Quantitative analysis of key periodontopathic bacteria in gestational diabetic and non-diabetic women

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Abstract

Purpose The aim of this study is to compare the periodontal status and quantify *Porphyromonas gingivalis* (*Pg*) and *Prevotella intermedia* (*Pi*) from plaque samples of both gestational diabetic mellitus (GDM) and non diabetic pregnant women.

Materials and methods Sixty first time pregnant women were selected after adjusting for age, duration of pregnancy and educational status. They were then categorized into gestational diabetic women (GDM) (Group A) and healthy pregnant women (non GDM) (Group B). Periodontal examination was done by assessing gingival index, periodontal disease index and probing depth. Microbial analysis on sub-gingival plaque was performed using polymerase chain reaction (PCR). Statistical analysis was done by student t test, chi square test and Fischer exact test.

Results Group A showed higher gingival index, probing depth and periodontal disease index scores than group B at $p < 0.001$. *Pg* was detected in 80% of group A and 40% of group B. Amongst these; it was measured over 2.0×10^4 in 33% of group A, while in the group B it never scored more than 1.0×10^4 . While *Pi* were also detected in 73% of group A women and 40% Group B women but quantification showed *Pi* $> 2.0 \times 10^4$ in more number of group A women.

Conclusion This study showed that there is significant association between the severity of periodontal disease and increased levels of *Pg* and *Pi* in gestational diabetic women.

Keywords Gestational diabetes · Periodontal disease · Pregnancy · Periodontopathogens

Introduction

Maternal glucose disorders (MGD) during pregnancy includes gestational diabetic mellitus (GDM), impaired glucose intolerance (IGT) and pre-pregnancy Type 2 diabetes [1]. GDM is defined as glucose intolerance with onset or first diagnosis during pregnancy; it is characterized by pancreatic cell function that is insufficient to meet the body's insulin needs [2]. Thus, GDM often holds great potential as a condition to study the

pathogenesis of diabetes and to develop test strategies for its prevention. Miscarriage, pre-eclampsia and preterm labour is more common in women with GDM [3, 4] Stillbirth, congenital malformations, macrosomia, birth injury, perinatal mortality and postnatal adaptation problems such as hypoglycaemia are more common in babies born to women with pre-existing diabetes [5]. Despite advances in prenatal care and increased public awareness, adverse pregnancy outcomes are still present as a major public health problem worldwide. Research on the impact of periodontal disease on pregnancy complications has been debated [6, 7]. Infection, therefore, represents an important and frequent contributor to complications of pregnancy. Key periodontopathic bacteria have been associated with various other systemic conditions such as cardiovascular disease, diabetes mellitus, and osteoporosis [8].

Various periodontal pathogens has been listed to be associated with poor pregnancy outcomes [9] and accumulated evidences have implicated the role of *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythensis*, *Treponema denticola* and *Prevotella intermedia* (*Pi*) in pregnancy related complication like preterm delivery [10–12]. Accurate quantification of

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Increased levels of *Pg* and *Pi* observed in GDM subjects with chronic periodontitis.

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individual bacterial species in dental plaque samples are needed to understand the bacterial etiology of periodontitis and for implicating periodontitis to systemic diseases. Hematogenous spread of *Pi* and *Pg* to distant sites away from oral cavity have been demonstrated in patients with chronic periodontitis and even after mastication, causing recurrent bacteremias [13]. The frequency of *Pg* isolation from periodontal healthy individuals is sufficiently rare and that is why *Pg* is not considered as a member of the normal healthy oral microbial flora [12, 13].

Polymerase Chain Reaction (PCR) allows the determination of the total number of bacterial cells present in a complex sample so that the percentage of the particular bacteria can be determined. Studies have evaluated the usefulness of detection and quantification of the bacterial DNA in plaque samples with different techniques. Conventional semi quantitative PCR demonstrated sufficient sensitivity and specificity for microbiological examination of periodontal disease [14]. With this background, we in this case control study aimed to compare the periodontal status and quantitatively analyze *Pg* and *Pi* amongst GDM and non-GDM pregnant women.

Materials and methods

Source of data

This study was carried out in the Department of Periodontics, Bangalore Institute of Dental Sciences and in the Department of Obstetrics and Gynecology (OBG) and Endocrinology, St. John's Hospital (October 2010 to June 2011). All pregnant women with similar socio economic status enrolled for prenatal program were screened for GDM. The individuals were explained in detail regarding the study protocol; follow up periods and the investigations. Sixty individuals who volunteered to be part of the study signed an informed consent and registered for the study.

