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Bacteria in the amniotic fluid without inflammation: Early colonization vs. contamination

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Abstract

Objectives: Intra-amniotic infection, defined by the presence of microorganisms in the amniotic cavity, is often accompanied by intra-amniotic inflammation. Occasionally, laboratories report the growth of bacteria or the presence of microbial nucleic acids in amniotic fluid in the absence of intra-amniotic inflammation. This study was conducted to determine the clinical significance of the presence of bacteria in amniotic fluid samples in the absence of intra-amniotic inflammation.

Methods: A retrospective cross-sectional study included 360 patients with preterm labor and intact membranes who underwent transabdominal amniocentesis for evaluation of the microbial state of the amniotic cavity as well as intra-amniotic inflammation. Cultivation techniques were used to isolate microorganisms, and broad-range polymerase chain reaction coupled with electrospray ionization mass spectrometry (PCR/ESI-MS) was utilized to detect the nucleic acids of bacteria, viruses, and fungi.

Results: Patients whose amniotic fluid samples evinced microorganisms but did not indicate inflammation had a similar perinatal outcome to those without microorganisms or inflammation [amniocentesis-to-delivery interval (p=0.31), spontaneous preterm birth before 34 weeks

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(p=0.83), acute placental inflammatory lesions (p=1), and composite neonatal morbidity (p=0.8)].

Conclusions: The isolation of microorganisms from a sample of amniotic fluid in the absence of intra-amniotic inflammation is indicative of a benign condition, which most likely represents contamination of the specimen during the collection procedure or laboratory processing rather than early colonization or infection.

Keywords: *Acinetobacter*; acute funisitis; acute histologic chorioamnionitis; amniocentesis; interleukin-6; intra-amniotic infection; intra-amniotic inflammation; microbial burden; preterm labor with intact membranes; *Propionibacterium acnes*.

Introduction

Infection is a major cause of morbidity and mortality for mothers and newborns [1–3] who represent relatively vulnerable hosts to the effects of microorganisms or microbial products (i.e. bacterial endotoxin). Therefore, the accurate identification of intra-amniotic infection has become important in patients at risk for adverse pregnancy outcome (preterm labor with intact membranes [4–13], preterm prelabor rupture of the membranes [14–25], clinical chorioamnionitis [26–29], idiopathic vaginal bleeding in the second or third trimester [30, 31], sonographic short cervix [32–34], cervical insufficiency [35]), and in patients who conceive despite having an intrauterine contraceptive device [36].

Amniotic fluid does not contain bacteria under normal circumstances [37–44]. However, microorganisms may gain access to the amniotic cavity through different pathways, of which the most frequent is an ascending route from the lower genital tract [1, 45–48]. Bacteria in the amniotic cavity, detectable by cultivation or molecular microbiologic techniques, may elicit a local inflammatory response [49–63], and this state is referred to as intra-amniotic infection. Persuasive evidence indicates that intra-amniotic infection is causally linked to spontaneous preterm labor and delivery [64–67], the development of acute histologic chorioamnionitis [65, 68–73] and funisitis [74, 75], a fetal inflammatory response [73, 76–85], and adverse perinatal outcomes [6, 65, 86–106]. Recent evidence suggests that maternal administration of antibiotics can eradicate intra-amniotic infection [32, 107–111].

The definition of intra-amniotic infection requires the presence of both microorganisms and an intra-amniotic inflammatory response [1, 45]. Occasionally, during clinical management, patients present with a situation for which the laboratory reports bacterial growth or the presence of microbial nucleic acids in amniotic fluid in the absence of intra-amniotic inflammation [6, 13, 34, 92, 112–118]. The interpretation of this finding is a challenge. Are bacteria the result of contamination of amniotic fluid at the time of specimen collection/laboratory processing, or the result of early colonization by pathogenic bacteria before an inflammatory process is established? This conundrum haunts clinicians in virtually every specialty of medicine dealing with a presumably sterile specimen, such as cerebrospinal, pleural-pericardial, or amniotic fluid, or blood. Erroneous interpretation of a laboratory report can lead to devastating consequences in obstetrics, including iatrogenic preterm delivery. This study was conducted to determine the clinical significance of the presence of bacteria in amniotic fluid in the absence of intra-amniotic inflammation.

Materials and methods

Study population

This retrospective cohort study was conducted by searching the clinical database and bank of biological samples of Wayne State University, the Detroit Medical Center, and the Perinatology Research Branch. Patients diagnosed with preterm labor and intact membranes at Hutzel Women's Hospital were included in the study when they met the following criteria: (1) a singleton gestation; (2) a transabdominal amniocentesis to assess the microbial state of the amniotic cavity between 20 and 35 weeks of gestation; (3) availability of amniotic fluid for the performance of molecular microbiologic studies; and (4) known pregnancy and neonatal outcomes. Patients were excluded from the study when they presented with (1) a rupture of the chorioamniotic membranes before amniotic fluid collection or (2) a chromosomal or structural fetal anomaly.

A transabdominal amniocentesis was offered to patients with the diagnosis of preterm labor and intact membranes at the discretion of the attending physician to identify the microbial status of the amniotic cavity. Women who agreed to undergo this procedure were asked to donate additional amniotic fluid and to allow the collection of clinical information for research purposes. The administration of antibiotics was left to the discretion of the physician.

