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Red Complex Bacteria in Preeclamptic Pregnant Women with and without Periodontal Disease: A Cross Sectional Microbiological Study.

Swetha Tanneeru[1], Jaideep Mahendra[2]*, Mahaboob Vali SK[3], and Little Mahendra[4].

[1]Swetha Tanneeru, Department of Periodontology, Narayana Dental College, Nellore, Andhra Pradesh, India.
[2]Jaideep Mahendra, Professor, Dept. of Periodontology, Meenakshi Dental College, Chennai, Tamilnadu, India.
[3]Mahaboob Vali SK, Professor and Head, Department of Advanced Research Centre, Narayana Medical College, Nellore, Andhra Pradesh, India.
[4]Dr. Little Mahendra, Dean, Maktoum bin Hamdan dental university college, Dubai.

ABSTRACT

Red complex bacteria have been associated significantly in the pathophysiology of various systemic ailments. The main objective of the study is Red Complex Bacteria in Preeclamptic Pregnant Women with and without Periodontal Disease. A Cross-Sectional study is designed on pregnant women with and without periodontal disease and preeclampsia, who are attending Narayana hospital, Nellore for prenatal check-up's. 445 women were recruited in the study after obtaining the consents. Based on systemic and periodontal health, subjects were grouped into Group-1 (systemically healthy and periodontally healthy), Group-2 (systemically healthy with periodontal disease), Group-3 (preeclampsia women with periodontal disease) and Group-4 (preeclampsia women without periodontal disease). Women with preeclampsia and periodontal disease had more adverse outcomes and expressed more bacterial load compared to other groups that is group 4. 2 and 1 respectively in both sub gingival plaque and placental tissue samples. There was a significant association between periodontal bacteria (Pg., Pi, Fn, Tf) with pregnancy outcomes and preeclampsia. Bacteria and their products have a definite role in the development of preeclampsia, and pregnancy outcomes as well.

Keywords: Preeclampsia, Pregnant women, periodontal disease.

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Corresponding author

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INTRODUCTION

Periodontal disease is a pathological inflammatory condition of periodontal tissues, initiated and sustained by several bacteria that colonize the sub-gingival area leading to connective tissue destruction. Evidence suggests that these diseases can serve as reservoirs for specific organisms, generating an inflammatory reaction leading to systemically elevated levels of cytokines.[1] The adverse pregnancy outcomes like preterm, still births, low birth weights are major medical, social and economic problems that account for large proportion of maternal and especially neonatal mortality and acute morbidity[2]. Maternal infections have been associated with pregnancy complications for many years and have hypothesized that infections outside the urogenital area could contribute for occurrence of this pregnancy complications.[1, 3, 4]

An estimated 10% of pregnancies end in preterm and they appear to increase despite significant advances in obstetrical medicine and improvements in prenatal care utilization.[5] 50% of spontaneous preterm births are said to associate with ascending genitourinary tract infections.[4] Periodontal diseases experience more frequent bacteraemia resulting in colonization of specific organisms or their end products in uterine cavity or/and, cytokines generated within diseased periodontal tissues eliciting an inflammatory cascade. This in turn is said to trigger local and systemic adaptive immune response. [1, 3]

Bacteria associated with periodontal disease are not dissimilar to those known to be associated with genitourinary bacterial infections and adverse pregnancy outcomes. Many studies have demonstrated the translocation of *Fusobacterium nucleatum*, *Prevotella nigrescens*, *Prevotella intermedia*, *Porphyromonus gingivalis*, *Treponema denticola* to the feto-placental unit whereby a maternal or foetal response has been shown resulting in premature birth or low birth weight. Since then more attention has been focused on oral infections and their effects on pregnancy.

The present study is aimed at identification of red complex bacteria in sub gingival plaque and placental tissue samples in preeclampsia pregnant women with and without periodontal diseases.

MATERIALS AND METHODS

Nine hundred pregnant women were screened for the study 210 subjects were excluded due to factors like age and systemic conditions, 245 women had refused to participate in the research study. After obtaining consents remaining 445 subjects who met the eligibility criteria were recruited for the study and were categorized into 4 groups based on the presence and absence of preeclampsia and periodontal disease.

Based on systemic and periodontal health, the subjects were grouped in to 4 groups.