Inclusion Criteria:

1. Females aged 20–30 yrs.
2. First pregnancy
3. Diagnosed with Diabetes mellitus during pregnancy.
4. Have more than 20 teeth excluding third molars.
5. Individuals willing to participate in the study.

Exclusion Criteria

1. Pre existing diabetic patient.
2. Previous history of chronic infection or acute infection.
3. History of miscarriage or medical termination of pregnancy.
4. Smoking and alcohol consumption.
5. Patients with recent previous history of periodontal therapy.
6. Patients with positive medical history for cardiovascular disease, bleeding disorders and coagulation disorders.

The physician in accordance with the current American Diabetes Association criteria diagnosed GDM. [15] The protocol followed was as follows.

- a) Screening at 24–48 weeks (for women not previously diagnosed with overt diabetes)
- b) Perform 75 g OGTT with plasma glucose measurement
- c) Test in the morning after patient has fasted for ≥ 8 h.
- d) Repeat test at 1 and 2 h after initial measurement.

Diagnosis is confirmed when the plasma glucose levels meet or exceed fasting 92 mg/dl, 1 h 180 mg/dl and 2 h 153 mg/dl.

After fulfilling the inclusion criteria and voluntarily agreeing to participate in the study the participants were grouped into

Group A: Gestational diabetic women.

Group B: non-diabetic pregnant women.

Collection of blood samples 5 ml of blood was collected from antecubital vein of all individuals and was transferred into anticoagulant coated vacutainers for analysis of fasting, post-prandial blood sugar and glycosylated hemoglobin levels.

Clinical examination Periodontal measurements included full mouth gingival index (GI) [16], probing pocket depth (PD), Periodontal disease index (PDI) by Ramfjord (1959) [17]. Oral examination was done by a single experienced periodontist (P) and PD was recorded using UNC 15 periodontal probe* (HuFriedy IL, Chicago) and sites with pseudo pocketing were not considered. The measurements were carried out at six sites per tooth (mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual and distolingual) of all teeth. Each pocket was probed twice to replicate measurements. A third measurement was made if there was a ≥ 0.5 -mm difference between the first two measurements. The median of the two or three measurement was used in the data analyses. Chronic periodontitis was defined using CDC working definition accordingly as PD ≥ 5 mm or CAL ≥ 4 mm in at least six proximal sites [18].

Collection of subgingival plaque samples Sub-gingival plaque samples were collected using a sterile gracey curette. Curette was inserted parallel to long axis of tooth sub gingivally, into the deepest portion of the periodontal pocket and then moved coronally by scraping along the root surface. The plaque samples were then transferred in vials containing thioglycollate transport media, for microbial analysis.

DNA extraction procedure from plaque sample

1. Plaque sample was crushed with sterile blade and transferred in tube containing Tris-Borate-EDTA buffer (T.E)
2. Centrifuged at 50000 rpm for 2 min.

Table 1 Specific primers used

Bacteria	Sequence	Amplicon size (base pairs)
<i>Pg</i>	AGG CAG CTT GCC ATA CTG CG	404
<i>Pi</i>	ACT GTT AGC AAC TAC CGA TGT	589
	AAC GGC ATT ATG TGC TTG CAC	
	CTC AAG TCC GCC AGT TCG CG	

- Supernatant was discarded and fresh 200 µl T.E. buffer was added and centrifuged for 3–4 min.
- Same procedure was repeated for three- four times with fresh T.E. buffer.
- Supernatant was discarded and 500 micro liter lysis buffer 1 was added and centrifuged at 5000 rpm.
- Supernatant was discarded and 50 micro liter lysis buffer 2 and 5 micro liter proteinase K was added and then kept in water bath for 2 h, after this it was kept in boiling water bath for 10 min at 90 °C.
- Obtained DNA was stored at -20 °C.

Detection of pathogens After extraction of the DNA it was amplified using DNA polymerase with a primer. The selection of suitable primers and PCR products was done using program PRIME (Genetic computer groups, Madison, Wis). The strains used for *Pg* were ATCC 33277 and for *Pi* was ATCC 25611. DNA primers (Bioserve molecular services U.S.A) that anneal with nucleotide base sequences specific for the target organism was used (Table 1). The sequenced DNA was detected using conventional semi quantitative PCR and visualized using agarose gel electrophoresis. DNA from the standard strain with and without template served as positive and negative controls. The loading amount for the PCR was set at 100 ng. The band size of test samples was identified by comparing it with DNA ladder.

