

Microbial invasion of the amniotic cavity in preeclampsia as assessed by cultivation and sequence-based methods

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Method of study: Amniotic fluid (AF) from 62 subjects with preeclampsia, not in labor, was analyzed with both cultivation and molecular methods. Broad-range and group-specific PCR assays targeting small subunit ribosomal DNA, or other gene sequences, from bacteria, fungi and archaea were used. Results were correlated with measurements of host inflammatory response, including AF white blood cell count and AF concentrations of glucose, interleukin-6 (IL-6) and MMP-8.

Results: 1) The rate of MIAC in preeclampsia was 1.6% (1/62) based on cultivation techniques, 8% (5/62) based on PCR, and 9.6% (6/62) based on the combined results of both methods; 2) among the six patients diagnosed with MIAC, three had a positive PCR for *Sneathia/Leptotrichia* spp.; and 3) patients with MIAC were more likely to have evidence of an inflammatory response in the amniotic cavity than those without MIAC, as determined by a higher median AF IL-6 [1.65 ng/mL interquartile range (IQR): 0.35–4.62 vs. 0.22 ng/mL IQR: 0.12–0.51; $P=0.002$].

Conclusion: The prevalence of MIAC in preeclampsia is low, suggesting that intra-amniotic infection plays only a limited role in preeclampsia. However, the unexpectedly high number of positive AF specimens for *Sneathia/Leptotrichia* warrants further investigation.

Keywords: IL-6; intra-amniotic infection; intra-amniotic inflammation; PCR; preeclampsia; pregnancy; 16S rRNA; *Sneathia/Leptotrichia* spp.; *Ureaplasma urealyticum*.

Abstract

Objective: Infection has been implicated in the pathogenesis of preeclampsia, yet the association between microbial invasion of the amniotic cavity (MIAC) and preeclampsia has not been determined. The aim of this study was to determine the prevalence, and microbial diversity associated with MIAC, as well as the nature of the host response to MIAC in patients with preeclampsia.

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Introduction

Preeclampsia, one of the “great obstetrical syndromes” [32, 100, 101], is a major cause of maternal and perinatal morbidity and mortality [30, 69, 74, 85, 114, 118]. Preeclampsia has been associated with several mechanisms of disease including defective spiral artery remodeling [15, 39, 55], endothelial cell dysfunction [13, 23, 51, 58, 84, 90, 95, 98, 112, 125], an anti-angiogenic state [4, 17, 19–21, 26, 37, 38, 40, 47, 57, 59–61, 64–68, 70, 73, 75, 78, 91, 99, 105, 115, 121, 122, 127, 130–133, 135, 146], an exaggerated intra-vascular inflammatory response [7, 18, 22, 45, 48, 71, 72, 76, 77, 93, 94, 97, 109, 119, 120, 128, 129, 134, 142], oxidative stress [28, 52, 82, 83, 138] and a predominantly T helper (Th1)-biased immune response [6, 10, 25, 27, 35, 62, 79, 110, 123, 126, 137, 139].

Several lines of evidence support a role for infection in preeclampsia: 1) women with asymptomatic bacteriuria are more likely to develop preeclampsia than those with a negative urine culture [136]. Indeed, urinary tract infection in pregnancy is associated with an odds ratio (OR) of 2.5 [95%

confidence interval (CI) 1.3–5.0] for the development of preeclampsia. This rate is even higher among primigravidae [OR 5.5 (95% CI 2.9–9.7)] [81]; 2) a HELLP syndrome-like state can be induced by stimulation of the celiac ganglion with lipopolysaccharide (LPS) in non-pregnant rats [53]; 3) exposure of pregnant rats to low doses of endotoxin in early pregnancy leads to the development of preeclampsia [41, 42, 111]; 4) the presence of periodontal disease at <26 weeks of gestation increases the risk for subsequent preeclampsia [OR 2.3 (95% CI 1.0–5.2)] [9]; 5) case reports have linked recurrent eclampsia with chronic pyelonephritis [108]; and 6) preeclampsia has been associated with chronic gastrointestinal infection with parasites, such as *Schistosoma japonicum* [87] and *Strongyloides stercoralis* [140].

Despite these observations, the association between microbial invasion of the amniotic cavity (MIAC) and the presence of preeclampsia has not been investigated.

