Oral Microbiology Immunology 2009: 24: 299–303
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY AND IMMUNOLOGY

Does the frequency of *Prevotella intermedia* increase during pregnancy?

M. Gürsoy[1,2], G. Haraldsson[1,3], M. Hyvönen[1], T. Sorsa[2], R. Pajukanta[2], E. Könönen[1,4]

[1]Department of Bacterial and Inflammatory Diseases, National Public Health Institute (KTL), Helsinki, Finland, [2]Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital, Institute of Dentistry, University of Helsinki, Helsinki, Finland, [3]Department of Clinical Microbiology, Landspitali University Hospital, and Faculty of Odontology, University of Iceland, Reykjavik, Iceland, [4]Department of Periodontology, Institute of Dentistry, University of Turku, Turku, Finland

Gürsoy M, Haraldsson G, Hyvönen M, Sorsa T, Pajukanta R, Könönen E. Does the frequency of Prevotella intermedia *increase during pregnancy? Oral Microbiol Immunol 2009: 24: 299–303.* © 2009 John Wiley & Sons A/S.

Introduction: The former *Bacteroides intermedius*, currently including *Prevotella intermedia* and *Prevotella nigrescens*, has been associated with hormone-induced pregnancy gingivitis. The aim of the present longitudinal study was to determine whether only *P. intermedia* or *P. nigrescens*, or both species, are involved in the demonstrated microbial shift during pregnancy.
Methods: Subgingival plaque and saliva samples, collected from 30 healthy pregnant women and 24 healthy non-pregnant women as their controls, were examined for the presence of pigmented gram-negative anaerobes. Altogether 2628 isolates were preliminarily identified as *P. intermedia sensu lato*, based on phenotypic testing. Their further identification was performed by using a 16S ribosomal DNA-based polymerase chain reaction (PCR).
Results: A mean of 8.3 *P. intermedia sensu lato* isolates from each subject/sampling was examined. During the second trimester, the mean number of *P. intermedia sensu lato* in plaque increased along with increasing signs of pregnancy gingivitis, and then both decreased. After delivery, gingival inflammation still decreased while the number of *P. intermedia sensu lato* transiently increased both in plaque and saliva. In the present study, the vast majority of isolates (95.3%) proved to be *P. nigrescens* and 2.5% were *P. intermedia*. The remaining 2.2% of the isolates could not be identified with PCR as *P. intermedia* or *P. nigrescens*. The corresponding percentages in the control population were 94.2%, 5.5%, and 0.3%.
Conclusion: In the oral cavity of relatively young women without periodontitis, *P. nigrescens*, unlike *P. intermedia*, is a frequent finding. Conceivably, pregnant women harbor increasing numbers of *P. nigrescens* associated with pregnancy gingivitis.

Key words: gingivitis; polymerase chain reaction; pregnancy; *Prevotella intermedia*; *Prevotella nigrescens*; saliva; subgingival plaque

Mervi Gürsoy, Department of Bacterial and Inflammatory Diseases, National Public Health Institute (KTL), Mannerheimintie 166, 00300 Helsinki, Finland
Tel.: +358 9 47448489;
fax: +358 9 47448238;
e-mail: mervi.latva-aho@ktl.fi

Accepted for publication December 30, 2008

Since the 1960s, the condition of periodontal tissues in pregnant women has been widely studied. It has been demonstrated that pregnancy increases the susceptibility to gingivitis, described as pregnancy gingivitis (1, 5, 17, 23, 27). Elevated levels of female sex hormones during pregnancy have been considered to play the major role in this phenomenon (8, 18). Besides the direct physiological effects on periodontal tissues, the increase in systemic levels of female sex hormones that occurs with pregnancy seems to correlate with the enhanced growth of certain gram-negative anaerobes in the oral cavity (9, 28). This also occurs during puberty (6, 21) and during the use of oral contraceptives (9). *Prevotella intermedia sensu lato* (formerly *Bacteroides intermedius*), in particular, has been associated with hormone-induced pregnancy gingivitis (9, 15, 20). *In vitro* results indicate that, instead of vitamin K, the capability of this organism to use female sex hormones for growth may, at least in part, explain its relation to pregnancy (16).

