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Relationship between *Campylobacter rectus* and periodontal status during pregnancy

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Introduction: In a previous study, we showed that the growth of *Campylobacter rectus* is stimulated by the presence of female sex hormones in the culture medium. In the present study, we examined the relationship between *C. rectus* levels in the saliva and the periodontal status of pregnant women.
Methods: Unstimulated whole saliva was collected from 22 pregnant and 15 non-pregnant women. Periodontal pocket depth (PD) and bleeding on probing (BOP) were recorded. A quantitative real-time polymerase chain reaction was performed to determine the concentrations of suspected periodontopathogenic bacteria in the saliva samples. In addition, the concentration of estradiol in the saliva samples was measured by enzyme immunoassay.
Results: The average age, number of teeth, and total number of bacteria in the saliva of subjects in both groups were similar. The percentage of sites with a PD = 4 mm and the salivary estradiol concentrations were significantly higher in pregnant women than in non-pregnant women. In addition, the percentage of BOP sites and the *C. rectus* levels in the saliva of the pregnant women tended to be higher than in non-pregnant women, although these differences were not statistically significant. There were positive correlations between *C. rectus* levels and estradiol concentrations, and between *C. rectus* levels and the percentage of sites with PD = 4 mm in the pregnant women.
Conclusion: These results indicate that *C. rectus* levels are higher in the oral flora of pregnant women and that this may be associated with increased salivary estradiol concentrations. This may contribute to periodontal disease progression during pregnancy.

Key words: *Campylobacter rectus;* periodontal disease; pregnancy

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The host response has long been considered to be in part responsible for periodontal conditions during pregnancy (11, 25). Prostaglandin E_2 (PGE_2) production in the gingiva is stimulated by female sex hormones, which are present in high concentrations during pregnancy (5). Several authors have suggested that female sex hormones play an important role in periodontal disease progression in pregnant women (16, 26). More specifically, female sex hormones stimulate the growth of *Prevotella intermedia* (9) and may thus contribute to the increased levels of this bacterium in the periodontal sites of pregnant women (8).

We previously reported that the growth of *Campylobacter rectus* is significantly stimulated when estradiol is added to the culture medium (32). *C. rectus* is a gram-negative motile rod associated with some forms of periodontal diseases (14, 22). It produces a variety of putative virulence factors, including lipopolysaccharide, crystalline surface layer, and GroEL-like protein (GroEL), which can induce the expression of various inflammatory mediators by host cells (7, 21, 27, 28, 31). More specifically, *C. rectus* lipopolysaccharide stimulates the production of PGE_2, interleukin-1β (IL-1β), and IL-6 by gingival fibroblasts (21, 27), whereas the crystalline surface layer stimulates the secretion of

IL-6, IL-8, and tumor necrosis factor-α by HEp-2 cells derived from a human pharyngeal cancer (31). *C. rectus* GroEL, which is a 64-kDa heat-shock protein, also stimulates the production of IL-6 and IL-8 by human gingival cells (7, 28).

The present study was conducted to verify our hypothesis that increased levels of estradiol in saliva are associated with higher *C. rectus* levels, which may in turn stimulate the progression of periodontal disease during pregnancy. We measured the levels of five suspected periodontal pathogens [*C. rectus*, *Porphyromonas gingivalis*, *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, *Fusobacterium nucleatum*, and *P. intermedia*] in saliva samples using a quantitative real-time polymerase chain reaction (PCR) technique; estradiol concentrations were measured using an enzyme immunoassay, and the periodontal status of the pregnant and non-pregnant women was noted.