Methods

Study population

A retrospective cohort study was conducted including patients with preeclampsia who met the following inclusion criteria: 1) singleton gestation; 2) gestational age (GA) between 20 and 40 weeks; 3) intact chorioamniotic membranes; and 4) amniocentesis fluid available for analysis. Patients were excluded from the study if: 1) spontaneous labor or labor was induced; 2) clinical metadata were unavailable; or 3) a major fetal chromosomal and/or congenital anomaly was present.

All women provided written informed consent prior to the collection of biological samples. The utilization of samples and clinical data for research purposes was approved by the Institutional Review Boards of Sotero del Rio Hospital, Azienda Ospedaliera of Padova, Stanford University, Wayne State University, and the National Institute of Child Health and Human Development (NICHD/NIH/DHHS).

Definitions

Preeclampsia was defined as the new onset of hypertension that developed after 20 weeks of gestation (systolic or diastolic blood pressure ≥ 140 or ≥ 90 mm Hg, respectively, measured at two different time points, 4 h–1 week apart) coupled with proteinuria (≥ 300 mg in a 24-h urine collection, or two random urine specimens obtained 4 h–1 week apart containing $\geq 1+$ by dipstick or one dipstick demonstrating $\geq 2+$ protein) [1, 117]. Severe preeclampsia was defined as systolic blood pressure ≥ 160 mm Hg or diastolic blood pressure ≥ 110 mm Hg and/or proteinuria > 5 g in a 24-h collection or $\geq 3+$ protein on dipstick, and it was also diagnosed in the presence of multi-organ involvement [1, 117]. HELLP syndrome was defined as hemolysis (serum LDH > 600 IU/L; bilirubin > 1.2 mg/dL; presence of schistocytes in peripheral blood), elevated liver enzymes (serum ALT and/or AST > 70 IU/L) and thrombocytopenia (platelet count $< 100,000/\text{mm}^3$) [8]. Histological chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or chorioamniotic membranes [56, 92]. Acute funisitis was diagnosed by the presence of neutrophils in the wall of the umbilical vessels and/or Wharton's jelly using criteria previously described [88].

Sampling procedures

Patients with preeclampsia were offered amniocentesis to assess fetal lung maturity in patients close to term. Amniotic fluid (AF) samples were also obtained at the time of cesarean delivery, using meticulous aseptic technique, in a subset of patients. AF was transported in a capped sterile syringe to the clinical laboratory where it was cultured for aerobic and anaerobic bacteria, including genital mycoplasmas, as described [33]. White blood cell (WBC) count [107] and Gram stain [102] of AF were also performed shortly after collection using methods previously described. Shortly after the amniocentesis, AF not required for clinical assessment was centrifuged at $1300 \times g$ for 10 min at 4°C , and the supernatant was aliquoted into gamma-irradiated non-pyrogenic DNase/RNase-free cryovials (Corning, Acton, MA, USA), and immediately frozen at -70°C . AF interleukin-6 (IL-6) concentrations were determined using a specific and sensitive immunoassay which had been validated for the analysis of AF as previously described [86]. IL-6 and MMP-8 determinations were performed after all patients were delivered and were not used in clinical management.

Genomic DNA extraction

AF that was not required for clinical purposes (200 μL of each AF sample) was shipped on dry ice to Stanford, CA, where genomic DNA was extracted as described [34]. Extracted DNA was eluted into a final volume of 100 μL of QIAamp[®] AE buffer and stored at -20°C or colder until thawing for molecular analyses. Strategies to prevent, detect and neutralize potential contamination were implemented at critical steps [12], according to a previously described protocol that included mock extraction blanks to monitor potential contamination (at least one mock per 17 processed samples) [33].

Qualitative analysis by end-point PCR

DNA from each AF sample was analyzed by end-point PCR using broad-range bacterial 16S ribosomal DNA (rDNA) primers, and by group-specific end-point PCR using primers for six taxonomic groups including *Candida* sp. (Table 1) [14, 31, 63, 89, 141, 145]. PCR reactions, screening of PCR products by gel electrophoresis, and purification and cloning of amplicons from broad-range PCR was performed as described [34]. Sequencing of amplicons directly from group-specific PCR and of recombinant clones from broad-range PCRs (up to 10 clones/reaction) was performed, as described [34].