The implementation of molecular biology methods has resulted in major changes in the taxonomy of *Bacteroides* species during the last decades, and two new genera, *Porphyromonas* and *Prevotella*, have been formed for oral *Bacteroides* species (24, 25). Furthermore, the former *B. intermedius*, renamed as *P. intermedia*, was shown to include two phenotypically identical species, *P. intermedia* and *Prevotella nigrescens* (26). These two species are easily and reliably separated from each other by using molecular

biology methods, such as a 16S ribosomal DNA-based polymerase chain reactions (PCR) (7, 22).

The aim of the present study, as part of our longitudinal investigation on pregnancy-related events in the oral cavity, was to obtain updated information on the involvement of *P. intermedia* and *P. nigrescens* in pregnancy gingivitis.

Materials and methods

Study population

The pregnant study population (Pr group) comprised 30 generally healthy, non-smoking Caucasian women (mean age 29.3 ± 2.8 years) who were at an early phase of their pregnancy at baseline. Twenty-four non-pregnant women (mean age of 30.4 ± 3.1 years) were recruited as their controls (N-Pr group). The groups have been described in detail by Gürsoy et al. (5). Our sampling strategy was modified according to the corresponding longitudinal study by Kornman and Loesche (15). Briefly, the women in the Pr group were examined three times during pregnancy (Pr Ex I at 12–14 weeks, Pr Ex II at 25–27 weeks, and Pr Ex III at 34–38 weeks of pregnancy) and twice postpartum [Pr Ex IV at 4–6 weeks after delivery and Pr Ex V after lactation had stopped (mean lactation time of 38.7 ± 19.2 weeks)]. The women in the N-Pr group were examined three times (N-Pr Ex I, N-Pr Ex II, and N-Pr Ex III), once per subsequent month.

Collection of specimens

During each visit, the periodontal examinations, including bleeding on probing (BOP) measurements, were performed as previously described (5), and then followed by sample collections. After the tooth surfaces were gently dried with air and kept dry with cotton wool rolls, a pooled subgingival dental plaque sample was taken with a sterile Mini Gracey 11/12 curette (LM-Instruments Oy, Parainen, Finland), from the mesiobuccal sites of all the first molars or, if these were missing, second molars. The plaque sample was placed into VMGA III transport medium (3) in a vial with glass beads. Second, paraffin-stimulated saliva was collected by expectoration for 5 min, and the sample was placed in a plastic Nunc Cryo Tube™ (Thermo Fisher Scientific, Roskilde, Denmark). All samples were transported to the National Public Health Institute (KTL) within 2 h and cultivated there immediately.

Culture and preliminary identification

Each sample was thoroughly mixed on a vortex mixer and serial dilutions (10^{-1}–10^{-5}) were made. An aliquot of 100 μl of the different dilutions was plated and cultured on non-selective Brucella agar, enriched with hemin and vitamin K, for total anaerobic growth, and on selective kanamycin vancomycin laked blood (KVLB-2) agar for the detection of pigmented *Prevotella* species (10). The plates were incubated at 37°C in anaerobic jars for 5–7 days until isolation and subcultivation of potential *P. intermedia* and *P. nigrescens* colonies on rabbit laked blood agar, egg yolk agar, and Brucella agar, which were anaerobically incubated for 3–5 days. A minimum of 10 black-pigmented isolates per specimen, if possible, were collected from the KVLB-2 agar plates. The preliminary identification of *P. intermedia sensu lato* was based on colony description, Gram-staining (gram-negative short rods), aerotolerance, and phenotypic testing, including pigment production (black) and fluorescence under ultraviolet light (red) on rabbit laked blood agar, lipase reaction (positive) on egg yolk agar, and spot tests for the reduction of nitrate (negative) and the production of catalase (negative) and indole (positive) (10).

