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Original Article

Dentistry (Periodontics) Section

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 poly-microbial 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 ≥ 5mm 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 ≤ 3mm 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 mMol/L of each dNTP (Medox Biotech India Pvt Ltd), 1.5mM $MgCl_2$(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 1min, 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 10min.

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 [8]. The primer for *A. actinomycetemcomitans* 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

The mean 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%

	Periodontitis	Health	p
Age ‡	34.26±10.23†	26.09/±7.70†	<0.0001**
Gender(%)males/ females*	108/92	115/85	= 0.5
Mean PPD(mm) ‡	7.52±1.57†	2.00±0.07†	<0.0001**
Mean CAL(mm) ‡	9.67±1.70†	3.00±0.00†	<0.0001**

[Table/Fig-1]: Clinical parameters (Mean ± Standard deviation) of periodontitis patients and healthy subjects

* Refers to Chi-square test; † Standard deviation (SD); ‡ Mann-Whitney test; ** significant *p* value

Gram negative anaerobes	ChP (n=128)	AgP (n=72)	Health (n=200)
P.gingivalis	103 (80.50%)	53 (73.60%)	22 (11.00%)
T.forsythia	94 (73.40%)	43 (59.70%)	21 (10.50%)
T.denticola	91 (71.1%)	24 (33.30%)	11 (5.50%)
A.actinomy-cetemcomitans	41 (32.00%)	44 (61.10%)	5 (2.50%)
C.rectus	22 (17.20%)	8 (11.10%)	17 (8.50%)
E.corrodens	20 (15.60%)	8 (11.10%)	12 (6.00%)
P.intermedia	21 (16.40%)	18 (25.00%)	15 (7.50%)
P.nigrescens	17 (13.30%)	10 (13.90%)	28 (14.00%)

[Table/Fig-2]: Percentage prevalence of 8 anaerobic gram negative bacilli

of the AgP 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.

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.intermedia* 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 periodontopathic 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

	P. gingivalis	*T. forsytha*	*T. denticola*	*A. actinomy-cetemcomitans*	*C. rectus*	*E. corrodens*	*P. intermedia*	*P. nigrescens*
P.gingivalis		1.63	2.62*	4.79*	20.09*	21.77*	14.63*	22.71*
T. forsythia			1.60	2.94*	12.32*	13.35*	8.97*	13.93*
T. denticola				1.83	7.66*	8.31*	5.58*	8.66*
A.actinomy-cetemcomitans					4.18*	4.54*	3.05*	4.73*
C.rectus						1.08	0.72	1.13
E. corrodens							0.67	1.04
P. intermedia								1.55
P.nigrescens								

[Table/Fig-3]: The odds ratio of associations among species tested from 200 periodontitis subjects.

*$P<0.01$.

Bacterial species	Odds Ratio	95% confidence interval		p-value
		Lower limit	Upper limit	
P.gingivalis	28.68	16.46	49.97	<0.0001**
T.forsythia	18.53	10.78	31.86	<0.0001**
T.denticola	23.24	11.90	45.40	<0.0001**
A.actinomycetemcomitans	28.82	11.36	73.12	<0.0001**
C.rectus	1.89	1.01	3.56	0.062491
E.corrodens	2.55	1.25	5.17	0.012419
P.intermedia	2.98	1.58	5.6	0.000763
P.nigrescens	0.95	0.54	1.69	1

[Table/Fig-4]: The odds ratio of bacterial species tested with periodontitis and health
** Significant p value

Bacterial species	Odds Ratio	95% confidence interval		p-value
		Lower limit	Upper limit	
P.gingivalis	1.47	0.75	2.92	0.345477
T.forsythia	1.86	1.01	3.44	0.064802
T.denticola	4.91	2.64	9.16	<0.0001**
A.actinomycetemcomitans	0.29	0.16	0.55	<0.000121**
C.rectus	1.66	0.69	3.95	0.342782
E.corrodens	1.48	0.62	3.55	0.502335
P.intermedia	0.58	0.29	1.19	0.197603
P.nigrescens	0.94	0.41	2.20	0.920344

[Table/Fig-5]: The odds ratio of bacterial species tested with ChP and AgP
**Significant p value.