Group 1: (Controls) systemically and periodontal healthy women. N=145
Group 2: Systemically healthy women with periodontal disease. N=100
Group 3: Preeclampsia women with periodontal disease. N=100
Group 4: Preeclampsia women without periodontal disease. N=100

Sample Collection and Storage: The sampling site was isolated using cotton rolls and supragingival plaques were removed with the help of sterile cotton. The sub gingival plaque was then collected using sterile Gracey curette and suspended in 100 µL of Tris–HCl buffer. These sub gingival plaque samples were then immediately incubated at 50°C for 10 minutes and then stored at –20°C freezer till further processing.

Placental samples were also collected and stored for the same patients and stored as above.

All the plaque samples and placental samples were subjected to PCR which was carried out at advanced research centre (Narayana Medical Institutions, Nellore, A.P, INDIA).

Materials

1. TE buffer (pH 8.0): 10 mm Tris HCl (pH 8.0), 1 mm EDTA (pH 8.0)(sigma , US)
2. 10% SDS: Dissolve 10 g of SDS in 100 ml autoclaved distilled water.
3. Proteinase K: Dissolve 10 mg of Proteinase K in 1 ml autoclaved distilled water.

4. Phenol-chloroform mixture: The pH is very important. For RNA purification, the pH is kept around pH 4, which retains RNA in the aqueous phase preferentially. For DNA purification, the pH is usually 7 to 8, at which point all nucleic acids are found in the aqueous phase. Mix equal volume of phenol with chloroform. Keep the mixture on ice and add 20 ml TE buffer, extract by shaking for 15 minutes. Remove the dust on the surface layer using a pipette. Repeat 4-5 times. Add 30-40 ml of TE buffer and store it on ice.
5. 5M Sodium Acetate (pH 5.2): Dissolve 41 g of sodium acetate in 100 ml distilled water and adjust pH with dilute acetic acid (pH 5.2).
6. Isopropanol
7. 70% ethanol
8. Eppendorf tubes: 2 ml
9. Micropipette- 1-10 µL; 20-100 µL; and 200-1000 µL.
10. Microtips - 1-10 µL; 20-100 µL; and 200-1000 µL.
11. Micro-centrifuge
12. 10X Luna Universal qPCR Master Mix (contains deoxynucleoside triphosphate (dATP, dTTP, dCTP, and dGTP; MgCl2, Taq buffer, SYBR Green, Polymerase enzyme)

Isolation of DNA from plaque samples

- Samples from deep freezer (-80^{0}C) allowed to thaw at room temperature.
- Plaque samples in micro centrifuge tubes were centrifuged at 10000 rpm for 5 minutes.
- 875 µl of TE buffer is added to the pellet and resuspended in the buffer by gentle mixing.
- 100 µl of 10% SDS and 5 µl of Proteinase-K are added to the cells.
- The above mixture is mixed well and incubated at 37^0 C for one hour in incubator. 1 ml of phenol-chloroform mixture (3:1) is added to the contents mixed well by inverting and incubated at room temperature for 5 minutes.
- The contents are centrifuged at 10,000 rpm for 10 minutes at 4^0 C.
- The highly viscous jelly like supernatant is collected using cut tips and is transferred to a fresh tube.
- The process is repeated once again with phenol-chloroform mixture and the supernatant is collected in a fresh tube.
- 100 µl of 5M sodium acetate is added to the contents and is mixed gently.
- 2 ml of isopropanol is added and mixed gently by inversion till white strands of DNA precipitates out.
- The contents are centrifuged at 5,000 rpm for 10 minutes.
- The supernatant is removed and 1ml 70% ethanol is added.
- The above contents are centrifuged at 10,000 rpm for 10 minutes.
- After air drying for 5 minutes 200 µl of TE buffer or distilled water is added to the pellet.

