal therapy, antibiotics/antimicrobials within 3 months before sampling, pregnant and lactating women, tobacco habitures were excluded in this study.

The subjects were scrutinized by the recording of probing pocket depth (PD), clinical attachment level (CAL), plaque index,[9] and gingival index (GI).[10]

According to study requirements, the tooth with pocket was first identified, isolated with cotton roll and cleared of the supragingival plaque. Sub-gingival pooled plaque sample was then collected by sterile Gracey curette from two deep periodontal pockets (PD ≥5 mm). This was transferred to a vial containing 1 ml of reduced transport fluid and sent to the laboratory for further processing.

DNA was isolated using the in-house laboratory protocol with Proteinase K. Samples were thoroughly vortexed and then centrifuged at 5000 rpm for 5 min. The supernatant fluid was discarded, and the pellet was washed with fresh Tris EDTA buffer 2–3 times. Bacterial cells were broken down using lysis buffer I (containing 10 mm Tris buffer and 1 mm EDTA) and lysis buffer II (containing 50 mm Tris HCl, 50 mm KCL, $MgCl_2$ 2.5 mm, Tween 20 0.45%, and nonident-P 0.45%). Following lysis, 5 µl of Proteinase K (10 mg/ml) was added to degrade the protein contaminants. The samples were incubated in a water bath at 60°C for 2 h followed by enzyme deactivation by keeping in boiling water for 10 min. Samples were centrifuged at 5000 rpm for 5 min, and the supernatant containing DNA was collected in a fresh microcentrifuge tube. These were stored at 20°C until further use in a polymerase chain reaction (PCR).[11]

Multiplex PCR was carried out in 0.2 ml PCR tubes in a Veriti Thermal Cycler (Applied Biosystems, California, USA) as described by D'Ercole *et al*.[12] Species-specific 16S rRNA gene of each bacterium was targeted for the PCR amplification. The primer sequences and their specific amplified lengths were as given in Table 1. PCR reaction mixture was prepared in total 25 µl volume. Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany) was used to prepare the reaction mixture which contained ×10 PCR buffer containing 15 mm $MgCl_2$, 10 mm dNTP mix and 2.5 units/reaction of Taq DNA polymerase. Primers were added at 8 pmol/µl in the mixture. 2 µl of DNA template with approximately 100 µg/ml concentration was added. The total volume to 25 µl was made up with PCR grade water. The tubes were kept in verity thermal cycler and run the thermal cycling conditions as follows; initial denaturation at 95°C for 15 min followed by 35 cycles of denaturation at 95°C, annealing at 60°C and extension at 72°C for 1 min each. Final extension was done at 72°C for 5 min.

Post-PCR analysis was carried out by running the amplified products on agarose gel electrophoresis at 70 V for 2 h in Tris-acetate buffer. The gel was stained with 0.5 µg/ml ethidium bromide, viewed under UV transilluminator and images were captured on a gel documentation system (Major Science, USA). The bands were detected and identified by comparing with NEX-GEN 100 bp DNA ladder (Genetix Asia Pvt. Ltd, New Delhi, India) which was run simultaneously in each gel.

Results

The study included 170 adult patients with chronic periodontitis in an age range of 21–85 years. Among them, 79 (46.47%) were males and 91 (53.52%) were females with a mean age range of 49.24 ± 13.09 and 44.5 ± 11.24, respectively. There was no significant difference between gender ratios [Table 2].

Various clinical indices were recorded in each participant. The mean GI, PD, and CAL were 1.97 ± 0.56, 5.21 ± 0.86, and 5.97 ± 0.90, respectively. When these readings were compared with the occurrence of red complex bacteria either alone or in different combinations, it could be seen that association of PD and CAL with different bacterial combinations was statistically significant [Table 3].

