

Quantification of *Porphyromonas gingivalis* in chronic periodontitis patients associated with diabetes mellitus using real-time polymerase chain reaction

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Abstract

Introduction: Periodontal diseases, if left untreated, can lead to tooth loss and affect at least one tooth in 80% of adults worldwide, with the main cause being a bacterial plaque. Among subgingival plaque bacterial species, *Porphyromonas gingivalis* has been implicated as a major etiological agent causing tooth loss. Diabetics and smokers are two patient groups at high risk for periodontal disease. The increase in the number of this organism with the coexistence of other pathogenic microbes leads to rapid destruction of the periodontium, premature loss of teeth and also because of its virulence has implications in systemic pathology. Our aim was to observe the involvement of *P. gingivalis* in diabetes mellitus (DM) patients associated with periodontitis with and without tobacco-associated habits and to compare them with periodontitis patients having no other systemic pathologies.

Materials and Methods: Subgingival plaque samples from a total of seventy subjects were included in the study. DNA was isolated from the collected sample and was quantified using spectrophotometer for standardizing the polymerase chain reaction. The quantity of the isolated DNA was checked in a ultraviolet-visible spectrophotometer.

Statistics: One-way ANOVA and Tukey's multiple *post hoc* procedures were carried out.

Results: The maximum score of *P. gingivalis* was seen in periodontitis patients having DM, whereas the least score was seen in periodontitis patients having DM with tobacco smoking habit compared to the other groups.

Conclusion: *P. gingivalis* count is significantly reduced in periodontitis patients having DM with smoking habit; it is concluded that *P. gingivalis* might not be a key causative organism responsible for the periodontal destruction in case of smokers despite the DM condition. The decrease in counts may be attributed to change in the local environment like chemical (tobacco nitrosamines) and physical changes preventing the growth of *P. gingivalis*.

Key Words: Chronic periodontitis, diabetes mellitus, *Porphyromonas gingivalis*, smokers

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INTRODUCTION

Periodontal diseases are among the most prevalent dental diseases affecting people worldwide as well as in the Indian community.^[1] Periodontal diseases cause loss of teeth and also affect systemic health with the oro-hematogenous spread of bacteria. The related systemic diseases include cardiovascular diseases, preterm delivery of low birth weight, osteoporosis, diabetes mellitus (DM) and respiratory infections.^[2]

Periodontal diseases are infectious in nature, and bacteria play a major role in its initiation and progression.^[3] It occurs as a result of complex interactions between periodontopathic microorganisms and the host tissues.^[4] This process is modified by the status of the host immune system, genetic factors and a complex array of environmental exposures.^[5]

Chronic periodontitis is associated with accumulation of plaque and calculus and has a slow to moderate rate of disease progression; however, periods of rapid destruction are also observed. The increase in the rate of disease progression is caused by the impact of local, systemic or environmental factors that influence the normal host-bacterial interaction.^[3,4,6]

As periodontitis is an infectious disease, it is mainly associated with a group of Gram-negative bacteria such as *Actinobacillus actinomycetemcomitans* (AA), *Porphyromonas gingivalis* and *Bacteroides forsythus*. Of these, *P. gingivalis* and *B. forsythus* are found in chronic periodontitis, whereas AA is found in cases of aggressive periodontitis.^[3,7]

P. gingivalis is an anaerobic, Gram-negative, rod-shaped and highly virulent organism implicated as a major pathogen in destructive periodontal disease with its ability to adhere and invade oral epithelium.^[8] It is also implicated to be involved in the development of systemic diseases due to systemic inflammation with increased circulating cytokines and mediators, direct infection and cross-reactivity/molecular mimicry between bacterial antigens and self-antigens. It has been detected in heart valve lesions and atheromatous plaque, amniotic fluid of pregnant women with threatened premature labor and placentas from cases of preterm delivery, DM, respiratory diseases and osteoporosis subjects.^[5]