Statistical analysis

Power analysis The sample size is estimated using G power software version 3.1.9.2, the effect size to be assessed between

the groups is 80% ($d = 0.80$), the margin of error was set at 5% (0.05), allocation ratio $N2/N1 = 1$. The total sample size obtained with the present estimation is 52 with 26 samples in each group, hence the sample size in each group were rounded off to 30.

In the present study descriptive statistical analysis was carried out by Student *t* test (two tailed, independent), to find the significance of study parameters on continuous scale between two groups (Inter group analysis) on metric parameters. Chi-square test for independence was used to determine the relationship between two variables of a sample. Fisher Exact test was used to test the significance of the association.

Results 80% of subjects with GDM in group A were in third trimester followed by 10% in first and second trimester. While in group B 63.3% of the subjects were in third trimester while 36.7% in second trimester. The mean age group was 28.07 ± 3.75 years and 24.67 ± 3.69 years for subjects in Group A and B respectively. The mean value of fasting blood glucose and random blood glucose in group A was 122.27 ± 14.11 mg/dl and 181.33 ± 38.74 mg/dl respectively where as in group B, it was 92.70 ± 13.11 mg/dl and 111.63 ± 18.99 mg/dl respectively. The mean value of glycosylated hemoglobin in group A was $7.63 \pm 2.31\%$ showing moderate glycemic control; the corresponding values in group B was 4.61 ± 1.32 (Table 2).

Gingival index showed a mean score of 0.84 ± 0.20 in group A and 0.71 ± 0.15 in group B and the difference was statically significant. Plaque component of periodontal index showed statistically significant difference in mean score 2.16 ± 0.51 in group A and 1.80 ± 0.42 in group B at a *P* value of 0.004. Mean score of calculus component did not show significant difference in both the groups. There was highly

Table 2 Age and blood sugar values between the two groups

Variable	Group A (mean $\pm$ SD)	Group B (mean $\pm$ SD)	<i>P</i> value
Age (years)	28.07 ± 3.75	$24.67 \pm 3.69^*$	$<0.001^*$
Fasting blood sugar (mg/dl)	122.27 ± 14.11	92.70 ± 13.11	$<0.001^*$
Random blood sugar (mg/dl)	181.33 ± 38.74	111.63 ± 18.99	$<0.001^*$
HbA1c (%)	7.63 ± 2.31	4.61 ± 1.32	$<0.001^*$

Group A: Gestational diabetic group

Group B: Healthy pregnant group

HbA1c: Glycosylated haemoglobin

* Statistically significant at *p* value <0.001

Table 3 Comparison of gingival index, periodontal index and probing depth in group A and B

Clinical parameters	Group A	Group B	P value
Gingival index	0.84 ± 0.20	0.71 ± 0.15	0.007**
Periodontal index			
Plaque component	2.16 ± 0.51	1.80 ± 0.42	0.004**
Calculus component	2.15 ± 0.55	1.92 ± 0.55	0.113
Gingival and periodontal component	2.01 ± 0.39	1.48 ± 0.32	<0.001**
Probing depth	2.29 ± 0.44	1.99 ± 0.44	0.011*

Periodontal index: includes three components in plaque, calculus and gingival-periodontal component

Group A: Gestational diabetic group

Group B: Healthy pregnant group

*Statistically significant

**Highly statistically significant

statistically significant difference in the mean score of gingival and periodontal component between group A and B at P value <0.001. The mean probing depth was significantly more in group A (2.29 ± 0.44) when compared with group B (1.99 ± 0.44) (Table 3).

Pg was detected in 80% of subjects in group A and 40% of subjects in group B. quantification of Pg showed that in group B it never exceeded 1×10^4 where as in group A majority of patients showed Pg in the range of $>2 \times 10^4$. The difference in the amount was significantly more in group A when compared to group B at P value of 0.007 (Table 4).

Pi was detected in 73.3% of subjects in group A and 40% of subjects in group B. quantification of Pi in both the groups never exceeded 1.0×10^4 in 50% of group A and 26.7% of group B. However counts of $>2.0 \times 10^4$ was seen significantly higher in group A as compared to group B at P value of $P = 0.175$ (Table 4).