Identification by 16S ribosomal DNA PCR

For the DNA isolation, one or two colonies of *P. intermedia sensu lato* were harvested from the Brucella agar plate using a sterile loop and suspended in 500 μl of 5% Chelex 100 (Bio-Rad Laboratories, Hercules, CA), heated to 100°C for 10 min, and centrifuged. The supernatant was used for PCR amplification using the species-specific primer pairs PI3 5′-CCC GAT GTT GTC CAC ATA TGG-3′ and PI4 5′-GCA TAC GTT GCG TGC ACT CAA G-3′ for *P. intermedia* and PN1-kort 5′-TTG AGT ACA CGC AGC GCA GGC G-3′ and PN3 5′-CCC GAT GGC AAC TGG GAA AGG-3′ for *P. nigrescens*, using the slightly modified method of Haraldsson and Holbrook (7). Briefly, to 3.1 μl DNA suspension was added 1 μM of each primer, 0.2 mM of each deoxynucleotide triphosphate, 1.0 unit of DyNAZyme polymerase (Finnzymes, Espoo, Finland), 2.5 μl of 10× DyNAZyme buffer, and water to make a total reaction volume of 25 μl. PCR was performed in a thermal cycler (Eppendorf, Hamburg, Germany) using initial denaturation at 95°C for 1 min, followed by 25 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for the PI3/PI4 primer pair and 66°C for the PN1-kort/PN3 primer pair for 1 min, and extension at 72°C for 1 min and a final extension at 72°C for 10 min. The amplified products were kept at 4°C, electrophoresed in 1.5% agarose gel, stained with ethidium bromide, and photographed in ultraviolet light (AlphaImager; Alpha Innotech Co, San Leandro, CA). A 100-base-pair (bp) ladder (Amersham Bioscience, Piscataway, NJ) served as a molecular weight marker. The universal primers KO-1 CCC GGG AAC GTA TTC AAC G and KO-2-st GAT TAG ATA CCC TGG TAG TCC were used as a positive control for PCR amplification, using the above programming and an annealing temperature of 60°C. Twenty reference strains from the collection of anaerobic bacteria at the National Public Health Institute (KTL), including *P. intermedia* ATCC 25611^T and *P. nigrescens* ATCC 33263^T, were used to confirm the species specificity of the primers.

Statistical analyses

Data are expressed as mean values and standard deviations. Statistical evaluation was performed using SPSS 14.0 (SPSS Inc., Chicago, IL). Statistical differences within the two groups were determined by the Friedman test, followed by the Wilcoxon signed ranks test for comparing the variations between the follow-up visits. In addition, the Mann–Whitney test was used for the data comparison between the groups. Data were considered as statistically significant at P-values <0.05.

Results

Altogether, 2628 isolates were identified as *P. intermedia sensu lato*. Table 1 presents the number of isolates examined per subject and specimen in detail. The gaps in the data for number of subjects or samplings and *P. intermedia sensu lato* isolates were mainly caused by missing visits during the follow-up period and loss of isolates during the culture procedures.

Changes in the mean growth [presented as colony forming units (CFU)/ml] of *P. intermedia sensu lato* in subgingival plaque and saliva during the follow-up are shown in Fig. 1. In the Pr group, the mean growth of *P. intermedia sensu lato* in the subgingival plaque transiently increased during the second trimester, being nearly twice as high as during the first trimester. After delivery, there was a second transient

Table 1. Prevotella intermedia sensu lato isolates from saliva and subgingival sites examined as divided according to subject and sampling occasion

Subject group	Sample	No. of subjects	No. of samplings	No. of isolates/ subject		No. of isolates/ sampling		Total no. of isolates
				mean	range	mean	range	
Pr	Saliva	27	105	30.9	1–52	7.9	1–14	835
	Plaque	29	104	33.9	2–71	9.4	1–21	982
N-Pr	Saliva	24	59	16.7	2–32	6.8	1–12	400
	Plaque	21	47	19.6	2–40	8.7	1–12	411

