Detection of *Porphyromonas gingivalis* in the Amniotic Fluid in Pregnant Women With a Diagnosis of Threatened Premature Labor

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Background: Epidemiologic and randomized controlled studies have shown that periodontal diseases may be associated with preterm labor and delivery of infants with low birth weights. The purpose of the present study was to determine the presence of microbial invasion of the amniotic cavity by periodontopathic bacteria in pregnant women with a diagnosis of threatened premature labor.

Methods: A periodontal examination and collection of amniotic fluid and subgingival plaque samples were performed on women identified as having threatened premature labor (preterm premature rupture of membranes without clinical infection or labor and preterm labor with intact membranes) and a gestational age ranging between 24 and 34 weeks. Samples collected from amniotic fluid and from the four deepest periodontal pockets in each patient were pooled in prereduced transport fluid and cultured. *Porphyromonas gingivalis* was identified primarily by colony morphology under stereoscopic microscope and rapid biochemical tests. Amniotic fluid or plaque samples were homogenized, DNA was extracted, and polymerase chain reaction (PCR) amplification of 16S rRNA with specific and universal primers was carried out.

Results: Twenty-six women with threatened premature labor were included: eight with preterm premature rupture of membranes and 18 with preterm labor with intact membranes. Eight women presented with gingivitis, 12 with chronic periodontitis, and six without periodontal disease. Microbial invasion of the amniotic cavity as detected by *P. gingivalis* PCR was 30.8% (eight of 26 patients). In these eight patients, *P. gingivalis* was present in both the subgingival samples and the respective amniotic fluid sample.

Conclusion: The presence of microbial invasion of the amniotic cavity by *P. gingivalis* could indicate a role for periodontal pathogenic bacteria in pregnant women with a diagnosis of threatened premature labor. *J Periodontol 2007;78:1249-1255.*

KEY WORDS

Periodontal diseases; *Porphyromonas gingivalis*; pregnant women; premature labor.

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Periodontal disease (gingivitis and periodontitis) is caused by an overgrowth of putative periodontal pathogens in the subgingival plaque followed by an immunoinflammatory response in a susceptible host. As a result of this interaction, attachment loss, periodontal pocket formation, active bone resorption, and inflammation occur.[1] Dental plaque is a complex microbial biofilm that is made up of as many as 500 different bacterial species organized in the supragingival and subgingival locations.[2] In contrast to the supragingival plaque that attaches to the tooth surface and is dominated by *Streptococci* and *Actinomyces*, which are Gram-positive facultative species,[3] subgingival plaque is tooth- and tissue-associated and is made up of a great variety of Gram-negative anaerobic bacteria.[4] Interestingly, *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythensis* have been strongly associated with periodontal disease, disease progression, and unsuccessful therapy.[5]

A study[6] has shown that maternal periodontal disease is associated with delivery of preterm infants with low birth weights, a finding confirmed by two large prospective studies.[7,8] Other authors have also shown that maternal periodontal disease is associated with delivery of significantly smaller infants[8] and the development of

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preeclampsia.[9,10] Treatment of periodontal disease within the context of randomized controlled trials has been shown to reduce the incidence of preterm birth.[11]

Systemic dissemination of oral microbes with subsequent maternal, fetal, and/or placental inflammatory responses has been suggested as a possible causal mechanism.[12] There is a growing body of evidence supporting a role for intra-amniotic infection in the pathogenesis of preterm labor.[13] Human studies in patients with periodontitis and animal studies have suggested *P. gingivalis* may be an important component in the underlying association in linking periodontitis to preterm birth.[14] Chorioamnionitis and fetal inflammation are considered key features in the origins of many cases of preterm delivery and fetal injury.[15]

In the present study, we investigated the prevalence and proportions of periodontopathic bacteria in the amniotic fluid and subgingival plaque samples in pregnant women with a diagnosis of threatened premature labor. Polymerase chain reaction (PCR) and bacteriologic culturing methods were used for detection and quantitative evaluation of *A. actinomycetemcomitans*, *P. gingivalis*, *Prevotella intermedia/nigrescens*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Capnocytophaga* species, *Campylobacter rectus*, and *Micromonas micros*. In this study, we showed the presence of *P. gingivalis* within the amniotic fluid among pregnant women with a diagnosis of threatened premature labor.