DM is a metabolic disorder characterized by hyperglycemia due to defective secretion or activity of insulin. Diabetes increases the glucose concentration in the gingival crevicular fluid and decreases the salivary levels of epidermal growth factor which plays an important role in wound healing. These modifications in GCF affect plaque composition which is supported by an increased amount of plaque and increased numbers of Gram-negative anaerobes. The consequences include impaired

cellular functions, impaired host defense, vascular alterations, prolonged inflammation, impaired bone formation or repair ultimately resulting in tooth mobility and premature loss of teeth.^[9]

There is a two-way relationship between DM and periodontitis, where local periodontal infection can exacerbate and help in the progression of systemic complications. The systemic exposure to periodontal pathogens in the periodontium worsens the low-grade systemic inflammation present in diabetes. The consequences are alterations in glucose metabolism and regulation resulting in difficulties in maintaining optimal glycemic control, which in turn increase the development and progression of diabetic-related complications such as periodontitis.^[9]

P. gingivalis is frequently detected in various distant organs such as the liver, cardiovascular tissue, cerebrospinal fluid and tubal-ovarian locations. *P. gingivalis* is also able to invade intracellularly without the signs of apoptosis and necrosis. It has virulence factors such as collagenase, aminopeptidase and trypsin-like enzyme activity. The structural components of *P. gingivalis* include lipopolysaccharide and fimbriae that trigger intracellular signaling events.^[10] *P. gingivalis* in the oral cavity moves to the liver and is responsible for glycogen synthesis through Akt/GSK-3 β signaling in the liver cells. Hence, *P. gingivalis* in the oral cavity contributes to the pathogenesis of DM by affecting hepatic glycogenesis through Akt/GSK-3 β signaling.^[11]

Local factors such as plaque with a complex array of environmental exposures also play an important role in the periodontal destruction. This involves cigarette smokers who develop severe periodontitis up to five times more than nonsmokers.^[2,12] Smokers have been associated with deeper pockets and greater attachment loss, more pronounced furcation involvement and increased alveolar bone loss.^[2,13]

As both DM and smoking are important and significant risk factors for periodontitis, it is very important to know the role of *P. gingivalis* and its implications. Hence, from our study, we intend to quantify the *P. gingivalis* count using real-time polymerase chain reaction (PCR). Accurate quantification of *P. gingivalis* is needed to understand bacterial etiology of periodontitis. Hence, we used real-time PCR which provides a sensitive, efficient and reliable method for quantification of *P. gingivalis*.^[14-16]

Our aim is to observe the relative involvement of *P. gingivalis* in DM patients having periodontitis with tobacco smoking habit and to compare them with Group IA (no periodontal diseases) and Group IB (periodontal disease with no systemic pathology).

MATERIALS AND METHODS

The study was conducted on the subgingival plaque of patients with chronic periodontitis, with and without type II DM associated with smoking habit from our institution. A total of seventy cases were considered.

Group I (Control): (a) Fifteen healthy individuals without periodontal and systemic pathologies. (b) Fifteen chronic periodontitis patients without systemic pathology. Group II (Study): (a) Twenty newly diagnosed cases of DM having chronic periodontitis without tobacco smoking habit. (b) Twenty newly diagnosed cases of DM having chronic periodontitis with tobacco smoking habit.

The random blood sugar levels of all the patients were taken. The subgingival plaque was collected under aseptic and sterile conditions using standard universal currettes. The sample was placed in a vial containing phosphate buffer at -4°C and transported to the laboratory for further procedures. DNA was isolated from samples by modified cetyltrimethylammonium bromide method by centrifuging at 10,000 rpm for 10 min to pellet down the bacterial cells. The quantity of the isolated DNA was checked in ultraviolet-visible spectrophotometer (Vivaspec Biophotometer, Germany). From the stock, 1 µl DNA was mixed with 49-µl sterile distilled water to get fifty times dilution. The A260/A280 ratio was recorded to check the purity of DNA preparation.