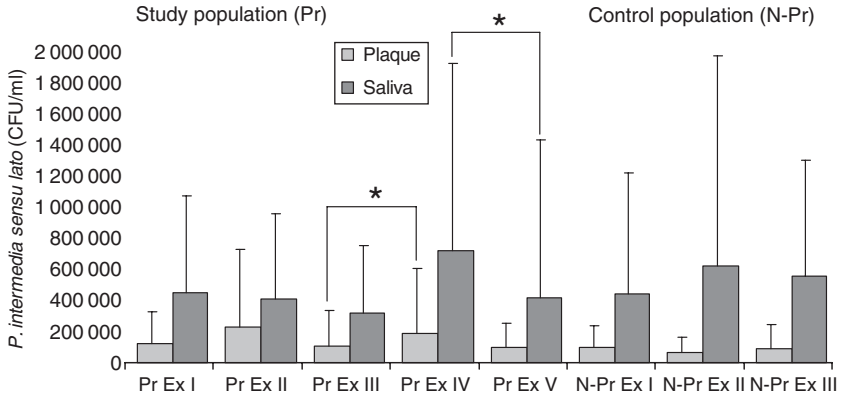

Fig. 1. The mean growth [colony-forming units (CFU)/ml] of *Prevotella intermedia sensu lato* in subgingival plaque and saliva samples from pregnant women at five visits (Pr Ex I–V) and non-pregnant women (N-Pr) at three visits (N-Pr Ex I–III). (Wilcoxon signed ranks test: $*P < 0.05$).

increase ($P < 0.05$) in the mean CFU, which decreased to the lowest point after lactation, reaching the same level as was found in the N-Pr group. In saliva, the mean CFUs of *P. intermedia sensu lato* were relatively invariable during pregnancy, then transiently increased after delivery, and decreased ($P < 0.05$) after lactation to the same level as those found in the N-Pr group.

When the proportions of *P. intermedia sensu lato*, presented as a percentage of the total anaerobic growth, were compared with the mean percentages of BOP during the follow-up, a trend was observed between its increased proportions in subgingival plaque and increased gingival inflammation in the Pr group (Fig. 2). The percentage of *P. intermedia sensu lato* in subgingival plaque reached its peak (6.3%) during the second trimester, while its proportion in saliva remained relatively stable during the follow-up in both subject groups.

The 16S ribosomal DNA PCR method correctly identified the 20 reference strains as *P. intermedia* ($n = 14$) or *P. nigrescens* ($n = 6$). Of the 1817 isolates in the Pr group, 95.3% were *P. nigrescens* and 2.5% were *P. intermedia*, whereas 2.2% remained unidentified. Of the 811 isolates in the N-Pr group, the corresponding percentages were 94.2%, 5.5%, and 0.3%. In the Pr group, five mothers harbored *P. intermedia* in their saliva and two of them also in the subgingival plaque. In the N-Pr group, *P. intermedia* was found in the saliva of five subjects and one of them harbored *P. intermedia* also in subgingival plaque. All subjects in both groups harbored *P. nigrescens* whether in saliva or in subgingival plaque during the follow-up.

Discussion

According to our knowledge, the present longitudinal study is the first to identify *P. intermedia* and *P. nigrescens* separately, providing new data on their occurrence in the oral cavity during pregnancy and relating this to pregnancy gingivitis. Our results confirm and further extend previous reports on the involvement of *P. intermedia sensu lato* with pregnancy gingivitis. In these periodontally healthy Finnish women, however, the microbial shift was the result of increased levels of *P. nigrescens*, but not of the increased levels of *P. intermedia*.

Taxonomical changes may cause confusion when comparing bacterial findings between different studies. After the identification of two separate species within the former *P. intermedia* (*B. intermedius*) by Shah and Gharbia in 1992 (26), the name *P. intermedia* has been used in many studies, despite the lack of proper methods for the separation of *P. intermedia* and *P. nigrescens* one from the other. '*P. intermedia sensu lato*', the term we have used here, is correct in situations where their identification is based solely on biochemical methods.

In the present study, to improve participants' commitment and to enable this longitudinal material to be handled by one investigator, the number of visits during pregnancy was limited to three. As shown in a longitudinal study by Kornman and Loesche (15), major changes in the bacteriology occur during each trimester. Also, our sampling strategy for the control group