MATERIALS AND METHODS

Patient Population

Patients were selected from those who attended San Borja Arriarán Hospital between July 2004 and December 2005. Patients identified as having threatened preterm, premature labor (preterm, premature rupture of membranes [PROM] without clinical infection or labor and preterm labor with intact membranes) and a gestational age ranging between 24 and 34 weeks were asked to participate in this study. Written informed consent was obtained from all women. This study was approved by the Institutional Review Board of San Borja Arriarán Hospital.

The diagnosis of PROM was made in the presence of evident loss of amniotic fluid by the cervix visualized with sterile speculum examination and/or nitrazine test. Preterm labor with intact membranes was defined as the presence of four or more uterine contractions in 30 minutes for ≥1 hour, a sonographic cervical length measurement <0.5 cm, and/or cervical dilatation of 1 to 3 cm.

Amniocentesis was offered to all patients. Because microbiologic studies of amniotic fluid were performed immediately after the procedure, only patients admitted during normal working hours were invited to participate in the study. Within of the same day, subgingival plaque samples were collected in each patient, and vials with samples were transported at 4°C to the Microbiological Laboratory, Faculty of Dentistry, University of Chile, and processed immediately.

Gestational-age assessment was based on menstrual history when a reliable, pelvic examination in the first trimester was consistent with the stated length of amenorrhea or ultrasonographic fetal biometry before 24 weeks. Assessment of cervical dilation was made, and samples of cervical and vaginal secretions were taken during speculum examination. Digital vaginal examinations were not performed on admission in women with preterm PROM. All patients underwent obstetrical ultrasound for fetal biometry and amniotic fluid assessment.

Exclusion criteria were as follows: significant hemorrhage; abruptio placentae; use of antibiotics within 30 days before screening for this study; fetal anomaly or death; multiple gestation; uterine anomalies; presence of an intrauterine device; fetal distress; clinical chorioamnionitis; preterm PROM with labor; previous cervical cerclage; maternal medical complications necessitating delivery or any condition precluding expectant management; intrauterine growth retardation (<10th percentile for gestational age); and documented allergy to clindamycin or gentamycin.

None of the subjects had received any periodontal treatment. Subjects did not have systemic illness. The protocol stated that all patients would be provided with periodontal treatment after the detection of periodontal diseases.

Clinical Measurements of Periodontal Diseases

Clinical parameters were taken by a skilled clinician (AC) at all teeth (excluding third molars) and included clinical attachment level (CAL), probing depth (PD), supragingival plaque accumulation (plaque index [PI]), and bleeding on probing (BOP). Six sites were examined in each tooth: mesio-buccal, buccal, disto-buccal, disto-lingual, lingual, and mesio-lingual. Patients with chronic periodontitis had moderate to advanced periodontitis (at least five or six teeth had sites with PD ≥5 mm, CAL ≥3 mm, and extensive bone loss in radiography) and had received no periodontal treatment at the time of examination. A woman had gingivitis if she had BOP at ≥50% of teeth and was not in either the chronic periodontitis or healthy category. Subjects having no pockets ≥4 mm or BOP occurring at <50% of teeth were regarded as periodontally healthy.

Microbiologic Samples

Amniotic fluid samples were obtained for microbiologic studies and transported to the laboratory immediately after collection. Microbial invasion of the

amniotic cavity was defined as the presence of a positive amniotic fluid by culture or PCR obtained by transabdominal amniocentesis.