Isolation of DNA from placental samples

- Samples from deep freezer (-80^{0}C) allowed to thaw at room temperature.
- After thawing, placental tissues were homogenized by standard protocol with homogenizer.
- Homogenized samples were added with 100 µl of lysis buffer and 100 µl of 10% SDS and 5 µl of Proteinase-K solution.
- The above mixture is mixed well and incubated at 37^0 C for one hour in incubator. 1 ml of phenol-chloroform mixture (3:1) is added to the contents mixed well by inverting and incubated at room temperature for 5 minutes.
- The contents are centrifuged at 10,000 rpm for 10 minutes at 4^0 C.
- The highly viscous jelly like supernatant is collected using cut tips and is transferred to a fresh tube.
- The process is repeated once again with phenol-chloroform mixture and the supernatant is collected in a fresh tube.
- 100 µl of 5M sodium acetate is added to the contents and is mixed gently.
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- The supernatant is removed and 1ml 70% ethanol is added.
- The above contents are centrifuged at 10,000 rpm for 10 minutes.
- After air drying for 5 minutes 200 µl of TE buffer or distilled water is added to the pellet.

Measurement of DNA concentration

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The concentration of DNA is determined using a NANODROP (Thermo Scientifics, US) spectrophotometer at 260/280 nm. The remaining samples are stored for PCR experiment.

This procedure was used to determine the amount, concentration and purity of the DNA sample. Turn on the Nanodrop, click on UV measure option in Nano drop software. Take 1 ul TE buffer to measure blank. Measure all the DNA samples (1 ul) separately.

The quality and yield obtained was measured by using a Nano Drop Spectrophotometer. Quantitation of DNA carried out 1µl of all plaque DNA samples using Nano Drop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific, US) at absorbance at 260 nm and 280 nm. The quality and yield obtained was measured by ratio of 260 nm and 280 nm using a Nano Drop Spectrophotometer.

PCR procedure

All mix was prepared in hard-shell PCR plate-96 well WHT-CLR (cat.no. HSP 9601)(Bio-Rad Laboratories Inc, US) with seal plates of optically transparent film(Bio-Rad Laboratories Inc., US). Care was taken to seal the plate edges and corners to prevent artifacts caused by evaporation. PCR amplification was performed in a real time thermo cycler (BIORAD-CFX100 (BIORAD, USA)). SYBR Green" channel of the real-time instrument BIORAD-CFX100 (BIORAD, USA) was used for the quantification of P.gingivalis using Luna Universal Master Mix.

Putative periodontopathic bacteria (*Porphyromonas gingivalis* [Pg.],*Fusobacteriumnucleatum*[Fn],*Prevotellaintermedia*[Pi], *Tannerella forsythia* [Tf], and *Treponema dent cola*[Td]) were detected using 16 S rRNA-based polymerase chainreactions(PCR). The primers of these microorganisms were designed based on the relevant literature and National Centre for Biotechnology Information BasicLocalAlignmentSearchTool(BLAST)andthensynthesizedby Bio serve Biotechnologies Pvt.Ltd. Hyderabad and IRA biotechnology Pvt.Ltd. Hyderabad, India. Primer sequences were shown in Table 1.

Luna Universal qPCR Master Mix and other reaction components were kept at room temperature to set room temperature, and then placed on ice. After thawing completely, all reagents were mixed by inversion, pipetting or gentle overtaxing.

Reaction Setup:

COMPONENT	25 µl REACTION	FINAL CONCENTRATION
Luna Universal qPCR Master Mix	10 µl	1X
Forward primer (10 µM)	1 µl	0.4 µM
Reverse primer (10 µM)	1 µl	0.4 µM
Template DNA	Variable	< 100 ng
Nuclease-free Water	to 25 µl	

For each batch of samples, negative control was set for PCR amplification using sterile deionized water. Duplicates were performed for all samples.

The reaction conditions of *Porphyromonas gingivalis*, *Fusobacteriumnucleatum*, and *Tannerella forsythia* were as follows: initial denaturation at 94^0 C for 5 min, followed by 30 cycles of denaturation at 94^0 C for 30 sec, annealing at 55^0 C for 30 sec, and extension at 72^0 C for 30 sec, with a last extension at 72^0 C for 5 min.

The reaction conditions of *Prevotellaintermedia*and *Treponema denticola* were as follows: initial denaturation at 94^0 C for 3 min, followed by 30 cycles of denaturation at 94^0 C for 30 sec, annealing at 55^0 C for 30 sec, and extension at 72^0 C for 20 sec, with a last extension at 72^0 C for 5 min.