Sequence alignment and phylogenetic analysis

Forward and reverse sequence reads were assembled into contigs as described [33]. Assembled sequences from group-specific PCR were queried against the NCBI's GenBank database using a basic local alignment search tool (BLAST) algorithm [5] to confirm specificity. Assembled sequences from broad-range end-point PCR were aligned and subjected to phylogenetic analysis as described [33]. After removal of vector, human, and poor-quality sequences from the alignment, a neighbor-joining tree was generated based on Felsenstein correction and 682 unambiguous filter positions. Phylogenies were defined using a 99% sequence similarity threshold, which approximates a species-level classification.

Quantitative analysis by real-time PCR

DNA from each sample was analyzed by means of two real-time PCR assays, each of which was designed to amplify in a specific

Table 1 PCR assays used in this study.

Approximate taxonomic level	End-point PCR taxonomic specificity	Lower detection limit (gene copies/ μ L)	Oligonucleotide name	Use	Sequence (5' $\rightarrow$ 3')	Gene target	Reference
Domain	<i>Bacteria</i>	100	Bact-8FM	FP	AGAGTTTGATCMTGGCTCAG	16S rDNA	[89]
Genus	<i>Ureaplasma</i>	10	Bact-806R	RP	GGACTACCAGGGTATCTAAT	Urease gene	[141]
			Urease185F	FP	GCTGCTGACGTTTGCAAGAAG		[34]
Genus	<i>Fusobacterium</i>	100	Urease756R	RP	CTCCTGGTTCAAAACGAATAGC		[34]
			Fuso-422F	FP	CGGAATGTAAAGTGCTTTC	16S rDNA	[34]
			Fuso-710R	RP	CCCATCGGCATTCTCTAC		[34]
Genus	<i>Sneathia/Leptotrichia</i>	10	SsLa-140F	FP	TAGACTGGGATAACAGAGG	16S rDNA	[34]
			SsLa-406R	RP	AGTCCTAAACCTTCTTCACAC		[34]
Species	<i>Streptococcus agalactiae</i>	10	Sag059F	FP	TTTCACCAAGCTGTATTAGAAGTA	<i>cfb</i>	[54]
			Sag190R	RP	GTTCCTGGAACATTATCTTTGAT		[54]
Species	<i>Mycoplasma hominis</i>	10	Mh-148F	FP	CAATGGCTAATGCCGGATACG	16S rDNA	mod. from [145]
			Mh-463R	RP	GGTACCGTCAGTCTGCAATC		mod. from [145]
Genus	<i>Candida</i>	10	Cand-ITS2-42F	FP	GGGTTTGCTTGAAAGACGGTA	ITS2	[34]
			Cand-ITS2-125R	RP	TTGAAGATATACGTGGTRGACGTTA		[34]
Real-time PCR taxonomic specificity		Dynamic range (gene copies/ μ L)					
Domain	<i>Bacteria</i>	15–1e8	Bact-8FM	FP	AGAGTTTGATCMTGGCTCAG	16S rDNA	[89]
			Bact-338K*	Probe	CCAKACTCCTACGGGAGGCAGCAG		[89]
			Bact-515R	RP	TTACCGCGGCKGCTGGCAC		[14]
Domain	<i>Archaea</i>	100–1e8	Arch333F	FP	TCCAGGCCCTACGGG	16S rDNA	[63]
			Univ-515F*	Probe	GTGCCAGCMGCCGCGGTAA		[14]
			Arch958R	RP	YCCGGGTTGAMTCCAATT		[31]

FP = forward primer, rDNA = ribosomal DNA, RP = reverse primer, Probe = TaqMan probe.

*Conjugated on the 5' end to 6-carboxyfluorescein, and on the 3' end to 6-carboxy-tetramethylrhodamine.

Table 2 Clinical and demographic characteristics of the study population.

Variables	Patients with preeclampsia (n=62)
Maternal age (years)	28 (23–35)
Ethnicity	
African-American	29 (47)
Caucasian	17 (27.5)
Hispanic	14 (22.5)
Others	2 (3)
BMI	27.8 (23.6–34.9)
Gestational age at amniocentesis (weeks)	34.7 (29.9–37.3)
Gestational age at delivery (weeks)	35 (30.1–37.4)
Birthweight (g)	2040 (1206–2998)

Data presented as median (interquartile range) or number (%).