Out of 170 patients studied, 106 (62.35%) showed the presence of red complex bacteria either singly or in different

Table 1: Primer sequence of red complex microorganisms

Organism	Primer sequence (5'–3')	Amplified product length (bp)
P. gingivalis	Forward: AGG CAG CTT GCC ATA CTG CG Reverse: ACT GTT AGC AAC TAC CGA TGT	404
T. denticola	Forward: TAA TAC CGA ATG TGC TCA TTT ACA T Reverse: TCA AAG AAG CAT TCC CTC TTC TTC TTA	316
T. forsythia	Forward: GCG TAT GTA ACC TGC CCG CA Reverse: TGC TTC AGT GTC AGT TAT ACC T	641

P. gingivalis: Porphyromonas gingivalis, T. denticola: Treponema denticola, T. forsythia: Tannerella forsythia

Table 2: Distribution of red complex bacteria in males and females

Microorganism	Gender	Negative (%)	Positive (%)	Total (%)	Fisher's exact test
T. forsythia	Male	41 (51.89)	38 (48.00)	79 (100.00)	0.67056687, not significant
	Female	51 (56.04)	40 (44.00)	91 (100.00)	
	Total	92 (54.12)	78 (45.88)	170 (100.00)	
P. gingivalis	Male	58 (73.42)	21 (26.58)	79 (100.00)	0.5353, not significant
	Female	62 (68.14)	29 (31.86)	91 (100.00)	
	Total	120 (70.58)	50 (29.41)	170 (100.00)	
T. denticola	Male	45 (56.97)	34 (43.03)	79 (100.00)	0.8844, not significant
	Female	49 (53.85)	42 (46.15)	91 (100.00)	
	Total	94 (55.30)	76 (44.70)	170 (100.00)	

P. gingivalis: Porphyromonas gingivalis, T. denticola: Treponema denticola, T. forsythia: Tannerella forsythia

Table 3: Correlation of clinical parameters with red complex bacteria in various combinations

Clinical parameters	*T. denticola*		*P. gingivalis*		*T. forsythia*		*T. denticola, P. gingivalis*		*T. denticola, T. forsythia*		*T. forsythia, P. gingivalis*		*T. denticola, P. gingivalis and T. forsythia*	
	R	p	r	p	r	p	r	p	r	p	r	p	r	p
Average GI	0.108	0.161	–0.066	0.393	0.054	0.482	0.009	0.898	0.089	0.247	0.011	0.883	0.080	0.300
Average PD	0.224	0.003	0.126	0.101	0.214	0.005	0.167	0.045	0.289	0.000	0.191	0.012	0.207	0.006
Average CAL	0.223	0.003	0.194	0.010	0.188	0.013	0.183	0.016	0.207	0.006	0.155	0.042	0.142	0.063

r: Correlation coefficient, $P \leq 0.05$ implies significant. *P. gingivalis: Porphyromonas gingivalis, T. denticola: Treponema denticola, T. forsythia: Tannerella forsythia*, GI: Gingival index, PD: Probing depth, CAL: Clinical attachment level

combinations. The analysis of the results of 106 positive samples showed that the prevalence of *P. gingivalis* was 43.48%, *T. denticola* was 66.09%, and *T. forsythia* was 67.84% [Figure 1]. There was no statistically significant difference in the distribution of these bacteria among males and females [Table 2]. In the positive samples, the prevalence of different combinations of red complex bacteria in chronic periodontitis patients is shown in Figure 2. It could be seen that among patients who showed the presence of bacteria, 64.15% were in different combinations of *P. gingivalis* and *T. denticola*, (32.17%), *T. denticola* and *T. forsythia* (45.22%), and *P. gingivalis* and *T. forsythia* (34.78%), or all the three together (25.22%). Samples from remaining patients (35.85%) were positive for any one of the bacteria. Among them, *P. gingivalis* was seen in only 3 patients whereas *T. denticola* and *T. forsythia* were present in 18 and 17 patients, respectively.

We also compared the prevalence of red complex organisms in different age groups of 21–30, 31–40, 41–50, 51–60, and >60 years. There was a statistically significant increase in the prevalence of these bacteria with increasing age, only within the age categories of 41–50, 51–60, 61, and above [Table 4].