Subgingival plaque samples were collected from four periodontally affected or healthy sites (one in each quadrant) in each patient. After isolating the area with cotton rolls and gently air drying, supragingival deposits were carefully removed with Gracey curets.[‖] Subgingival microbial samples, the deepest sites in each quadrant, were obtained by inserting two standardized number 30 sterile paper points[¶] in the deepest part of the periodontal pocket for 20 seconds. The samples from each patient were pooled in a vial containing 2 ml cold sterilized, prereduced transport fluid (RTF) without EDTA. Vials with samples of amniotic fluid and subgingival plaque were transported at 4°C to the Microbiological Laboratory, Faculty of Dentistry, University of Chile.

Microbiologic Procedures

Amniotic fluid and subgingival plaque samples were dispersed by mixing[#] for 45 seconds followed by a 10-fold serial dilution of the bacterial suspension in RTF. Aliquots of 100 μl of the appropriate dilution (10^{-3}, 10^{-4}, and 10^{-5}) were plated on non-selective Columbia blood-agar (5% defibrinated sheep blood, 1 mg/l hemin, and 0.5 mg/l menadione) for total anaerobic cell counts (colony forming units [CFU]/ml) and for detection and quantification of *P. gingivalis*, *P. intermedia/nigrescens*, *E. corrodens*, *Capnocytophaga* species, *C. rectus*, and *M. micros*. Plates were incubated anaerobically at 35°C for 5 to 7 days in a jar containing gas generator envelopes for the production of an anaerobic atmosphere.[**]

Bacteria were primarily identified by colony morphology under a stereoscopic microscope,[††] pigment production, Gram stain, and rapid enzymatic tests for oxidase, trypsin-like (N-benzoyl-DL-arginine-naphthylamide [BANA]), and α-glucosidase detection. In addition, black pigmented colonies of *P. gingivalis* and *P. intermedia/nigrescens* were tested for red fluorescence under ultraviolet (UV) light (360 nm) as negative for *P. gingivalis* and positive for *P. intermedia/nigrescens*. *A. actinomycetemcomitans* was also primarily identified by colony morphology (starlike inner structure or ridges) but also on selective trypticase, 10% horse serum, bacitracin, and vancomycin (TSBV) medium. One hundred microliters of undiluted and 10^{-1} diluted samples were inoculated on the agar plates and incubated at 35°C for 2 to 3 days in CO_2 candle jars. The percentage of *A. actinomycetemcomitans* was obtained using the number of CFUs on TSBV as a percentage of total anaerobic counts. Gram stain and catalase production were also used for *A. actinomycetemcomitans* identification. To obtain *F. nucleatum* counts, selective trypticase, crystal violet, trytophane, erythromycin, 5% defibrinated sheep blood (CVE) medium was used. Plates were incubated anaerobically as mentioned above for 5 days. Colony morphology, pigmentation, Gram stain, and direct sheep red blood cell hemaglutination were used for identification. The anaerobic isolates were further identified by an identification system.[‡‡]

PCR

PCR was used for detection of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia/nigrescens*, *E. corrodens*, *F. nucleatum*, *Capnocytophaga* species, *C. rectus*, and *M. micros* in the amniotic fluid and subgingival plaque samples. PCR assays were performed in 25-μl tubes containing 2.5 μl 10× PCR buffer (10 mM Tris-HCl [pH 9.0 at 25°C], 50 mM KCl, and 0.1% Triton X-100), 2.0 μl 25 mM $MgCl_2$, 0.5 μl 10 mM deoxynucleoside triphosphates, 0.5 U Taq DNA polymerase, 1.0 μl 25 μM primers, and 10 or 5 μl amniotic fluid or subgingival samples, respectively.[16] A 15-μl aliquot of each PCR was electrophoresed through a 2.0% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer, and the PCR products were visualized by staining with ethidium bromide.[17] PCR assays were performed with a cycler.[§§]

After denaturation at 96°C for 3 minutes, a total of 40 PCR cycles were performed; each cycle consisted of 30 seconds of denaturation at 95°C, 30 seconds of annealing at 55°C, and 180 seconds of extension at 72°C.