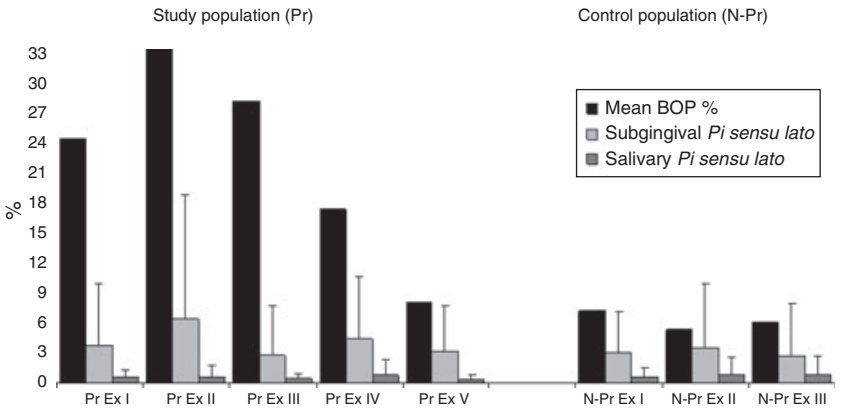

Fig. 2. The mean percentage of bleeding on probing (BOP; data available from ref. 5) in relation to the proportions (%) of *Prevotella intermedia* (*Pi*) *sensu lato* in subgingival plaque and saliva samples from pregnant women at five visits (Pr Ex I–V) and non-pregnant women (N-Pr) at three visits (N-Pr Ex I–III).

was modified according to Kornman and Loesche (15), i.e. the non-pregnant women were examined three times, once per subsequent month. This sampling frequency was considered sufficient for evaluating the stability over time of both clinical and microbiological measurements in non-pregnant women.

Recently, we reported on the clinical changes that occurred in the periodontium in these women during pregnancy and postpartum (5). Indeed, periodontal tissues proved to be susceptible to inflammation during pregnancy because both the tendency and severity of gingivitis increased significantly. This increase was most obvious during the second trimester but, after lactation, the BOP levels returned to a level similar to that found in the matched non-pregnant control group. The amount of dental plaque in pregnant women decreased visit by visit, being at the lowest level after lactation (5). Also, qualitative changes in plaque composition could be observed, as shown in the present study. In subgingival plaque, both the amount and proportion of *P. intermedia sensu lato* increased transiently twice in the Pr group, reaching the highest peaks during the second trimester and again 4–6 weeks after delivery. No such changes in subgingival or salivary microbial flora were detected among non-pregnant women. The present findings are consistent with observations in other longitudinal studies (15, 20). Kornman and Loesche (15) were the first to report statistically significant increases in gingivitis scores and in the proportion of *Bacteroides melaninogenicus* ss. *intermedius* (i.e., *P. intermedia sensu lato*) during the second trimester. Later, Muramatsu and Takaesu (20) demonstrated increasing levels of *P. intermedia* (here: *P. intermedia sensu lato*) from the fourth month to the eighth month of pregnancy. They also found a second peak in the proportions of *P. intermedia sensu lato* during the last visit, 1 month after delivery, as we did in the present study. A strong correlation between the growth of *P. intermedia sensu lato* and elevated levels of female sex hormones may explain the peak during the second trimester but not postpartum. Muramatsu and Takaesu (20) speculated that the second peak in the proportions of *P. intermedia sensu lato* could be related to advanced inflammation in periodontal tissues with prolonged healing. Our findings do not support this suggestion, because the mean probing pocket depths decreased gradually after the highest peak was reached during the second trimester.

In the present study, the mean percentage of *P. intermedia sensu lato* in subgingival plaque during the second trimester was 6.3%, whereas in previous studies (15, 20, 23) it has been around 10%. One reason for the discrepancy may be the result of standardizing; in the present study, a pooled subgingival plaque sample was always taken from the mesiobuccal sites of the same molar teeth. The sampling was independent of the degree of inflammation around the other teeth, whereas the sampling sites during the follow-up by Muramatsu and Takaesu (20) were selected based on the most inflamed sites from the anterior area.

In previous pregnancy-related studies on oral microbes, subgingival plaque as a study specimen and phenotypic methods for identification have been used. This makes the comparison of our salivary findings with other studies somewhat difficult. A cross-sectional case–control study by Yokoyama et al. (28) used real-time PCR to detect periodontopathogens, including *P. intermedia*, in unstimulated saliva of pregnant women. The authors failed to find any correlation either between the levels of *P. intermedia* and signs of gingival inflammation or between the levels of *P. intermedia* and salivary estradiol concentrations. The latter disagreement with the report by Kornman and Loesche (15) was suggested to be caused by the use of saliva as their study specimen. Indeed, the occurrence rate of *P. intermedia sensu lato* seems to be slightly higher in subgingival sites than in saliva (11, 12). One possible explanation for the missing correlation between the levels of *P. intermedia* and the increased gingivitis or salivary estradiol concentrations in the study by Yokoyama et al. (28) might be that, after all, the *P. intermedia* levels remain unaffected in pregnant women without attachment loss or periodontal pockets >4 mm. Besides, Yokoyama et al. (28) did not investigate *P. nigrescens* and its correlation to increased gingivitis. In our study, the proportions of salivary *P. intermedia sensu lato* did not differ significantly either within the subject group or between the two groups. However, an increase in the mean growth of *P. intermedia sensu lato* was seen 4–6 weeks after delivery both in saliva and in subgingival plaque.