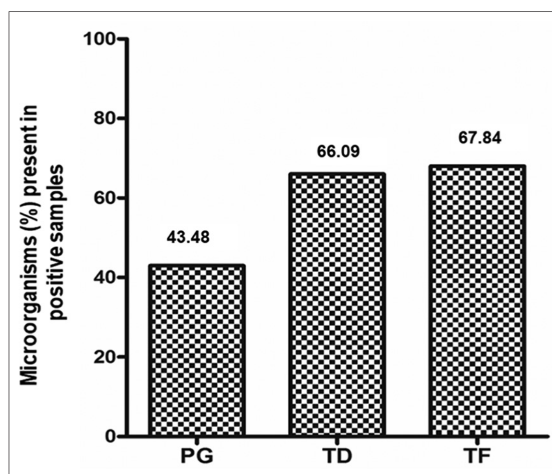

Figure 1: Prevalence of individual microorganisms seen in positive samples (106)

Discussion

The etiology of chronic periodontitis is highly complex and varied. Among the nearly 700 bacterial species that inhabit the subgingival plaque, several have been found to be associated with chronic periodontitis. Among them, *P. gingivalis*, *T. denticola,* and *T. forsythia* which form the red complex are implicated as most common periodontopathogens. These bacteria are usually not found alone but frequently in combination in periodontal pockets suggestive of the cooperative manner of disease production and tissue destruction by them.[13] Several animal studies conducted over the years have also confirmed the synergistic pathogenicity of mixed infections by these bacteria.[14]

All the three members of the red complex are known to produce a range of virulence factors that aid them in survival

in the host and lead to the initiation and progression of the disease. The virulence factors produced by *P. gingivalis* include cysteine proteases such as gingipains, hemagglutinins, lipopolysaccharides, fimbriae, and heat shock proteins.[15] Some of the factors responsible for the pathogenicity of *T. forsythia* are proteases, sialidases, hemagglutinins, and surface-layer associated glycoproteins.[16] *T. denticola* is known to produce dentilisin, major outer sheath protein OppA, cystalysin, and heme binding proteins as virulence factors.[17] Studies have also shown that interspecies interactions between these bacteria occur *in vivo* indicating nutritional interdependency and modulation of virulence factors of each other.[13] Since all the three members of red complex are anaerobes and nutritionally demanding and fastidious, it is difficult to isolate and identify by culture. Hence, PCR is the most commonly used technique for their identification from clinical samples.[18]

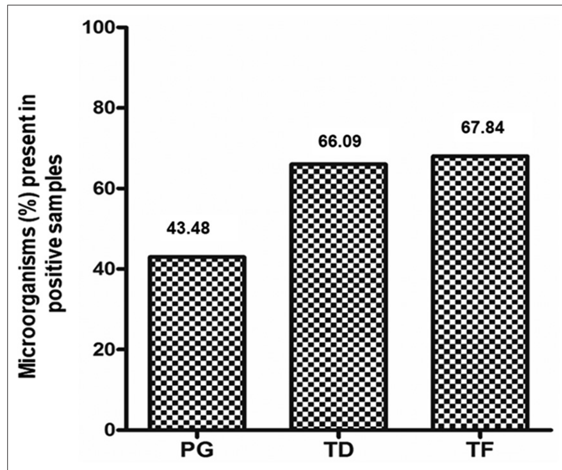

Figure 2: Combination of microorganisms among positive samples (106)

In the present study, out of 170 subgingival plaque samples tested from patients with chronic periodontitis, 106 (62.35%) showed the presence of one or the other red complex bacteria either singly or in different mixtures. The occurrence of *P. gingivalis*, *T. denticola,* or *T. forsythia* in these patients was 43.48%, 66.09%, and 67.84%, respectively. These prevalence rates are in agreement with findings of other workers.[19-21]

When the clinical indices in all 170 patients were compared with the prevalence of these bacteria, a significant correlation could be seen with pocket depth and clinical attachment loss. Similar findings have also been reported by other investigators.[1,22] Surprisingly, the results with GI were non-significant.