Data Analysis

Clinical parameters and presence of periodontal pathogens were calculated for each subject (mean ± SD). Patients were used as the experimental unit of observation. Comparisons between subjects with gingivitis, periodontitis, and without periodontal disease were performed by analysis of χ^2 and Student t tests. Two-tailed t tests were used to compare the parameters between the clinical periodontitis groups and microbiologic data. $P < 0.05$ was considered statistically significant.

RESULTS

Twenty-six women with threatened premature labor were enrolled in this study: eight patients with preterm PROM and 18 with preterm labor with intact membranes. Clinical data of patients and sites selected for bacterial sampling are shown in Table 1. Eight women with gingivitis (mean age, 20.6 years), 12 with periodontitis (mean age, 31.2 years), and six without

‖ Hu-Friedy, Chicago, IL.
¶ Johnson & Johnson, Tokyo, Japan.
# Maxi Mix II Type 37600, Thermolyne, Dubuque, IA.
** Oxoid, Basingstoke, Hampshire, U.K.
†† Stemi 2000-C, Zeiss, Jena, Germany.
‡‡ BBL-Crystal Anaerobe ID System, Becton Dickinson, Meylan, France.
§§ iCycler Thermal Cycler, Bio-Rad Laboratories, Hercules, CA.

Table 1.

Clinical Data and Microbiologic Findings in Amniotic Fluid and Subgingival Plaque From Pregnant Women

	Gingivitis (n = 8)	Periodontitis (n = 12)	Gingivitis + Periodontitis (n = 20)	Without Periodontal Disease (n = 6)
Age (years; mean [range])	20.6 (18-29)*	31.2 (16-42)*	27.0 (16-42)	22.7 (16-38)
Parity				
Nulliparous	6	4	10 (50.0%)	5 (83.3%)
Multiparous	2	8	10 (50.0%)	1 (16.7%)
Gestational age at admission (weeks; mean [range])	30.3 (26.3-34.0)	29.8 (26.5-32.1)	30.0 (26.3-34.0)	31.3 (29.3-33.1)
Preterm PROM	3	5	8†	0†
Preterm labor and intact membranes	5	7	12 (60.0%)	6 (100%)
Preterm delivery	4	8	12 (60.0%)	3 (50.0%)
MIAC by *P. gingivalis* (PCR)	2 (25.0%)	3 (25.0%)	5 (25.0%)	3 (50.0%)
P. gingivalis in subgingival plaque				
Bacterial culture	4 (50.0%)	6 (50.0%)	10 (50.0%)	3 (50.0%)
Percent of total count	2-18	1.6-48.3	1.6-48.3	1-16

MIAC = microbial invasion of amniotic cavity.
* Age of gingivitis versus periodontitis subjects: $P<0.05$.
† Preterm PROM periodontal diseases versus subjects without periodontal diseases: $P<0.05$.

periodontal disease (mean age, 22.7 years) were studied. Table 1 describes the microbiologic findings in amniotic fluid and subgingival plaque samples of pregnant women with a diagnosis of threatened premature labor.

Subgingival plaque samples including *P. gingivalis* were found in 50.0% (13/26) of patients. The prevalence of patients with periodontal disease with *P. gingivalis* (bacterial culture in subgingival samples) was 50.0% (10/20). In women with gingivitis, the occurrence of *P. gingivalis* was 50.0% (four of eight patients). In patients with chronic periodontitis, the prevalence was 50.0% (six of 12 patients). Table 1 also depicts the level of *P. gingivalis* expressed as mean percentages of the total anaerobic counts per group of patients. In this study, all amniotic fluid samples examined by bacterial culture were negative for *P. gingivalis*.

The prevalence of patients with microbial invasion of amniotic cavity (MIAC) by *P. gingivalis* detected by PCR was 30.8% (eight of 26 patients; Table 2). All colonies were studied by PCR. Figure 1 shows the identification of periodontal pathogens by PCR in the amniotic cavity.