Table 1. Species-specific primers for real-time PCR analysis

Target	Sequence of primer (5′ to 3′)	GenBank accession no. or reference no.
C. rectus		L04317
Forward	AGC GCA ACC CAC GTC ATT A	
Reverse	CGC CAT TGT AGC ACG TGT GT	
P. gingivalis		(1)
Forward	AGG CAG CTT GCC ATA CTG CG	
Reverse	ACT GTT AGC AAC TAC CGA TGT	
A. actinomycetemcomitans		(1)
Forward	AAA CCC ATC TCT GAG TTC TTC TTC	
Reverse	ATG CCA ACT TGA CGT TAA AT	
F. nucleatum		AJ133496
Forward	GGA ACC TTA AAC CAG CGT TTG A	
Reverse	CAG CGT ATA AGG GGC ATG AT	
P. intermedia		L16468
Forward	CAG TTC GGA CTG AGG TCT GCA A	
Reverse	ATG GCT GAT GCG CGA TTA CTA	
Universal (Total bacteria)		(18)
Forward	TCC TAC GGG AGG CAG CAG T	
Reverse	GGA CTA CCA GGG TAT CTA ATC CTG TT	

Species-specific primers were designed from the regions of the bacterial 16S rRNA gene sequence.

Materials and methods

Study subjects

Twenty-two pregnant women aged 25–41 years (mean age 31.9 ± 4.4 years) at a gestational stage between 15 and 37 weeks (mean gestational period 27.4 ± 5.1 weeks) and 15 non-pregnant women aged 24–40 years (mean age 31.6 ± 5.4 years), who visited the Clinic of Dental Hygiene Section, Tokushima University Medical and Dental Hospital were recruited for the study. None had received periodontal treatments or taken antibiotics in the previous 3 months. Periodontal pocket depth (PD) and bleeding on probing (BOP) were assessed at six sites around each tooth using a periodontal pocket probe. The Ethics Committee of Tokushima University Medical and Dental Hospital approved the study and participants provided written informed consent.

Determination of levels of periodontopathogenic bacteria in the saliva by real-time PCR

C. rectus ATCC 33238 (American Type Culture Collection, Rockville, MD), *P. gingivalis* ATCC 33277, *A. actinomycetemcomitans* ATCC 29523, *F. nucleatum* ATCC 23726, and *P. intermedia* 163 were grown under appropriate conditions as described previously (32) to prepare bacterial suspensions for real-time PCR. Briefly, *C. rectus* was grown in Trypticase soy broth (Becton Dickinson, Sparks, MD) supplemented with 0.2% yeast extract (Becton Dickinson), 0.3% phytone-peptone (Becton Dickinson), 0.2% NaCl, 0.3% ammonium formate, 0.4% sodium fumarate, and 0.4% L-asparagine, adjusted to pH 7.8. The other bacterial species were grown in brain–heart infusion broth (Becton Dickinson) supplemented with 0.5% yeast extract, 0.001% vitamin K1, and 0.0005% hemin, adjusted to pH 7.8. All cultures were incubated at 37°C in an anaerobic chamber (80% N_2, 10% H_2, 10% CO_2) to mid-log phase.

The bacteria were washed in phosphate-buffered saline and counted using a Petroff–Hauser chamber. DNA was extracted using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instruction. Bacterial standards (10^2–10^8 cells/200 μl) were prepared by 10-fold serial dilutions. They were incubated at 56°C for 30 min, vortexed for 30 s, incubated at 100°C for 8 min, and then stored at –20°C until used for the real-time PCR analysis.

Unstimulated whole saliva samples were collected in sterile plastic tubes before the assessments of PD and BOP. The samples (200 μl) were centrifuged at 10,000 ***g*** for 10 min at 4°C, and the pellets were washed in phosphate-buffered saline and suspended in 200 μl InstaGene Matrix. The suspensions were incubated at 56°C for 30 min, vortexed for 30 s, incubated at 100°C for 8 min, and then stored at –20°C until used for the real-time PCR analysis.