The relative quantification of bacterial load was achieved by comparison with a standard amplification curve obtained from the (standard) genomic DNA corresponding to 6×10^6 colony founding units (CFUs) of bacterial pure cultures. The standard Bacterial DNA concentrations: 1×10^9, 1×10^7, 1×10^5, $1x\ 10^3$, and 1×10^1 respectively. Finally,

the expected amplicon was analysed along with standard 1 kb DNA ladder on 1.5% agarose gel under ultraviolet (UV) Tran's illumination.

Table 1. Primer sequences of Putative Periodontopathicmicroorganisms and ThreeHerpes viruses.

Periodontapothic microorganisms	Primer sequence (5'-3')	Primer length	Amplified fragment length (bp)
*Fusobacteriumnucleatum*FP	ATTGTGGCTAAAAATTATAGTT	22	817
*Fusobacteriumnucleatum*RP	ACCCTCACTTTGAGGATTATAG	22	
Porphyromonas gingivalis FP	AGGCAGCTTGCCATACTGCG	20	404
Porphyromonas gingivalis RP	ACTGTTAGCAACTACCGATGT	21	
*Prevotellaintermedia*FP	CGTGGACCAAAGATTCATCGGTGGA	26	259
*Prevotellaintermedia*RP	CCGCTTTACTCCCCAACAAA	20	
Tannerella forsythia FP	GCGTATGTAACCTGCCCGCA	21	641
Tannerella forsythia RP	TGCTTCAGTGTCAGTTATACCT	22	
Treponema denticola FP	TAATACCGAATGTGCTCATTTACAT	26	316
Treponema denticola RP	TCAAAGAAGCATTCCCTCTTCTTCTTA	27	

RESULTS

Statistical Analysis: The descriptive statistics (mean, standard deviation or percentage) of all variables were recorded. Chi-square tests or two sample t tests were performed to compare differences in periodontal clinical parameters and prevalence of periodontopathic microorganisms between the case group and the control group. The difference of the average bacterial counts among the study groups analysed by ANOVA test. $P < 0.05$ was considered statistically significant and all P-values were two-sided. Statistical analysis was performed using SPSS version 23.0 (IBM, Armonk, NY, USA).

The quality and yield obtained was measured by using a Nano Drop Spectrophotometer (Figure 1).The NonoDrop data given mean DNA yield obtained was 120 ng/µl (range 51-225 ng/µl) and purity (A260/A280 ratios) ranged between 1.55 to 1.90.

PCR Data Analysis: In our approach, each bacterial species was targeted by individual qPCR reactions containing specific primer pairs. PCR experiment with primers of Putative periodontopathic bacteria (*Porphyromonas gingivalis* [Pg.],*Fusobacteriumnucleatum*[Fn],*Prevotellaintermedia*[Pi], *Tannerella forsythia* [Tf], and *Treponema denticola* [Td]) were amplifying the 16S ribosomal RNA gene was set up with the extracted genomic DNA from each sample. Real-time PCR experiment using serial diluted templated over 4 order of magnitude indicate the absence of PCR inhibitors in the extracted DNA.

The real-time PCR using the SYBR Green chemistry was applied to the clinical plaque samples has been described in method. The original 10 µl of extracted DNA samples were run in the assay. The 16S ribosomal RNA gene in the plaque samples was evaluated by the SYBR Green assay using the standard of the DNA of each bacterium.

In our approach, each microbial species was targeted by individual qPCR reactions containing specific primer pairs.

The Biorad CFX 96 software computed the Ct values that were compared with the Ct inferred from the amplification curve of a standard sample containing genomic DNA equivalent to $6x10^6$ CFUs of *each* microorganism. The target amplicon corresponding to *P. gingivalis* have 404 basepair lengths, *Fusobacteriumnucleatum* have 817 basepair length, *Prevotellaintermedia* have 259 base pair length, *Tannerella forsythia* have 641 base pair length, *Treponema denticola* have 314 base pair lengths. This feature could impact the amplification efficiency, due to their increased size that enhances the assay sensitivity. However, the fact that *Ct* values are estimated in the early cycles of exponential amplification, and considering that errors of up to few hundred percent may be tolerated when trends

or relative big changes in amounts are measured; PCR efficiencies are adequately comparable for the study's purpose.