DISCUSSION

This was a cross-sectional study of 26 pregnant women with a diagnosis of threatened premature labor: 20 with periodontal disease (eight with gingivitis and 12 with chronic periodontitis) and six without periodontal disease. Our results show MIAC by *P. gingivalis* in eight women (eight of 26 patients; 30.8%). *P. gingivalis* was detected by PCR in the subgingival samples and the respective amniotic cavity.

Several studies[18,19] have shown an association between maternal periodontal disease and preterm birth and fetal growth restriction, but the underlying mechanisms are unknown. To begin to elucidate possible causal pathways between maternal periodontal disease and adverse pregnancy outcome, Boggess et al.[20] developed a rabbit model of chronic maternal exposure to *P. gingivalis*, an oral pathogen. The model documented not only placental but fetal exposure after maternal inoculation with *P. gingivalis* that was distant from the uterus.[20] The same authors[21] showed that kid and placental weights are not diminished with chronic *P. gingivalis* exposure and that there are no differences in serum interleukin (IL)-1β, insulin-like growth factor (IGF), or IGF binding proteins (IGFBPs) between exposed and unexposed *P. gingivalis*. The lack of changes in maternal IL-1β suggested that this level of exposure may be cleared quickly by maternal and/or fetal immune responses without inducing an inflammatory response.

Table 2.

Bacterial Species Present in the Periodontal and Amniotic Fluid Samples

Patient Number	Diagnosis	Periodontal Samples		Amniotic Fluid	
		Culture	PCR	Culture	PCR
1	Chronic periodontitis	*E. corrodens*	*E. corrodens*	ND	ND
		P. gingivalis	*P. gingivalis*	ND	*P. gingivalis*
2	Chronic periodontitis	*P. gingivalis*	*P. gingivalis*	ND	*P. gingivalis*
		E. corrodens	*E. corrodens*	ND	ND
		P. micros	*P. micros*	ND	ND
3	Healthy	*P. gingivalis*	*P. gingivalis*	ND	*P. gingivalis*
		E. corrodens	*E. corrodens*	ND	ND
		P. micros	*P. micros*	ND	ND
4	Healthy	*P. gingivalis*	*P. gingivalis*	ND	*P. gingivalis*
		E. corrodens	*E. corrodens*	ND	ND
5	Chronic periodontitis	*F. nucleatum*	*F. nucleatum*	ND	ND
		ND	*P. gingivalis*	ND	*P. gingivalis*
6	Gingivitis	*E. corrodens*	*E. corrodens*	ND	ND
		F. nucleatum	*F. nucleatum*	ND	ND
		ND	*P. gingivalis*	ND	*P. gingivalis*
7	Healthy	*P. gingivalis*	*P. gingivalis*	ND	*P. gingivalis*
		F. nucleatum	*F. nucleatum*	ND	ND
		P. micros	*P. micros*	ND	ND
8	Gingivitis	*P. gingivalis*	*P. gingivalis*	ND	*P. gingivalis*
		F. nucleatum	*F. nucleatum*	ND	ND
		E. corrodens	*E. corrodens*	ND	ND

ND = not detected, indicating that the samples were found to be negative.

However, the lack of significant differences in placental and kid weights may also be attributed to the small sample size of this study and the large intralitter correlation of kid weight among littermates.[21]

Data from a pregnant mouse model of infection[22] showed that maternal *P. gingivalis* challenge can result in *P. gingivalis* translocation to placental tissue and induction of fetal growth restriction (FGR), whereas non-affected littermates showed no evidence of *P. gingivalis* signals in their corresponding placentas. However, *P. gingivalis* signal was not detected in 61% of placentas from FGR fetuses.

In a large cohort study,[23] Madianos et al. found that 317 (58%) of 546 umbilical cord samples collected were positive for immunoglobulin M (IgM) to one or more specific oral pathogens, suggesting systemic dissemination and fetal exposure. A previous study[20] showed exposure of the fetal and placental compartments to *P. gingivalis* after maternal exposure at a site distant from the reproductive tract in the rabbit. Microbial DNA was recovered from one-third of maternal livers, one-third of placentas, and almost one-half of fetuses, suggesting maternal systemic dissemination and transplacental passage to the fetal compartment.