In the present study, the vast majority of the more than 2600 *P. intermedia sensu lato* isolates, collected from subgingival plaque and saliva, proved to be *P. nigrescens*. *P. intermedia sensu stricto* was detected only occasionally. Despite the typical characteristics of *P. intermedia sensu lato* in phenotypic tests, the PCR method failed to identify a small proportion (<3%) of the isolates. The reason for this remained unclear.

In the present study population, the salivary carriage rate of *P. intermedia sensu stricto* was 17% in pregnant women and 21% in non-pregnant women. In a recent population-based study, the salivary carriage of periodontal pathogens among 1294 adults living in southern Finland was examined by PCR (13). The detection rate of *P. intermedia sensu stricto* varied between 12 and 23% according to background variables, such as gender, age, education, marital status, smoking history, the number of teeth, and the number of teeth with periodontal pockets. In the group for 30–34 years, the prevalence of *P. intermedia* was 13%. In dentate adults (n = 1216), *P. intermedia* associated significantly with smoking history (highest in daily smokers), marital status (more prevalent in singles than in married/cohabiting couples), and the number of teeth with pockets ≥4 mm (13). In another Finnish study, presenting separate data for *P. intermedia* and *P. nigrescens* in 23 mothers, prevalence rates of 22% for *P. intermedia* and 87% for *P. nigrescens* were reported (14). The study population included also mothers with initial or advanced periodontitis, which may explain the slightly higher prevalence of *P. intermedia* in comparison with our present results. Indeed, *P. intermedia* has been associated with periodontal infections (4, 13, 19), whereas *P. nigrescens* has been frequently isolated from the oral cavity of periodontally healthy individuals (2, 4, 14, 19).

In conclusion, *P. nigrescens*, unlike *P. intermedia*, was a common finding in these relatively young Finnish women with signs of pregnancy gingivitis but without periodontitis. Furthermore, our findings indicate that increased levels of *P. nigrescens* are associated with pregnancy gingivitis.

Acknowledgments

This study has been supported by grants from the Finnish Dental Society Apollonia, the Finnish Women Dentists' Association, the Academy of Finland, and the HUCH-EVO-Foundation.

References

1. Cohen DW, Friedman L, Shapiro J, Kyle GC. A longitudinal investigation of the periodontal changes during pregnancy. J Periodontol 1969: **40**: 563–570.