BMI=body mass index.

manner and quantify 16S rDNA of domain *Bacteria* or domain *Archaea* (Table 1). Reactions were carried out as described [34].

Statistical analysis

Differences in continuous variables between patients with and without MIAC (as determined by either a positive culture or a positive PCR) were computed using the Mann-Whitney *U*-test. Descriptive statistics were used to report the prevalence of MIAC. A $P < 0.05$ was considered statistically significant. Analysis was performed with SPSS, version 14 (SPSS Inc., Chicago, IL, USA).

Results

Study population

Table 2 displays the demographic and clinical characteristics of the study group. Twenty-eight patients were diagnosed with mild preeclampsia, twenty with severe preeclampsia, nine with superimposed preeclampsia and five with HELLP syndrome. Maternal age, body mass index (BMI), GA at amniocentesis, GA at delivery and birthweight did not differ significantly between patients who tested negative by both

PCR and culture and those who tested positive by either method (culture or PCR).

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Table 3 displays the clinical characteristics of the six patients that were positive by either culture or PCR. The rate of MIAC in preeclampsia was 1.6% (1/62) based on cultivation, 8% (5/62) based on PCR, and 9.6% (6/62) based on the combined results of both methods. The patient that was positive by culture was negative by PCR, and the five patients that were positive by PCR were negative by culture. Among patients who were negative by both methods (culture and PCR), one had elevated IL-6 and one had elevated MMP-8 AF concentration.

Cultivation methods recovered one species (*Ureaplasma urealyticum*), whereas molecular methods revealed five phylotypes. Of these five, three were detected by broad-range PCR: *Lactobacillus iners* (one patient; eight clones; 99.9% identity to type strain CCUG 28746^T); *Streptococcus* sp. (one patient; two clones; 100% identity to *Streptococcus anginosus*, GenBank AF30688.1) and *Corynebacterium tuberculos-tearicum* (one patient; five clones; 99.9% identity to type strain CCUG 45418^T). Two phylotypes were detected by group-specific PCR, although the targeted gene and/or size of the amplified sequences limited taxonomic resolution to approximately the genus level: *Ureaplasma* spp. (two patients) and *Sneathia/Leptotrichia* (two patients). No members of domain *Archaea* or genus *Candida* were detected.

Assessment of the intra-amniotic inflammatory response

Women with MIAC had a median AF IL-6 concentration that was significantly higher than those without MIAC [1.65 ng/mL interquartile range (IQR): 0.35–4.62 vs. 0.22 ng/mL IQR: 0.12–0.51; $P = 0.002$]. There were no significant differences in the rate of positive Gram stain, AF WBC count, and AF concentrations of glucose, and MMP-8 (Table 4).

Table 3 Clinical characteristics of six patients with a positive amniotic fluid PCR or culture.

Patient	Microbe	Method	GA at AC (weeks)	AF (cells/mm ³)	AF glucose (mg/dL)	AF IL-6 (ng/mL)	AF MMP-8 (ng/mL)	Placental pathology	GA at delivery (weeks)	Birthweight
1	<i>Ureaplasma urealyticum</i>	Culture	26.3	360	5	2.21	1.27	No inflammation	26.3	620
2	<i>Sneathia/Leptotrichia</i> spp.	PCR	28.9	2	19	1.11	2.49	No inflammation	28.9	1120
3	<i>Sneathia/Leptotrichia</i> spp.	PCR	36.7	0	20	0.36	3.40	No inflammation	36.7	2665
4	<i>Corynebacterium tuberculos-tearicum</i>	PCR	25.1	93	27	0.35	8.18	No inflammation	25.1	474
5	<i>Ureaplasma</i> sp.	PCR	39.7	0	40	3.02	43.96	No inflammation	39.7	3100
6	<i>Sneathia/Leptotrichia</i> spp.	PCR	37.6	50	9	9.44	20.19	No inflammation	37.6	2720
	<i>Lactobacillus iners</i>									
	<i>Streptococcus anginosus</i>									

GA = gestational age, AC = amniocentesis, AF = amniotic fluid, IL-6 = interleukin-6.