The red complex bacteria are known to occur together in plaque samples often adjacent to the epithelial lining of the periodontal pocket in deeper areas. This is mainly due to interspecies interaction, coaggregation, and metabolic interdependency among these three bacterial species.[13,18] In our study as well, among positive samples (106), 64.15% showed the presence of one or the other red complex bacteria in different combinations. Another interesting observation was that *P. gingivalis* alone could be detected in only three patients whereas the numbers of other two bacteria as the sole organisms detected were much higher. It has been documented that *P. gingivalis*

Table 4: Distribution of red complex bacteria in various age categories

Age categorized (Years)	Microorganisms	PCR		Total	Fisher's exact test
		Negative	Positive		
21–30	*T. denticola*	13 (61.91)	8 (38.09)	21 (100.00)	<0.0924, not significant
	P. gingivalis	15 (71.43)	6 (28.57)	21 (100.00)	
	T. forsythia	16 (76.20)	5 (23.80)	21 (100.00)	
31–40	*T. denticola*	19 (55.89)	15 (44.11)	34 (100.00)	<0.1000, not significant
	P. gingivalis	21 (61.77)	13 (38.23)	34 (100.00)	
	T. forsythia	16 (47.06)	18 (52.94)	34 (100.00)	
41–50	*T. denticola*	27 (48.22)	29 (51.78)	56 (100.00)	<0.0026, Significant
	P. gingivalis	38 (67.86)	18 (32.14)	56 (100.00)	
	T. forsythia	26 (46.43)	30 (53.57)	56 (100.00)	
51–60	*T. denticola*	17 (47.23)	19 (52.77)	36 (100.00)	<0.0003, significant
	P. gingivalis	27 (75.00)	9 (25.00)	36 (100.00)	
	T. forsythia	20 (55.56)	16 (44.44)	36 (100.00)	
>61	*T. denticola*	18 (78.27)	5 (21.73)	23 (100.00)	<0.001, significant
	P. gingivalis	19 (82.61)	4 (17.39)	23 (100.00)	
	T. forsythia	14 (60.87)	9 (39.13)	23 (100.00)	

Interpretation: Statistically significant concentrations of *P. gingivalis, T. forsythia*, and *T. denticola* were seen in chronic periodontitis patients only within the age categories of 41–50, 51–60, 61, and above when compared by Fisher's exact test. *P. gingivalis: Porphyromonas gingivalis, T. denticola: Treponema denticola, T. forsythia: Tannerella forsythia*, and PCR: Polymerase chain reaction

usually does not occur alone in periodontal pockets in the absence of *T. forsythia*.[18]

When the results of our study of 170 participants were analyzed based on the prevalence in different age groups, it was observed that there was an increase in the prevalence of red complex bacteria with advancing age and this association was highly significant. Similar observation about periodontopathogens has been made by earlier investigators.[23]

There is a scarcity of adequate data on the prevalence of red complex organisms in chronic periodontitis in our country. It is established as well, that periodontitis has a multifactorial etiology, the microbial being albeit an important one. Results of our multiplex PCR study sheds some light on the manifestation of the red complex organisms, their interdependency on each other and their ability to elicit pathological changes in the periodontium. Within the limits of our study, as we could not collect data from healthy individuals, comparative assessment between healthy participants and those with chronic periodontitis could not be done. More light can be shed if healthy sample data can be compared with chronic periodontitis and this could certainly fortify conclusions that can be drawn about the role of the red complex bacteria on periodontal health.

It could be fair to say that not only can there be antagonist interactions between microbial species but also collaborative ventures too, which run in parallel streams. The host with the defenses is also an integral part of the scheme, and the eventual outcome of these relations is responsible for either a healthy periodontium or an ailing one. It releases questions whether other microbes apart from the red complex also play a pivotal role in eliciting a pathological change in periodontally healthy tissues.