On comparing the clinical parameters to Pg and Pi it was seen than all the components of periodontal index and PD were slightly higher in subjects whom Pg were detected although not significant statistically. Whereas the difference in

values of gingival and periodontal component of Pi in both the groups between the subjects having Pi and the subjects not having Pi in their plaque samples was statistically significant at p value of 0.016 (Tables 5 and 6).

Discussion There is a large body of evidence pointing to infection as a key factor in adverse pregnancy outcomes, [19–24]. Literature evidence [25–30] suggests possible association between periodontal pathogens and adverse pregnancy outcomes in gestational diabetic individuals thus suggesting the importance of periodontal health in pregnant women. However, till date, it is yet not clearly justified whether mere presence or increased number of these key perio-pathogens like Pg and Pi have an impact on the outcome of pregnancy. Hence in this study we decided to quantify both Pg and Pi in healthy and gestational diabetic women and found that 85% of gestational diabetic women showed presence of Pg in sub-gingival microflora and amongst those 53.3% of individuals presented with very high titers of Pg (greater than 1×10^4). While amongst non-diabetic it was not detected in 60% of individuals and even in those detected the titers were lower than 1×10^4 . While for Pi it was detected in 74% of the Gestational diabetic and 40% of the non diabetic, of those detected 20% of gestational diabetic showed very higher titers (2×10^4).

We assessed fasting, random glucose and glycosylated hemoglobin levels (HbA1c) for both groups, as HbA1c is considered to be a sensitive predictor for the risk for developing gestational diabetes [31]. The average levels of HbA1c of subjects in group A was $7.63 \pm 2.31\%$ which showed moderate control of diabetes mellitus (7–8%) and in group B it was 4.61 ± 1.32 . The mean age of the subjects in our study was 28.07 ± 3.75 yrs. in group A and 24.67 ± 3.69 yrs. in group B. Maternal age is an established risk factor for GDM, but there is no consensus on the age group mentioned above having significantly increased risk of GDM [28]. The American Diabetes Association has mentioned the age group in which the lowest cut off is ≥ 25 years as most predictive of GDM [32]. It was also noted in our

Table 4 Detection and quantification of *Porphyromonas gingivalis* and *Prevotella intermedia* by polymerase chain reaction between the groups

Bacteria	<i>Porphyromonas gingivalis</i>		<i>Prevotella intermedia</i>	
	Group A	Group B	Group A	Group B
Not detected	20.0%	60.0%	26.7%	60.0%
$<1.0 \times 10^4$	26.7%	40.0%	53.3%	26.7%
$1.0\text{--}2.1 \times 10^4$	20.0%	0	0	6.7%
$>2.0 \times 10^4$	33.3%	0	20.0%	6.7%
Total	100.0%	100.0%	100.0%	100.0%
P value	$p = 0.007^{**}$		$p = 0.175$	

Group A: Gestational diabetic group

Group B: Healthy pregnant group

**Highly statistically significant

Table 5 Comparison of Clinical parameters with the Positivity of *Porphyromonas gingivalis* in group A and B

Clinical parameters	Positivity of Porphyromonas gingivalis		P value
	Not detected	Detected	
Group A			
Gingival index	0.86 ± 0.10	0.87 ± 0.20	0.947
Periodontal index			
Plaque component	1.99 ± 0.17	2.04 ± 0.31	0.816
Calculus component	1.77 ± 0.54	2.04 ± 0.39	0.337
Gingival and periodontal component	2.39 ± 0.48	1.98 ± 0.46	0.191
Probing pocket depth	2.39 ± 0.17	2.52 ± 0.37	0.583
Group B			
Gingival index	0.76 ± 0.17	0.69 ± 0.19	0.494
Periodontal index			
Plaque component	1.72 ± 0.33	1.94 ± 0.39	0.245
Calculus component	1.83 ± 0.36	1.84 ± 0.53	0.979
Gingival and periodontal component	1.51 ± 0.45	1.53 ± 0.16	0.949
Probing pocket depth	2.26 ± 0.34	2.33 ± 0.26	0.646

study that GDM occurred 80% in the third trimester 10% in first and second trimester as in accordance with the study by Seshiah [33].