The primers for quantification analysis were designed using Perkin-Elmer Primer Express® software. The melting temperature was calculated, and the synthesized primers were purified by high-performance liquid chromatography. The quantified DNA was used to detect the presence of *P. gingivalis* using the specific primers, and primer optimization was done in a gradient PCR at the annealing temperature of 55°C [Tables 1 and 2]. Amplified products were resolved on 2% ethidium bromide-stained agarose gel along with a 100 bp DNA ladder

Electrophoresis results were analyzed in gel documentation system and photographed.

RESULTS AND OBSERVATIONS

Of 70 samples, 46 were male (65.71%) and 24 were female (34.28%) [Table 3].

The study subjects were found to be in the age group between 2nd to 5th decades [Table 4].

The maximum chronic generalized periodontitis were seen in Group IIA and localized periodontitis in Group IIB [Table 5].

Table 1: Polymerase chain reaction Design and synthesis

Name	Sequence 5-3	GC (%)	Temperature (°C)
<i>P. gingivalis</i> FP	GTAAGTCAGCGGTGAAACCTG	52	55.9
<i>P. gingivalis</i> RP	TCAGTGTGAGTCGCAGTATGG	52	56.6

P. gingivalis: *Porphyromonas gingivalis*, GC: Gingival crevicular, FP: Forward primer, RP: Reverse primer

Table 2: Polymerase chain reaction Temperature profile

Initial denaturation	35 cycles			Melt curve
	Denaturation	Annealing	Extension	
94°C for 10 (min)	94°C for 15 (s)	55°C for 30 (s)	60°C for 30 s	60°C

Table 3: Distribution of study subjects by gender

Gender of the subjects in both groups	Number of subjects	Percentage of subjects
Male	46	65.71
Female	24	34.28
Total	70	100

The maximum *P. gingivalis* count were observed in Group IIA and minimum count in Group IA [Table 6 and Graph 1]. It was also observed that the *P. gingivalis* count was significantly reduced in Group IIB compared to nonsmokers.

Pair-wise comparison of all the groups with respect to *P. gingivalis* scores as given by Tukey’s multiple *post hoc* procedures is presented in Table 7.

DISCUSSION

Periodontitis is a disease that affects the periodontium which is characterized by the loss of periodontal attachment including the alveolar bone. The bacterial etiology of periodontal disease is complex, with a variety of organisms responsible for the initiation and progression of the disease. The microbe which causes periodontitis contains primarily Gram-negative rods and cocci, filaments, flagellated rods and spirochetes. Many of these organisms are also present in periodontally healthy individuals and can exist in commensal harmony with the host.^[14] The mere presence of pathogen alone is not sufficient to cause disease but should be above a determined threshold to cause periodontal disease.

P. gingivalis has been implicated to be associated with many systemic diseases, where multiple fold increase is seen. These pathogens gain entry into circulation through the ulcerated epithelium and exposed capillaries during periodontal inflammation and may induce systemic symptoms.^[5]

DM is commonly associated with periodontal diseases. The interrelationship between periodontitis and DM provides an example of systemic disease predisposing to oral infection, and once that infection is established, the oral infection exacerbates systemic disease.^[9]

Table 4: Distribution of study subjects in each group by age

Age groups (years)	Group IA	Percentage	Group IB	Percentage	Group IIA	Percentage	Group IIB	Percentage	Total
21-30	5	33.33	2	13.33	2	10.00	3	15.00	12
31-40	8	53.33	6	40.00	7	35.00	6	30.00	27
41-50	2	13.33	7	46.67	11	55.00	11	55.00	31
Total	15	100.0	15	100.0	20	100.00	20	100.00	70
Mean age	33.80		39.00		40.35		38.40		38.10

Table 5: Distribution of study subjects based on their periodontal status

Periodontal status	Group IA	Percentage	Group IB	Percentage	Group IIA	Percentage	Group IIB	Percentage	Total
Healthy gingival	15	100.00	0	0	0	0	0	0	15
CGP	0	0	8	53.33	12	60.00	8	40.00	28
LP	0	0	7	46.67	8	40.00	12	60.0	27
Total	15	100	15	100	20	100	20	100	70