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 *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 ($p< 0.0001$) for *T. denticola*. 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 Moter *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 Botero *et al*'s findings [16].

A statistical significance was observed between the patients and the healthy group for *P.intermedia* ($p=0.044$), but not 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].

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/ P.intermedia* and *E.corrodens / P.intermedia* suggested their negative association.

CONCLUSION

The odds of detecting *P. gingivalis, T.forsythia, T.denticola* and *A.actinomycetemcomitans* 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.actinomycetemcomitans* suggested a strong association between them and periodontitis (ChP and AgP). In aggressive periodontitis, in addition to *A.actinomycetemcomitans,* the prevalence of *P.gingivalis* and *T.forsythia* was also high. The significant odds ratio for *T.denticola* and *A.actinomycetemcomitans* 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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REFERENCES

[1] Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, *et al*. The bacterial diversity in the human subgingival plaque. J Bacteriol 2001; 183: 3770-83.
[2] Socransky SS. Haffajee AD. Evidence of the bacterial etiology: a historical perspective. Periodontol 2000 1994; 5: 7-25.
[3] Marcotte H. Lavoie MC. Oral microbial ecology and the role of the salivary immunoglobulin A. Microbiol Mol Biol Rev 1998; 62: 71–109.
[4] Slots J. Rams TE. New views on the periodontal microbiota in special patient categories. J Clin Periodontol 1991; 18: 411-20.
[5] Slots J. The 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 RLJr. The microbial complexes in the subgingival plaque. J Clin Periodontol 1998; 25: 134-44.
[7] Wu Y, Yan J, Chen L, Gu Z. Association between infection of different strains of *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* in subgingival plaque and the clinical parameters in chronic periodontitis. J Zhejiang Univ Sci *B* 2007; 8: 121-31.
[8] Ashimoto A, Chen C, Bakker I Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in the subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol Immunol 1996; 11: 266-73.
[9] Slots J, Ashimoto A, Flynn MJ, Li GL, Chen C. Detection of putative periodontal pathogens in subgingival specimens by 16S ribosomal DNA amplification with the polymerase chain reaction. Clin Infect Dis 1995; 20: 304-07.
[10] Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Microbial composition of the supra- and the subgingival plaque in subjects with adult periodontitis. J Clin Periodontol 2000b; 27: 722-32.
[11] Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. J Clin Periodontol 2000a; 27: 648-57.
[12] Nishihara T. Koseki T. Microbial Aetiology of periodontitis. Periodontol 2000. 2004; 36: 14–26.
[13] Noiri Y, Li L, Ebisu S. The localization of periodontal disease which was associated bacteria in the human periodontal pockets. J Dent Res 2001; 80: 1930–34.
[14] Socransky SS. Haffajee AD. Periodontal microbial ecology. Periodontol 2000. 2005; 38: 135–87.
[15] Takeuchi Y, Umeda M, Ishizuka M, Huang Y, Ishikawa I. Prevalence of periodontopathic bacteria in the aggressive periodontitis patients in a Japanese population. J Periodontol 2003; 74: 1460-69.