Table 4 Comparison of intra-amniotic inflammatory response between patients with and without microbial invasion of the amniotic cavity.

	Culture and PCR negative (n = 56)	Culture or PCR positive (n = 6)	P-value
WBC count (cells/mm ³)	3 (0–10)	26 (0–159)	0.5
AF Glucose (mg/dL)	29 (20–39)	19 (8–30)	0.09
AF MMP-8 (ng/mL)	4.09 (1.71–7.67)	5.8 (2.18–26.13)	0.4
AF IL-6 (ng/mL)	0.22 (0.12–0.51)	1.65 (0.35–4.62)	0.002

Data are presented as median and interquartile range.

WBC = white blood cells, AF = amniotic fluid, IL-6 = interleukin-6.

Short-term neonatal outcome

In the case with a positive culture for *Ureaplasma urealyticum* (26.3 weeks of gestation), the neonate was admitted to the neonatal intensive care unit (NICU) and was diagnosed with pneumonia on day 7 of life. The neonate was treated with oxacillin. Of note, *Ureaplasma urealyticum* was isolated from the nasopharynx on day 20 of life. The neonate developed respiratory distress syndrome (RDS), bronchopulmonary dysplasia, intraventricular hemorrhage grade I, retinopathy of prematurity, and necrotizing enterocolitis. The second preterm neonate (28.9 weeks of gestation; AF positive for *Sneathia/Leptotrichia* spp. by PCR only) did not have a proven neonatal sepsis. Subsequently, the newborn developed RDS and hyperbilirubinemia. The third preterm neonate (25.1 weeks, AF positive for *Corynebacterium tuberculostearicum* by PCR only) had RDS, hyperbilirubinemia, patent ductus arteriosus, anemia, thrombocytopenia and renal failure. Although blood cultures were negative, broad-spectrum antibiotics were started immediately after delivery. On day 20 of life, the neonate died of respiratory failure. None of the newborns that were born at term or close to term and were diagnosed with MIAC by PCR required NICU admission or developed short-term complications.

Discussion

Principal findings of the study

1) The rate of MIAC in preeclampsia was 1.6% (1/62) based on cultivation techniques, 8% (5/62) based on PCR, and 9.6% (6/62) based on the combined results of both methods; 2) among the six patients diagnosed with MIAC, three had a positive PCR for *Sneathia/Leptotrichia* spp.; and 3) patients with preeclampsia and MIAC were more likely to have evidence of an inflammatory response in the amniotic cavity, as determined by a higher median AF IL-6 concentration, than those without MIAC.

Detection of microbial invasion of the amniotic cavity

The AF in normal pregnancy is considered sterile in the majority of cases. However, MIAC has been demonstrated with cultivation techniques in 18% of patients in spontaneous labor at term with intact membranes [106], 34% of women with prelabor rupture of membranes (PROM) at term [104],

13% of women presenting with an episode of preterm labor [46], 32% of women with preterm PROM [46], and 9% of women with a short cervix [50]. Among women with cervical insufficiency, the prevalence of MIAC is about 50% [103]. However, all these estimates are based upon cultivation techniques and rely on the ability to provide adequate conditions required for the growth of microorganisms in the laboratory.

Molecular methods offer a sensitive, cultivation-independent approach for detecting microbes. In particular, broad-range PCR assays that target rDNA allow for detection and characterization of diverse microbial taxa, including unknown species [96]. These methods have been used to assess diversity within the human indigenous microbiota [2, 36] and to characterize microbes associated with a wide range of clinical syndromes [33, 43]. In addition, it is possible to use specific primers that target microorganisms, such as *Ureaplasma urealyticum* and fungi. Specific assays appear to have a greater sensitivity than “universal” assays for the detection of targeted microorganisms.

Our group has previously reported that specific PCR assays for *Ureaplasma urealyticum* are more sensitive than cultivation for this species in patients with preterm labor and intact membranes [144], preterm PROM [143] and cervical insufficiency [16]. We have also employed a combination of broad-range and specific PCR assays for bacteria and fungi, and have demonstrated that the combination of culture and molecular methods allows improved detection of MIAC [34]. Indeed, the impetus for including a *Sneathia/Leptotrichia* spp. group-specific PCR was our prior finding of an unexpectedly high rate of MIAC due to those two related genera [33]. Importantly, an intrauterine inflammatory response is associated with the presence of microbial DNA in the AF, even in the presence of a negative culture [34, 143, 144]. Such findings provide evidence that a positive PCR-based assay has biological significance.