CGP: Chronic generalized periodontitis, LP: Localized periodontitis

Table 6: Comparison of *Porphyromonas gingivalis* scores in all the study groups

Treatment	IA	IB	IIA	IIB	Pooled total
N	15	15	20	20	70
Mean \bar{x}	525.0667	4178.6000	19,140.4500	1238.5000	6830.4857
Sum $\sum xi$	7876.0000	62,679.0000	382,809.0000	24,770.0000	478,134.0000
Sum of squares $\sum xi^2$	8,353,780.0000	673,600,039.0000	19,449,661,915.0000	104,690,908.0000	2,023,636,642.0000
Sample variance s^2	301,311.0667	29,406,397.8286	638,027,652.1553	3895,434.8947	245,948,104.1375
Sample SD	548.9181	5422.7666	25,259.2093	1973.6856	15,682.7327
SD of mean	141.7300	1400.1523	5648.1309	441.3295	1874.451

Descriptive statistics of $k=4$ independent treatments. SD: Standard deviation

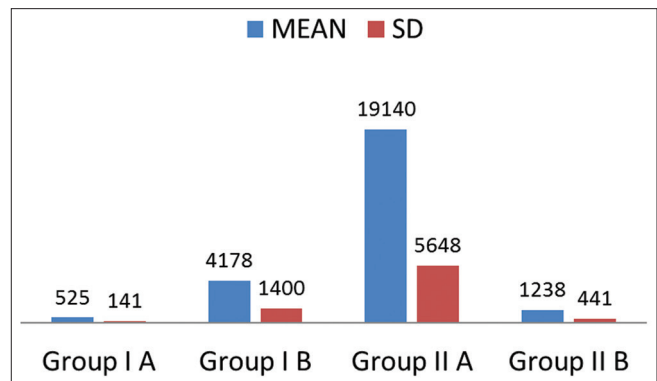
Table 7: Pair-wise comparison of four groups with respect to log *Porphyromonas gingivalis* scores by Tukey's multiple post hoc procedures

Groups	Group IA	Group IB	Group IIA	Group IIB
Mean	525.0667	4178.6000	19,140.4500	1238.5000
SD	141.7300	1400.1523	5648.1309	441.3295
Group IA	-			
Group IB (P)	0.0025*	-		
Group IIA (P)	0.0002*	0.6726	-	
Group IIB (P)	0.9991	0.0018*	0.0002*	-

* $P < 0.05$. SD: Standard deviation

The effects of smoking in oral cavity include bad breath, tooth discoloration, inflammation of the salivary gland openings, increased plaque and tartar on the teeth, risk of leukoplakia and gum diseases leading to tooth loss.^[17] Nicotine, a major smoke component, induces periodontal collagen degradation by increasing expression of the collagenase matrix metalloproteases which results in detachment of the periodontal ligaments. *P. gingivalis* persistence in the oral cavity of smokers is attributed to a compromised immune response and/or increased bacterial virulence. Despite increased *P. gingivalis*, smokers consistently display reduced clinical inflammation due to lower levels of proinflammatory cytokines at diseased sites.^[17,18]

As periodontal diseases are polymicrobial infections with various etiologic factors, accurate quantitation of the number of cells of individual bacterial species in dental plaque samples is needed for understanding the bacterial



Graph 1: Comparison of *Porphyromonas gingivalis* scores in all the study groups (Groups IA, IB, IIA and IIB)

etiology of periodontitis. Detection and quantification of this microorganism is relevant for diagnosis and treatment planning.^[19] The real-time PCR provides precise counts through direct monitoring of the increasing amount of PCR product throughout the enzymatic assay and is the most sensitive method with detection limits of $10^{[2]}$ genome copies.^[20]

Hence, we used real-time PCR method for quantification of *P. gingivalis*.