2. Conrads G, Mutters R, Fischer J, Brauner A, Lütticken R, Lampert F. PCR reaction and dot-blot hybridization to monitor the distribution of oral pathogens within plaque samples of periodontally healthy individuals. J Periodontol 1996: **67**: 994–1003.
3. Dahlén G, Pipattanagovit P, Rosling B, Möller AJ. A comparison of two transport media for saliva and subgingival samples. Oral Microbiol Immunol 1993: **8**: 375–382.
4. Gharbia SE, Haapasalo M, Shah NH et al. Characterization of *Prevotella intermedia* and *Prevotella nigrescens* isolates from periodontic and endodontic infections. J Periodontol 1994: **65**: 56–61.
5. Gürsoy M, Pajukanta R, Sorsa T, Könönen E. Clinical changes in periodontium during pregnancy and post-partum. J Clin Periodontol 2008: **35**: 576–583.
6. Gusberti FA, Mombelli A, Lang NP, Minder CE. Changes in subgingival microbiota during puberty. A 4-year longitudinal study. J Clin Periodontol 1990: **17**: 685–692.
7. Haraldsson G, Holbrook W. Identifying clinically important gram-negative anaerobes from the oral cavity. Eur J Oral Sci 1999: **107**: 429–436.
8. Hugoson A. Gingival inflammation and female sex hormones. A clinical investigation of pregnant women and experimental studies in dogs. J Periodontal Res 1970: **5**(Suppl): 1–18.
9. Jensen J, Liljemark W, Bloomquist C. The effect of female sex hormones on subgingival plaque. J Periodontol 1981: **53**: 599–602.
10. Jousimies-Somer HR, Summanen P, Citron DM et al. Anaerobic bacteriology manual, 6th edn. Belmont, CA: Star Publishing, 2002.
11. Könönen E. Pigmented *Prevotella* species in the periodontally healthy oral cavity. FEMS Immunol Med Microbiol 1993: **6**: 201–205.
12. Könönen E, Jousimies-Somer H, Asikainen S. The most frequently isolated gram-negative anaerobes in saliva and subgingival samples taken from young women. Oral Microbiol Immunol 1994: **9**: 126–128.
13. Könönen E, Paju S, Pussinen PJ et al. Population-based study of salivary carriage of periodontal pathogens in adults. J Clin Microbiol 2007: **45**: 2446–2451.
14. Könönen E, Wolf J, Mättö J et al. The *Prevotella intermedia* group organisms in young children and their mothers as related to maternal periodontal status. J Periodontal Res 2000: **35**: 329–334.
15. Kornman KS, Loesche WJ. The subgingival microbial flora during pregnancy. J Periodontal Res 1980: **15**: 111–122.
16. Kornman KS, Loesche WJ. Effects of estradiol and progesterone on *Bacteroides melaninogenicus* and *Bacteroides gingivalis*. Infect Immun 1982: **35**: 256–263.
17. Löe H, Silness J. Periodontal disease in pregnancy. I. Prevalence and severity. Acta Odontol Scand 1963: **21**: 533–551.
18. Mascarenhas P, Gapski R, Al-Shammari K, Wang HL. Influence of sex hormones on the periodontium. J Clin Periodontol 2003: **30**: 671–681.
19. Mättö J, Saarela M, von Troil-Lindén B et al. Distribution and genetic analysis of oral *Prevotella intermedia* and *Prevotella nigrescens*. Oral Microbiol Immunol 1996: **11**: 96–102.
20. Muramatsu Y, Takaesu Y. Oral health status related to subgingival bacterial flora and sex hormones in saliva during pregnancy. Bull Tokyo Dent Coll 1994: **35**: 139–151.
21. Nakagawa S, Fujii H, Machida Y, Okuda K. A longitudinal study from prepuberty to puberty of gingivitis. Correlation between the occurrence of *Prevotella intermedia* and sex hormones. J Clin Periodontol 1994: **21**: 658–665.
22. Paster BJ, Dewhirst FE, Olsen I, Fraser GJ. Phylogeny of *Bacteroides*, *Prevotella*, and *Porphyromonas* spp. and related bacteria. J Bacteriol 1994: **176**: 725–732.
23. Raber-Durlacher JE, van Steenbergen TJM, van der Velden U, de Graaff J, Abraham-Inpijn L. Experimental gingivitis during pregnancy and post-partum: clinical, endocrinological, and microbiological aspects. J Clin Periodontol 1994: **21**: 549–558.
24. Shah HN, Collins MD. Proposal for reclassification of *Bacteroides asaccharolyticus*, *Bacteroides gingivalis*, and *Bacteroides endodontalis* in a new genus, *Porphyromonas*. Int J Syst Bacteriol 1988: **38**: 128–131.
25. Shah HN, Collins MD. *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. Int J Syst Bacteriol 1990: **40**: 205–208.
26. Shah HN, Gharbia SE. Biochemical and chemical studies on strains designated *Prevotella intermedia* and proposal of a new pigmented species, *Prevotella nigrescens* sp. nov. Int J Syst Bacteriol 1992: **42**: 542–546.
27. Tilakaratne A, Soory M, Ranasinghe AW, Corea SM, Ekanayake SL, de Silva M. Periodontal disease status during pregnancy and 3 months post-partum, in a rural population of Sri-Lankan women. J Clin Periodontol 2000: **27**: 787–792.
28. Yokoyama M, Hinode D, Yoshiyoka M et al. Relationship between *Campylobacter rectus* and periodontal status during pregnancy. Oral Microbiol Immunol 2008: **23**: 55–59.