The real-time PCR analyses were performed using a MiniOpticon system (Bio-Rad) with SYBR Green I dye and species-specific primers designed from the regions of the bacterial 16S ribosomal RNA gene sequences registered in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). The species-specific primers are listed in Table 1. The *C. rectus*-specific and *P. intermedia*-specific primers were designed and prepared for this study by Applied Biosystems Japan (Tokyo, Japan). The *F. nucleatum*-specific primers were designed using PRIMER3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (24) while the *P. gingivalis*-specific and *A. actinomycetemcomitans*-specific primers were designed according to Ashimoto et al. (1). The universal primers used to determine the total number of bacterial cells were designed according to Nadkarni et al. (18). These four primer pairs were prepared by Hokkaido System Science (Sapporo, Japan). The specificity of the primers was confirmed by BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/). The primers were checked for primer–dimer formation, melting temperature, and G + C content. A standard curve prepared using the bacterial standards was used to determine the number of bacteria in the saliva samples. The standard curve for total bacteria was generated from the average of five bacterial species according to the method of Lyons et al. (13).

The PCR mixtures contained diethylpyrocarbonate-treated water, iQ SYBR Green Supermix (Bio-Rad), forward and reverse primers, and DNA from a bacterial standard or sample. The amplification conditions were as follows: an initial denaturation (3 min at 95°C) and 50 cycles of denaturation (20 s at 95°C), annealing (20 s at 60°C), and extension (20 s at

72°C). Fluorescent products were analyzed before and after each denaturation. A melting curve covering 60–95°C at 0.5°C intervals was constructed. Melting peaks were used to confirm the specificity of the PCR. The data were analyzed using OPTICON MONITOR Version 3.1 software (Bio-Rad), and the concentrations of each bacterial species in the saliva samples were calculated from the number of copies of the target sequence.

Determination of salivary estradiol concentrations by enzyme immunoassay

Following the real-time PCR analysis, the remaining saliva in the samples was centrifuged at 3500 ***g*** for 30 min at 4°C, and the supernatants were collected. Estradiol and transferrin concentrations in the saliva samples were measured using commercial enzyme immunoassay kits (Salimetrics, State College, PA) according to the manufacturer's instructions. Transferrin, which is present in abundance in the blood but in only trace amounts in the saliva, served as a marker for blood contamination. The chromogenic reactions were recorded by measuring absorbance at 450 nm using an automatic microplate reader (Bio-Rad, Model 680).

Statistical analysis

Statistical significance was assessed by either an unpaired Student's *t*-test or by Pearson's correlation coefficient (*r*) using the SPSS 11.0 FOR WINDOWS statistical software package (SPSS Inc., Chicago, IL). Differences were considered statistically significant at $P < 0.05$.

Results

The age, number of teeth, periodontal status of the subjects, salivary estradiol concentrations, and results of the real-time PCR microbiological analyses are shown in Table 2. The average estradiol concentration of the pregnant women (115.2 ± 57.5 pg/ml) was 10.2-fold higher ($P < 0.001$) than that of non-pregnant women. Both groups were comparable in terms of age, number of teeth, and level of blood contamination of saliva samples. All the subjects in this study had no attachment loss, nor PD >4 mm. No correlation was found between estradiol concentration and blood contamination in either group (data not shown). The percentage of sites in the pregnant women with a PD = 4 mm (14.5 ± 16.7%) was 5.4-fold higher ($P = 0.004$) than that in the non-pregnant women. In addition, the percentage of BOP sites and the *C. rectus* levels in the saliva of the pregnant women (69.5 ± 29.2% and 0.531 ± 0.522%) tended to be higher than those in non-pregnant women, although these differences were not statistically significant. Both groups were comparable in terms of total bacteria and levels of other bacterial species.