Both pregnancy and hyperglycemic conditions are known to have effect on plaque ecology [3] and host response [32]. Association of *Pg* and *Pi* in periodontal disease and maternal health is well documented and researched [34–36]. *Pg* employs a variety of strategies to control commensal microbiota and direct disease progression and hence regarded as a key-stone species [37]. Recent animal studies have shown *Pg* to be associated with fetal loss in pregnant mice, reduction in fetal weight and increase fetal resorption [38]. In humans *Pg*

DNA has been found in chorionic tissues of high-risk pregnant women [39, 40] and in the amniotic fluid of women bearing pre-term and/or low birth weight infants [39]. One cross-sectional study demonstrated the presence of *Pg* antigens in five cell types in the placental tissues [12] suggesting that *Pg* may commonly colonize placental tissue, leading to an inflammatory response contributing to preterm delivery [41]. Studies have shown relationship between *Pi* and pregnancy associated periodontal disease as it has shown the ability to thrive in progesterone rich environment. Study by Ning-Yan Yang et al. [42] has shown it to be associated with

Table 6 Comparison of Clinical parameters with the Positivity of *Prevotella intermedia* in group A and B

Clinical parameters	Positivity of <i>Prevotella intermedia</i>		<i>P</i> value
	Not detected	Detected	
Group A			
Gingival index	0.77 ± 0.17	0.89 ± 0.18	0.221
Periodontal index			
• Plaque component	1.92 ± 0.21	2.07 ± 0.30	0.350
• Calculus component	1.96 ± 0.34	1.99 ± 0.46	0.872
• Gingival and periodontal compo	1.66 ± 0.14	2.21 ± 0.47	0.044*
Probing pocket depth	2.39 ± 0.17	2.53 ± 0.39	0.493
Group B			
Gingival index	0.65 ± 0.13	0.87 ± 0.15	0.011*
Periodontal index			
• Plaque component	1.73 ± 0.39	1.91 ± 0.30	0.363
• Calculus component	1.85 ± 0.45	1.80 ± 0.40	0.823
• Gingival and periodontal component	1.35 ± 0.27	1.78 ± 0.32	0.016*
Probing pocket depth	2.41 ± 0.28	2.11 ± 0.26	0.064+

* statistically significant

increased progression of periodontal inflammation in adolescents.

Conventional semi quantitative PCR used in our study has shown to be sensitive enough to detect pathogens from clinical specimens, which are below the detection limit by culture technique [43]. Both *Pg* and *Pi* were increased in the group A compared to group B. The detection levels of *Pg* were almost double in the Group A when compared to group B which are similar to studies which demonstrates increased levels of *Pg* in periodontal tissues of pregnant women. [35, 44, 45]. Levels of *Pi* were lower than *Pg* in both the groups however, in-group A *Pi* show increased detection and values.

GDM patients exhibited higher values of gingival index, periodontal disease index and higher mean PD as compared to non-diabetic control subjects [42, 43]. It was also found that women with GDM had higher periodontal destruction (50%) when compared with healthy pregnant women (37.3%) [43, 44] although there was no statistical difference between the groups. Similar results were shown by Ruiz et al. and Novak et al. [46, 47] Both GDM and periodontal disease can independently affect the outcome of pregnancy and when they are present together, a synergic exposure to inflammatory components can have a greater impact on fetal health [48].

The strength of this study would be its strict inclusion and exclusion criteria and quantitative analysis of both *Pg* and *Pi* rather than merely detecting their presence or absence. Limitations include smaller sample size owing to lack of funds, and not correlating these bacteria with the bacterial profile of amniotic fluid of the gestational diabetic individuals. Evaluating these individuals on a longitudinal basis and checking for any adverse pregnancy outcomes if any, quantifying the inflammatory burden, and gathering evidence for hematogenous dissemination could possibly be the next step in this research.

Conclusion The present study showed that, there is significant association between the severity of periodontal disease and increased levels of *Pg* and *Pi* in gestational diabetic women.

Incidence of GDM is more in the third trimester, higher quantity of *Pg* was seen in GDM group when compared with non- GDM group, which could hypothetically imply that *Pg* could play a greater role than *Pi* in adverse pregnancy outcomes and therapeutic strategies thus should target in reducing the numbers of this keystone pathogen.

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Compliance with ethical standards

Conflict of interest The authors claim no conflict of interest in this study.

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