[16] Botero JE, Contreras A, Lafaurie G, Jaramillo A, Betancourt M, Arce RM. Occurrence of periodontopathic and superinfecting bacteria in the chronic and aggressive periodontitis subjects in a Colombian population. J Periodontol 2007; 78: 696-704.
[17] Griffen AL, Becker MR, Lyons SR, Moeschberger ML, Leys EJ. Prevalence of *Porphyromonas gingivalis* and the periodontal health status. J Clin Microbiol 1998; 36: 3239-42.
[18] Kumar PS, Griffen AL, Barton JA, Paster BJ, Moeschberger ML, Leys EJ. New bacterial species which are associated with chronic periodontitis. J Dent Res 2003; 82: 338–44.
[19] Yang HW, Huang YF, Chou MY. Occurrence of *Porphyromonas gingivalis* and *Tannerella forsythensis* in periodontally diseased and healthy subjects. J Periodontol 2004a; 75: 1077-83.
[20] Chen L, Wu Y, Yan J, Sun W, Sun Y, David, O. Association between the co infection of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Treponema denticola* and the periodontal tissue destruction in chronic periodontitis. Chin Med J 2005; 118: 915-21.
[21] Lopez NJ. Occurrence of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia* in progressive adult periodontitis. J Clin Periodontol 2000; 71: 948–54.
[22] Faghri J, Moghim SH, Abed AM, Rezaei F, Chalabi M. Prevalence of *Porphyromonas gingivalis* and *Bacteroides forsythus* in chronic periodontitis, which was detected by multiplex PCR. Pak J Biol Sci 2007; 10: 4123-27.
[23] Lee JW, Choi BK, Yoo YJ, Choi SH, Cho KS, Chai JK. Distribution of periodontal pathogens in Korean aggressive periodontitis. J Periodontol 2003; 74: 1329-35.
[24] Mullally BH, Dace B, Shelburne CE, Wolff LF, Coulter WA. Prevalence of periodontal pathogens in localized and generalized forms of early-onset periodontitis. J Periodontal Res 2000; 3: 232–41.
[25] Haraszthy VI, Hariharan G, Tinoco EMB, Cortelli JR, Lally ET, Davis E, *et al*. Evidence for the role of highly laeukotoxic *Actinobacillus actinomycetemcomitans* in the pathogenesis of localized juvenile and other forms of early-onset periodontitis. J Periodontol 2000; 71: 912–22.
[26] Haubek D, Ennibi O, Abdellaoui L, Benzarti N, Poulsen S. Attachment loss in Moroccan early onset periodontitis patients and infection with the JP2-type of *Actinobacillus actinomycetemcomitans*. J Clin Periodontol 2002; 29: 657-60.
[27] Leung WK, Ngai VK, Yau JY, Cheung BP, Tsang PW, Corbet EF. Characterization of the *Actinobacillus actinomycetemcomitans* which was isolated from young Chinese aggressive periodontitis patients. J Periodontal Res 2005; 40: 258–68.
[28] Schacher B, Baron F, Roßberg M, Wohlfeil M, Arndt R, Eickholz P. *Aggregatibacter* actinomycetemcomitans as an indicator for aggressive periodontitis by two analysing strategies. J Clin Periodontol 2007; 34: 566-73.
[29] Meng S, Zhao L, Yang H, Wu Y, Ouyang Y. Prevalence of *Actinobacillus actinomycetemcomitans* in Chinese chronic periodontitis patients and periodontally healthy adults. Quintessence Int 2009; 40: 53-60.
[30] Vieira EM, Raslan SA, Wahasugui TC, Avila-Campos MJ, Marvulle V, Gaetti-Jardim Júnior E. Occurrence of *Aggregatibacter actinomycetemcomitans* in Brazilian Indians from Umutina Reservation, Mato Grosso, Brazil. J Appl Oral Sci 2009; 17: 440-45.
[31] Junior WR, Souza RC, Andrade AFB, Colombo APV. Analysis of the laeukotoxin gene types of *A. actinomycetemcomitans* in Brazilians with aggressive periodontitis. Braz J Microbiol 2006; 37: 127-34.