Table 2. Comparison between pregnant and non-pregnant women

	Pregnant women	Non-pregnant women	*P*-value[6]
Number of study subjects	22	15	–
Age (years)	31.9 ± 4.4[5]	31.6 ± 5.4	0.855
Gestational period (weeks)	27.4 ± 5.1	–	–
Number of teeth present	28.2 ± 2.6	28.3 ± 1.1	0.892
PD = 4 mm (%)[1]	14.5 ± 16.7	2.7 ± 3.5	0.004
BOP (%)[2]	69.5 ± 29.2	53.8 ± 28.8	0.117
Estradiol concentration (pg/ml)	115.2 ± 57.5	11.2 ± 8.6	< 0.001
Transferrin concentration (mg/dl)[3]	3.24 ± 8.4	1.45 ± 1.3	0.436
C. rectus (%)[4]	0.531 ± 0.522	0.316 ± 0.208	0.093
P. gingivalis (%)	0.099 ± 0.331	0.077 ± 0.163	0.791
A. actinomycetemcomitans (%)	0.046 ± 0.212	0.002 ± 0.005	0.340
F. nucleatum (%)	3.007 ± 6.675	3.162 ± 6.262	0.943
P. intermedia (%)	8.563 ± 6.415	7.675 ± 9.119	0.747
Total bacterial number (cells/ml)	$1.69 \times 10^8 \pm 1.36 \times 10^8$	$1.73 \times 10^8 \pm 1.95 \times 10^8$	0.947

[1]Percentage of sites with PD = 4 mm. The subjects in this study had no attachment loss, nor PD > 4 mm.
[2]Percentage of sites with BOP.
[3]The concentration of transferrin present in the saliva is indicative of blood contamination.
[4]Each species was expressed as a percentage of total bacteria.
[5]The values represent the mean ± SD.
[6]Statistical significance was assessed by unpaired Student's *t*-test. Probabilities (*P*-value) of < 0.05 were considered statistically significant.

Table 3. Correlation between levels of each bacterial species and estradiol concentrations or periodontal status in all subjects

Bacterial species	Estradiol *r* (*P*)	PD = 4 mm *r* (*P*)	BOP *r* (*P*)
C. rectus	0.443 (0.006)	0.563 (< 0.001)	0.419 (0.010)
P. gingivalis	0.229 (0.072)	0.212 (0.208)	0.202 (0.231)
A. actinomycetemcomitans	0.157 (0.354)	0.182 (0.281)	0.174 (0.303)
F. nucleatum	0.214 (0.203)	0.339 (0.040)	0.288 (0.084)
P. intermedia	0.075 (0.659)	0.144 (0.396)	0.170 (0.315)

Each species was expressed as a percentage of total bacteria. Estradiol: concentration of estradiol in the saliva; PD = 4 mm: percentage of sites with PD = 4 mm; BOP: percentage of sites with BOP. Pearson's correlation coefficient (*r*) was used to assess the relationship. $P < 0.05$ was considered statistically significant.

There were positive correlations between estradiol concentrations and the percentage of PD = 4 mm sites ($r = 0.362$, $P = 0.028$) and between *C. rectus* levels as a percentage of total bacteria and estradiol concentrations ($r = 0.443$, $P = 0.006$), the percentage of PD = 4 mm ($r = 0.563$, $P < 0.001$), and BOP sites ($r = 0.419$, $P = 0.010$) in all subjects (Table 3 and Fig. 1). There were positive correlations between *C. rectus* levels and estradiol concentrations ($r = 0.434$, $P = 0.044$) and between *C. rectus* levels and the percentage of PD = 4 mm sites ($r = 0.568$, $P = 0.006$) in pregnant women (Table 4). In addition, there were positive correlations between *P. gingivalis* and *F. nucleatum* levels as a percentage of total bacteria and estradiol concentrations in pregnant women ($r = 0.468$, $P = 0.028$ and $r = 0.452$, $P = 0.035$, respectively), but not in all subjects. There was no correlation between *A. actinomycetemcomitans* and *P. intermedia* levels and the other parameters in either group.