DISCUSSION

Uslu TH et al in 2007 have postulated that the host response to a long term exposure of periodontal pathogens may provoke systemic maternal and placental pro-inflammatory endothelial activation and dysfunction, representing a significant risk for diseases of vascular origin, such as preeclampsia, premature rupture of membranes' posing a threat to fatal-placental unit.[7] Many studies evidenced fatal antibody seropositivity to oral organisms, and exposure of foetus to these organisms or their end products. And it has been identified that fatal seropositivity to oral organisms is more frequently associated with preterm babies. These findings point to a blood borne infectious pathway leading to direct fatal exposure as a major pathogenic mechanism of periodontitis associated with prematurity.[8, 9, 10, 11]

Bacteria associated with periodontal disease are not dissimilar to those known to be associated with genito-urinary bacterial infections and adverse pregnancy outcomes. Many studies have demonstrated the translocation of *Fusobacterium nucleatum*, *Prevotella nigrescens*, *Prevotella intermedia*, *Porphyromonus gingivalis*, *Treponema denticola* to the feto-placental unit whereby a maternal or foetal response has been detected resulting in premature birth or low birth weight.

In the present study, bacteria were seen in more number in both sub gingival plaque and placental samples in women with preeclampsia associated with periodontal disease compared to women without periodontal disease. These women have experienced more number of preterm and lowbitth weight babies compared to women in other groups.

The sub gingival microbiota role in the development of periodontal diseases has been extensively documented. More frequently higher levels of Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythensis, and Treponema denticola are seen in periodontitis sites.[15, 16, 17] A periodontal microbes Fusobacterium nucleatum (F n), has been linked with adverse pregnancy outcomes. Many inflammatory markers like C-Reactive protein, IL-1, 6, TNF-α markers of systemic inflammation and are associated with periodontal disease.[18, 19]Elevated immunoglobulin G and CRP induced by bacterial species were associated with adverse pregnancy outcomes.[20] From this it is said that the infection enter the uterine cavity either by an ascendant route coming up through the genital tract or through bloodstream making its way down from the oral cavity.

Current research suggests periodontal disease a new established risk factor for adverse pregnancy outcomes. Systemic dissemination of periodontal infection through its chemical mediators plays a major role in the pathogenesis of adverse pregnancy outcomes. Furthermore, periodontal disease has been associated with increased risk of preterm delivery[21, 22] pre-eclampsia[23], low birth weight[24, 25],, intrauterine growth restrictions.[25] Therefore these chemical mediators might be a plausible mediator in the development of adverse pregnancy outcomes. According to a five-year study, pregnant women with moderate to severe periodontal disease may be seven times at more risk to deliver a premature child.

The immune system alters slightly during pregnancy so as not to harm the fetus, which helps in multiplication of periodontal bacteria. In addition, increased bleeding due to hormonal influence will aid in the entry of bacteria to the uterus through blood stream.

S. Offenbacher in a 5-year prospective study, among periodontally healthy mothers the unadjusted prevalence of births of GA and showed periodontal disease are significantly associated with a higher prevalence rate of preterm births, BW. The two complexes more associated in periodontal pathogenesis are orange complex (Campylobacter rectus, Fusobacterium nucleatum, Peptostreptococcus micros, Prevotella nigrescens, and Prevotella intermedia) and red complex (Porphyromonas gingivalis, Bacteroides forsythus, and Treponema denticola). When these complexes were checked for their association with adverse pregnancy outcomes, 2.9-fold higher prevalence of IgM seropositivity for one or more organisms of the Orange or Red complex were encountered in preterm babies compared to term babies. Further, lack of maternal IgG antibody to Red complex organisms was associated with an increased rate of PTB. This states that maternal antibody protects the fetus from exposure and resultant PTLBW with the high rate of PTB was seen in subjects without a protective Red complex IgG response with a fetal IgM response to microbes belonging to Orange complex. These data support the concept that maternal periodontal infection in

the absence of a protective maternal antibody response is associated with systemic dissemination of oral organisms which might translocate to the fetus. [26]

CONCLUSION

In the current study, periodontal bacteria (Pg., Pi, Tf, Fn) and their products were significantly seen to be associated to adverse pregnancy outcomes like preterm low birthweight deliveries and preeclampsia. This is implying periodontal disease is a potential risk for PTLBW and PE. Thus, including periodontal screening in prenatal check-up's schedule and explaining the importance of oral hygiene on developing foetus would be beneficial.

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