MIAC in patients with preeclampsia

We have not been able to identify any prior study that has systematically examined the presence of MIAC in preeclampsia with either cultivation or molecular methods. The findings reported herein indicate that 9% of women with preeclampsia have MIAC detected with cultivation and molecular techniques. The organisms identified included *Ureaplasma urealyticum*, *Sneathia/Leptotrichia* spp., *Lactobacillus* spp. and *Streptococcus* spp.

The most common organisms detected in the AF of patients with preeclampsia were *Sneathia/Leptotrichia* spp. (50% of all MIAC cases) which was identified only by PCR, and *Ureaplasma* spp. (50%) which was determined by both cultivation techniques (one case) and PCR (two cases). Two of these cases, both detected only by PCR, had normal AF WBC counts, as well as normal AF concentrations of glucose, IL-6 and MMP-8, while the other four cases had at least one abnormal value of intra-amniotic infection/inflammation (IAI) indices.

The taxonomy of the related genera *Leptotrichia* and *Sneathia* and our understanding of their potential roles in human disease are still evolving. Both groups are Gram-negative, strictly-anaerobic, fastidious members of the family *Fusobacteriaceae* [29]. Based on phenotypic and phylogenetic analysis, Collins et al. [24] proposed in 2001 that "*Leptotrichia sanguinegens*" and three related clinical isolates be assigned to a new genus, *Sneathia*, as *Sneathia sanguinegens* gen. nov. sp. nov [24]. Six species of *Leptotrichia* and one of *Sneathia* are currently recognized with valid published names.

Leptotrichia sp. are members of the health-associated microbiota of the oropharynx [3]. *Sneathia* and *Leptotrichia* have been associated with bacterial vaginosis [80, 124] as well as with other conditions especially in immunosuppressed patients [113, 116]. *Sneathia* and *Leptotrichia* are rarely detected during pregnancy. An association with human disease is strongest for four species – *L. buccalis*, *L. trevisanii*, *L. goodfellowii* and *S. sanguinegens*. Another taxon that has been isolated in culture, "*Leptotrichia amnionii*" was first described in a case of maternal bacteremia and fetal demise [116].

Sneathia and *Leptotrichia* are rarely detected as pathogens in association with pregnancy. Indeed, a PubMed search in January 2010 using the key words "*Leptotrichia*," and "*pregnancy*" revealed only nine original reports. This has been attributed to the fastidious nature of these organisms. Hanff et al. [49] reported four cases of postpartum bacteremia due to *Sneathia sanguinegens*. Subsequently, De Martino et al. [29] reported three cases of maternal postpartum bacteremia with *Leptotrichia amnionii* or *Sneathia sanguinegens*, and Boennelycke et al. [11] reported the detection of *Leptotrichia amnionii* in a blood culture of a patient with septic abortion at 16 weeks of gestation. Gardella et al. [44] identified two cases of *Sneathia sanguinegens* in culture-negative AF of patients with preterm labor (n=132). Recently, we have reported the results of a large retrospective cohort study in which cultivation methods, as well as broad-range end-point and real-time PCR were used to define the prevalence, diversity and abundance of microbes invading the amniotic cavity in 166 patients with preterm labor. *Sneathia sanguinegens* and *Leptotrichia amnionii* were among the taxa detected by PCR only. Four patients had a positive PCR for one or both taxa. Some phylotypes appeared to be as-yet uncultivated and uncharacterized species of *Leptotrichia*, or to represent a novel sequence type (<94% nearest-neighbor similarity) that clustered with the genus *Leptotrichia* [34]; this suggests that the diversity of disease-associated *Leptotrichia* spp. is greater than currently recognized.

In conclusion, we were able to report that the rate of MIAC among patients with preeclampsia is 9.6% using both cultivation and molecular methods. *Sneathia/Leptotrichia* was detected by PCR in half of the patients with MIAC. Collectively, our findings do not support a prominent role for intra-amniotic infection in the pathophysiology of preeclampsia. The unexpectedly high prevalence of *Sneathia/Leptotrichia* warrants further investigation.

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