Discussion

To our knowledge, this is the first time that the levels of periodontopathogenic bacteria in the saliva of pregnant women have been measured using real-time PCR. Saliva samples were used to measure the number of bacteria by real-time PCR rather than subgingival plaque samples, which are a very sensitive method (3, 10). Saliva samples have been reported to contain subgingival periodontopathogens and thus

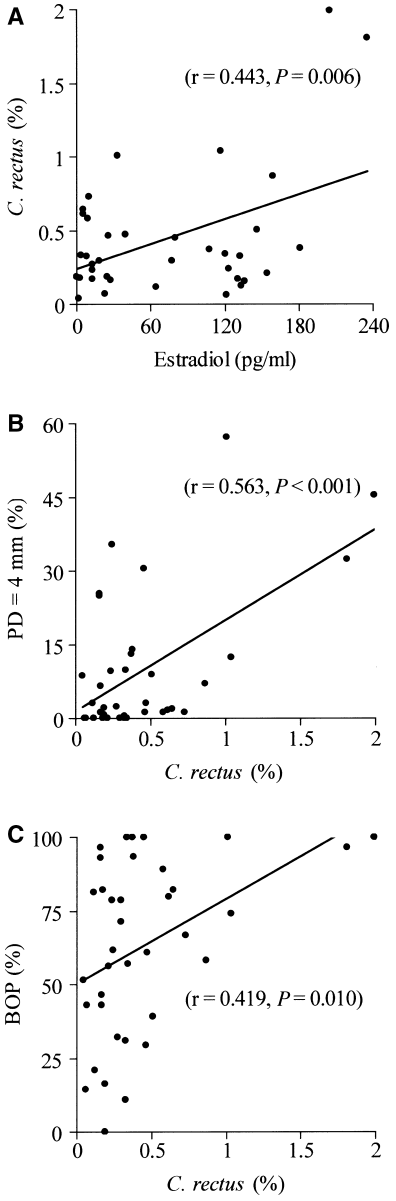

Fig. 1. Correlation between levels of *Campylobacter rectus* as a percentage of total bacteria and estradiol concentrations in the saliva (A) or periodontal status [the percentage of sites with PD = 4 mm (B) and BOP (C)]. Correlations were performed in all subjects ($n = 37$). Pearson's correlation coefficient (r) and probabilities (P) are shown in parentheses.

represent an excellent alternative to sampling individual periodontal pockets (30). In addition, saliva sampling is technically easy and less of a burden for subjects than subgingival plaque sampling using paper points. In the present study, real-time PCR was performed using SYBR Green I dye instead of the TaqMan probe. The specificity of the SYBR Green assay has already been confirmed by the single peak of the melting curve (23).

Table 4. Correlation between levels of each bacterial species and estradiol concentrations or periodontal status in pregnant women

Bacterial species	Estradiol r (P)	PD = 4 mm r (P)	BOP r (P)
C. rectus	0.434 (0.044)	0.568 (0.006)	0.416 (0.054)
P. gingivalis	0.468 (0.028)	0.242 (0.278)	0.133 (0.556)
A. actinomycetemcomitans	0.086 (0.703)	0.142 (0.528)	0.181 (0.420)
F. nucleatum	0.452 (0.035)	0.489 (0.021)	0.289 (0.193)
P. intermedia	0.046 (0.839)	0.197 (0.380)	0.117 (0.605)

Each species was expressed as a percentage of total bacteria. Estradiol: concentration of estradiol in the saliva; PD = 4 mm: percentage of sites with PD = 4 mm, BOP: percentage of sites with BOP. Pearson's correlation coefficient (r) was used to assess the relationship. $P < 0.05$ was considered statistically significant.