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Conflicts of Interest and Sources of Funding

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References

1. Mahalakshmi K, Krishnan P, Chandrasekaran SC, Panishankar KH, Subashini N. Prevalence of periodontopathic bacteria in the south subgingival plaque of a South Indian population with periodontitis. J Clin Diagn Res 2012;6 Suppl 2:747-52.
2. Paster BJ, Olsen I, Aas JA, Dewhirst FE. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. Periodontol 2000 2006;42:80-7.
3. Marcotte H, Lavoie MC. Oral microbial ecology and the role of salivary immunoglobulin A. Microbiol Mol Biol Rev 1998; 62:71-109.
4. Slots J, Rams TE. New views on periodontal microbiota in special patient categories. J Clin Periodontol 1991;18:411-20.
5. Slots J. Microflora in the healthy gingival sulcus in man. Scand J Dent Res 1977;85:247-54.
6. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998; 25:134-44.
7. Darveau RP, Tanner A, Page RC. The microbial challenge in periodontitis. Periodontol 2000 1997;14:12-32.
8. Offenbacher S. Periodontal diseases: Pathogenesis. Ann Periodontol 1996;1:821-78.
9. Loe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. Acta Odontol Scand 1963;21:533-51.
10. Löe H. The gingival index, the plaque index and the retention index systems. J Periodontol 1967;38:610-6.
11. Kugaji MS, Bhat KG, Joshi VM, Pujar M, Mavani PT. Simplified method of detection of *Dialister invisus* and *Olsenella uli* in oral cavity samples by polymerase chain reaction. J Adv Oral Res 2017;8:47-52.
12. D'Ercole S, Piccolomini R, Capaldo G, Catamo G, Perinetti G, Guida L, *et al.* Effectiveness of ultrasonic instruments in the therapy of severe periodontitis: A comparative clinical-microbiological assessment with curettes. New Microbiol 2006; 29:101-10.
13. Suzuki N, Yoneda M, Hirofuji T. Mixed red-complex bacterial infection in periodontitis. Int J Dent 2013;2013:587279.
14. Kesavalu L, Sathishkumar S, Bakthavatchalu V, Matthews C, Dawson D, Steffen M, *et al.* Rat model of polymicrobial infection, immunity, and alveolar bone resorption in periodontal disease. Infect Immun 2007;75:1704-12.
15. Zenobia C, Hajishengallis G. *Porphyromonas gingivalis* virulence factors involved in subversion of leukocytes and microbial dysbiosis. Virulence 2015;6:236-43.
16. Sharma A. Virulence mechanisms of *Tannerella forsythia*. Periodontol 2000 2010;54:106-16.
17. Ishihara K. Virulence factors of *Treponema denticola*. Periodontol 2000 2010;54:117-35.
18. Holt SC, Ebersole JL. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: The "red complex", a prototype polybacterial pathogenic consortium in periodontitis. Periodontol 2000 2005;38:72-122.
19. Joshi VM, Bhat KG, Kugaji MS, Ingalagi PS. Prevalence of *Porphyromonas gingivalis* and its relationship with herpes virus in Indian subjects with chronic periodontitis: A cross-sectional study. J Int Clin Dent Res Organ 2016;8:106-10.
20. Wadhwani RB, Chaudhary MS, Tharani DA, Chandak SA. Effect of scaling and root planing on detection of *Tannerella forsythia* in chronic periodontitis. J Oral Dis 2013;2013:6.
21. Kishi M, Ohara-Nemoto Y, Takahashi M, Kishi K, Kimura S, Yonemitsu M, *et al.* Relationship between oral status and prevalence of periodontopathic bacteria on the tongues of elderly individuals. J Med Microbiol 2010;59:1354-9.
22. Medina AC, Garces AA, Zuluaga AC. Co-existence of *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema*

denticola in the red bacterial complex in chronic periodontitis subjects. Int J Odontostomatol 2014;8:359-64.

23. Griffen AL, Becker MR, Lefons SR, Moeschberger ML, Leys EU. Prevalence of *Porphyromonas gingivalis* and periodontal health status. J Clin Microbiol 1998;36:3239-42.

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