[32] Cortelli JR, Roman-Torres CVG, Aquino DR, Franco GCN, Costa FO, Cortelli SC. Occurrence of *Aggregatibacter actinomycetemcomitans* in Brazilians with chronic periodontitis. Braz Oral Res 2010; 24: 217-23.
[33] Herrera D, Contreras A, Gamonal J, Oteo A, Jaramillo A, Silva N, *et al*. Sub-gingival microbial profiles in chronic periodontitis patients from Chile, Colombia and Spain. J Clin Periodontol 2008; 35: 106-13.
[34] Lafaurie GI, Contreras A, Barón A, Botero J, Mayorga-Fayad I, Jaramillo A, *et al*. Demographic, clinical, and microbial aspects of chronic and aggressive periodontitis in Colombia: a multi-center study. J Periodontol 2007; 78: 629-39.
[35] Yoshida Y, Suzuki N, Nakano Y, Shibuya K, Ogawa Y, Koga T. Distribution of the *Actinobacillus actinomycetemcomitans* serotypes and *Porphyromonas gingivalis* in Japanese adults. Oral Microbiol Immunol 2003; 18: 135-39.
[36] Faveri M, Figueiredo LC, Duarte PM, Mestnik MJ, Mayer MPA, Feres M. Microbiological profile of untreated subjects with localized aggressive periodontitis. J Clin Periodontol 2009; 36: 739–49.
[37] Leys EJ, Lyons SR, Moeschberger ML, Rumpf RW, Griffen AL. Association of *Bacteroides forsythus* and a novel *Bacteroides* phylotype with periodontitis. J Clin Microbiol 2002; 40: 821–25.
[38] Lillo AD, Booth V, Kyriacou L, Weightman AJ, Wade WG. The culture-independent identification of periodontitis-associated *Porphyromonas* and *Tannerella* populations by targeted molecular analysis. J Clin Microbiol 2004; 42: 5523–27.
[39] Conrads G, Flemmig TF, Seyfarth I, Lampert F, Lutticken R. Simultaneous detection of *Bacteroides forsythus* and *Prevotella intermedia* by the *16S rRNA* Gene-Directed Multiplex PCR. J Clin Microbiol 1999; 37: 1621–24.
[40] Simonson LG, Robinson PJ, Pranger RJ, Cohen ME, Morton HE. *Treponema denticola* and *Porphyromonas gingivalis* as prognostic markers following a periodontal treatment. J Periodontol 1992; 63: 270-73.
[41] Moter A, Hoenig C, Choi B, Riep B, Go¨bel UB. Molecular epidemiology of the oral treponemes which were associated with periodontal disease. J Clin Microbiol 1998; 36: 1399–1403.
[42] Asai Y, Jinno T, Igarashi H, Ohyama Y, Ogawa T. Detection and quantification of oral treponemes in subgingival plaque by real-time PCR. J Clin Microbiol 2002; 40: 3334–40.
[43] Colombo AV, Silva CM, Haffajee A, Colombo APV. Identification of the oral bacteria which were associated with the crevicular epithelial cells from chronic periodontitis lesions. J Med Microbiol 2006; 55: 609-15.
[44] Rivere GR, Smith KS, Carranza Jr N, Tzagaroulaki E, Kay SL, Dock M, *et al*. Associations between *Porphyromonas gingivalis* and oral treponemes in subgingival plaque. Oral Microbiol Immunol 1996; 11: 150-55.
[45] Simonson LG, McMahon KT, Childers DW, Morton HE. Bacterial synergy of *Treponema denticola* and *Porphyromonas gingivalis* in a multinational population. Oral Microbiol Immunol 1992; 7: 111-12.
[46] Ali RW, Skaug N, Nilsen R, Bakken V. Microbial associations of 4 putative periodontal pathogens in Sudanese adult periodontitis patients, which were determined by DNA probe analysis. J Periodontol 1994; 65: 1053-57.
[47] Lotufo RFM, Flynn J, Chen C, Slots J. Molecular detection of *Bacteroides forsythus* in human periodontitis. Oral Microbiol Immunol 1994; 9: 154-60.

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