Berg et al. (2) reported that estradiol concentrations in saliva increase to approximately 100 pg/ml during pregnancy while Lu et al. (12) found that estradiol concentrations in saliva range from 5 to 12 pg/ml during the menstrual cycle. In this study, the estradiol concentration in the saliva of pregnant women averaged 115.2 ± 57.5 pg/ml, which was approximately 10-fold higher than the average in non-pregnant women (11.2 ± 8.6 pg/ml). These results are thus consistent with those in previous studies (2, 12). During pregnancy, the estradiol concentration in the plasma increases to approximately 15,000 pg/ml (29), which is almost 150 times the concentration observed in the saliva. The contamination of saliva by blood is thus a potential problem. In the present study, however, such contamination was negligible in both groups. This indicated that the high concentrations of estradiol in the saliva of pregnant woman were not the result of contamination by blood.

Recent studies have suggested that periodontal diseases in pregnant women may be a significant risk factor for preterm low-birth weight (PLBW) infants (19) and that *C. rectus* may be associated with PLBW infants (4, 15, 17). More specifically, the degree of seropositivity of fetal immunoglobulin M to *C. rectus* is significantly higher in preterm neonates than in full-term neonates (15). It has also been reported that mothers who have PLBW infants have significantly higher levels of *C. rectus* in their subgingival plaque than women with normal birth outcomes (17). A case–control study evaluating the subgingival bacteria of women within 3 days postpartum suggested that the presence of *C. rectus* increases the risk of a PLBW infant with an odds ratio of 7.15 (4). Furthermore, Offenbacher et al. (20) have shown in a mouse model that maternal *C. rectus* infections can cause placental inflammation and decidual hyperplasia as well as a concomitant increase in fetal brain interferon-γ. *C. rectus* may thus increase the risk for PLBW infants.

In the present study, the level of *C. rectus* as a percentage of the total oral microflora tended to be higher in pregnant women than in non-pregnant women and was positively correlated with the estradiol concentration, indicating that higher levels of *C. rectus* in pregnant women may be associated with PLBW infants. Steroid hormones can modulate the microbial ecology in the gingival sulcus and may be responsible for the changes observed in the oral microflora of pregnant women (8). Female sex hormones interact directly with the fumarate reductase system and stimulate the growth of *P. intermedia* (9), which can produce large amounts of formate. As formate enhances the growth of *C. rectus* (6), female sex hormones can thus indirectly stimulate the growth of *C. rectus*. However, in contrast to the previous report that demonstrated the involvement of *P. intermedia* in the progression of gingivitis in pregnant women (8), we did not found a correlation between the levels of this microorganism and estradiol concentrations in the saliva. This discrepancy could be because different types of samples (saliva compared with subgingival plaque) were used. In addition, adding estradiol to the culture medium significantly stimulates the growth of *C. rectus* (32). This suggests that female sex hormones are likely to stimulate the growth of *C. rectus* directly, although further research is needed to clarify the mechanism of this stimulation. *C. rectus* levels of the pregnant women tended to be higher than those in non-pregnant women but the difference was not statistically significant. A *post hoc* power calculation suggests that a total of 130 subjects would be required to achieve a power of 80% and $P < 0.05$.

It was interesting to note that the correlations between *F. nucleatum* and

the parameters under study, such as estradiol concentrations and sites where PD = 4 mm, became stronger when only pregnant subjects were used compared to when all the subjects were used for the correlation analyses. Since female sex hormones did not promote the growth of *F. nucleatum* (32), one may hypothesize that the increased number of PD = 4 mm sites in the pregnant women may have grown *F. nucleatum*. However, this hypothesis contradicts our finding that both the pregnant and non-pregnant women were comparable in terms of their *F. nucleatum* levels (Table 2). Further studies are needed to clarify this.

In conclusion, the level of *C. rectus* as a percentage of the total microflora in the saliva was positively correlated with the concentration of estradiol in the saliva and with the percentage of PD = 4 mm and BOP positive sites. These results are in agreement with our previous *in vitro* study (32), and suggest that the population of *C. rectus* increases in the oral microflora of pregnant women and may contribute to periodontal disease progression during pregnancy.

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