In Group IA, *P. gingivalis* counts ranged from 0 to 1561 with a mean of 525. It implies that *P. gingivalis* exists in commensal harmony with other organisms present in the periodontally

healthy individuals. The study is supported by other studies, where Lyons *et al.*^[19] and Van Winkelhoff *et al.*^[21] showed the presence of *P. gingivalis* in healthy subjects by PCR assay and concluded that this organism may also be a normal inhabitant of a periodontally healthy dentition.

In Group IB, *P. gingivalis* counts ranged from 288 to 21697 with a mean of 4178. It implies that *P. gingivalis* is one of the major pathogens implicated in the development and progression of the periodontal disease. Hence, we can observe the substantial increase in the *P. gingivalis* count when compared to healthy individuals from our results. Our study results are supported by Kirakodu *et al.*^[20] and Socransky *et al.*^[22] who conducted a study on quantification of *P. gingivalis* from subgingival plaque by quantitative PCR assay which showed an increased prevalence of *P. gingivalis* organisms ranging from $6 \times 10^2 - 8.6 \times 10^4$ in chronic periodontitis patients.

In Group IIA, *P. gingivalis* count ranged from 204 to 81691 with a mean of 19140. It shows that high glucose levels in type II DM patients lead to the development of periodontogenic flora due to reduced oxygen production and defense cells. Hence, diabetic patients have an increased susceptibility for more severe periodontal disease due to increased prevalence of *P. gingivalis*. In our study, Group IIA had statistically significant increase in the *P. gingivalis* counts by 40 folds than Group IA. However, it was increased only by 4 folds when compared to Group IB. Our study results are supported by Campus *et al.*^[23] and Ebersole *et al.*^[24] who conducted a study using PCR assay and concluded that these patients have an increased susceptibility for severe periodontal disease and increased prevalence of *P. gingivalis*.

In Group IIB, the *P. gingivalis* counts ranged from 0 to 8684 with a mean of 1238. The counts of *P. gingivalis* have significantly decreased by 16-fold ($P = 0.0002$) when compared to Group IIA. This is because the organism cannot thrive in the altered environment that is attributed to heat and tobacco smoke extracts despite increased blood glucose levels. Hence, the destruction of periodontium might be caused by other virulent periodontal pathogens. The studies done by Bagaitkar^[25] and Zeller *et al.*^[26] showed increased prevalence for *P. gingivalis* as compared to nonsmokers; whereas studies done by Zambon^[27] and Kinane and Chestnutt^[28] gave inconclusive results about the prevalence of *P. gingivalis* in smokers, where they concluded that no statistically significant difference was found between smokers and nonsmokers. Although the previous studies have shown increased prevalence of this organism in smokers compared to nonsmokers, our study showed conflicting results where the count of this organism was significantly reduced. However, none of the previous studies have included both the parameters of diabetes and smoking together for assessing *P. gingivalis* count in periodontitis subjects. Hence,

further studies need to be done with a large sample size. We chose quantitative real-time PCR as it is more efficient and sensitive compared to other methods and provides precise counts through direct monitoring of the increasing amount of PCR product throughout the enzymatic assay. Subgingival plaque samples were chosen over other samples such as saliva and gingival crevicular fluid for better yield and to understand bacterial etiology of periodontitis.

CONCLUSION

P. gingivalis is one of the major etiological agents in periodontal destruction and in recent years, it is gaining importance because of its implications in the systemic pathologies such as DM. It is also considered a risk factor associated with tobacco smoking habit. Thus, by assessing its quantity, we can analyze its role as a pathogen and the risk associated with this organism as studies have shown that it exacerbates the systemic pathology. A quantitative real-time PCR is an efficient and sensitive method which allows us to study specific organisms and their role in various pathologies. Larger studies of this kind can throw light on a variety of etiological microorganisms. Further studies with larger sample size should be conducted to confirm the findings of our study.

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Conflicts of interest

There are no conflicts of interest.

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