Different mechanisms have been proposed to explain *P. gingivalis*–induced systemic responses. Intrachamber *P. gingivalis* immunization may induce the production of T-helper type 1 (Th1) cells specific for *P. gingivalis* locally and systemically,[24,25] and a low-dose subsequent challenge with live *P. gingivalis*

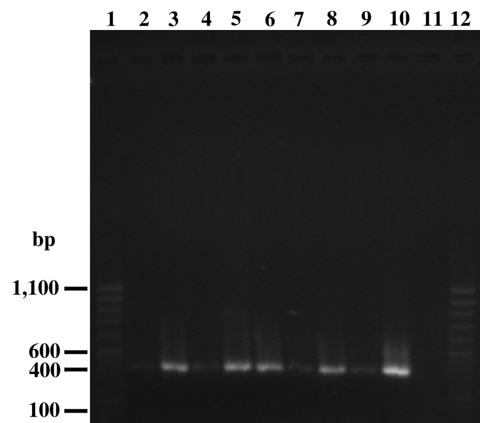

Figure 1.
Identification of P. gingivalis *in samples of amniotic fluid by PCR. Lanes 1 and 12, molecular weight marker (100-base pair [bp] DNA ladder); lanes 2 through 10, PCR products of* P. gingivalis *(404 bp) in samples of amniotic fluid; lane 10, amplification of* P. gingivalis *ATCC 33277; and lane 11, negative control.*

could activate these Th1 cells to secrete higher levels of proinflammatory cytokines (e.g., tumor necrosis factor [TNF]-α) and suppress the production of antiinflammatory cytokines (e.g., IL-10). It is also possible that the activated maternal innate immune system (such as monocyte/macrophage activation) secretes TNF-α and IL-6 after infection or lipopolysaccharide (LPS) stimulation.[26,27]

When injected into the amniotic cavity of pregnant sheep, periodontopathic LPSs caused a mild, transient metabolic acidosis in the fetus, similar to that which generally accompanies sepsis.[28] Rates of mortality and morbidity were considerably greater than have been observed after intra-amniotic injection of *E. coli* LPS where a single injection in doses that ranged from 1 to 100 mg did not jeopardize fetal survival, and any changes in acid-based balance were transient.[28,29] Consistent rates of survival were seen after intra-amniotic injection of 1 mg *A. actinomycetemcomitans* LPS but not after *P. gingivalis* LPSD, even in doses as low as 0.1 mg.[30] These findings suggest that this group of periodontopathic LPSs is highly toxic to the fetus, with potencies up to ≥100-fold than *E. coli* LPS.[30] The strain of *P. gingivalis* (ATCC 33277) used in this study has been shown to induce higher rates of bone loss in rat mandibles than other strains.[31]

It is not known whether the source of *P. gingivalis* is of oral or possibly vaginal origin. However, the presence of *P. gingivalis* being simultaneously detected in the oral cavity is consistent with oral amniotic dissemination. We showed the presence of *P. gingivalis* within the amniotic fluid, which may contribute to the pathology in pregnant women with a diagnosis of threatened premature labor. That observation is consistent with recent findings with mice where hematogenous infection with orally related *Fusobacterium nucleatum* resulted in localized infection in the placenta, causing preterm and term stillbirths of the fetal pups.[32] Direct evidence detecting and matching the microorganisms in intrauterine infections with those in the oral cavities of the pregnant women was recently examined.[33] Han et al.[33] reported the identification of uncultivated oral *Bergeyella* sp. strain associated with preterm birth and the possible source of the infection. It is plausible that periodontal disease may facilitate the oral-utero transfer because of the increased bacterial load in the oral cavity and the altered host immune responses during disease.

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