All patients provided written informed consent prior to the procedure and the collection of samples. The use of biological specimens as well as clinical and ultrasound data for research purposes was approved by the Human Investigation Committee of Wayne State University.

Clinical definitions

Preterm labor was defined by the presence of at least two regular uterine contractions every 10 min associated with cervical changes in patients with a gestational age between 20 and 36 6/7 weeks. *Neonatal death* was defined as an infant death before 28 days of age [119].

Composite neonatal morbidity included two or more of the following: a 5-min Apgar score <7, bronchopulmonary dysplasia, respiratory distress syndrome, requirement for ventilation, necrotizing enterocolitis, intraventricular hemorrhage, retinopathy of prematurity, neonatal bacteremia, or neonatal intensive care unit admission.

Intra-amniotic inflammation was diagnosed when the amniotic fluid interleukin (IL)-6 concentration was ≥2.6 ng/mL [6, 120]. Based on the results of amniotic fluid culture, polymerase chain reaction with electrospray ionization mass spectrometry (PCR/ESI-MS) (Ibis® Technology-Athogen, Carlsbad, CA, USA) testing [20, 121–123], and the amniotic fluid IL-6 concentration, patients were classified into four subgroups: (1) *no intra-amniotic infection and/or inflammation*: negative amniotic fluid by both culture and PCR/ESI-MS in the absence of intra-amniotic inflammation (IL-6 <2.6 ng/mL); (2) *microorganisms in the amniotic fluid without intra-amniotic inflammation*: positive amniotic fluid by either culture or PCR/ESI-MS in the absence of intra-amniotic inflammation (IL-6 <2.6 ng/mL); (3) *sterile intra-amniotic inflammation*: negative amniotic fluid, by both culture and PCR/ESI-MS, in the presence of intra-amniotic inflammation (IL-6 ≥2.6 ng/mL); and (4) *intra-amniotic infection*: positive amniotic fluid, by either culture or PCR/ESI-MS, in the presence of intra-amniotic inflammation (IL-6 ≥2.6 ng/mL).

Sample collection

Amniotic fluid, upon collection, was immediately transported in a capped, sterile syringe to the clinical laboratory where it was cultured for aerobic and anaerobic bacteria as well as genital mycoplasmas. Evaluations of the white blood cell (WBC) count [124], glucose concentration [125], and Gram stain [126] in amniotic fluid were also performed shortly after collection. Amniotic fluid not required for clinical assessment was centrifuged at 1,300×*g* for 10 min at 4 °C, shortly after amniocentesis, and the supernatant was aliquoted and stored at −80 °C until analysis.

Detection of microorganisms with cultivation and molecular microbiologic methods

Amniotic fluid was analyzed utilizing aerobic and anaerobic cultures, an assay for genital mycoplasmas, and broad-range real-time PCR/ESI-MS. The methods have been previously described in detail [11, 48, 127]. We included molecular microbiologic techniques for the detection of bacteria, fungi, and select viruses. PCR/ESI-MS identified 3,400 bacteria and 40 *Candida* spp., represented in the platform's signature database [127–129]. Fourteen primer pairs detected the following viruses: Human herpesvirus 1 (HHV-1), Human herpesvirus 2 (HHV-2), Human herpesvirus 3 (HHV-3), Human herpesvirus 4 (HHV-4), Human herpesvirus 5 (HHV-5), Human herpesvirus 8 (HHV-8), Human adenovirus, Human enteroviruses, BK polyomavirus, JC polyomavirus, and Human parvovirus B19 [130].

The microbial burden was assessed by calculating the genome equivalents per PCR well (GE/well). The microbial genome load per mL of amniotic fluid (GE/mL) is equal to the GE/well multiplied by 133.33. The sensitivity, or limit of detection (LOD), of PCR/ESI-MS for the detection of bacteria in the blood is, on average, 100 CFU/mL [95% confidence interval (CI), 6–600 CFU/mL] [129]. A comparison of detection limits between blood and amniotic fluid showed that the assays have similar detection limits (100 CFU/mL) [11]. The LOD for the broad viral load in plasma ranges from 400 copies/mL to 6,600 copies/mL [131]. An LOD comparison of the samples of amniotic fluid and plasma was performed by spiking known amounts of a DNA virus (HHV-5) and an RNA virus (Human enterovirus) into these fluids. The LODs in amniotic fluid, similar to those in plasma, ranged from approximately 800 copies/mL to 1,600 copies/mL (depending upon the specific microorganism) [11].

Determination of IL-6 in amniotic fluid

IL-6 concentrations were determined to assess the presence and magnitude of the intra-amniotic inflammatory response, by using a sensitive and specific enzyme immunoassay obtained from R&D Systems (Minneapolis, MN, USA). The details of the assay, including sensitivity and the inter- and intra-assay coefficients of variation, have been previously reported [11, 127]. The cut-off value of 2.6 ng/mL has been previously reported for the diagnosis of intra-amniotic inflammation [11, 31, 127, 132].

Placental histopathologic examination

Placentas, collected in the Labor and Delivery Unit or Operating Room at Hutzel Women's Hospital of the Detroit Medical Center, were transferred to the clinical laboratory of the Perinatology Research Branch. Placental sampling was conducted in compliance with protocols of the Perinatology Research Branch, as previously described [133–138]. A minimum of 5 full-thickness sections of the chorionic plate, three sections of the umbilical cord, and three chorioamniotic membrane rolls, collected from each patient, were examined by placental pathologists who were blinded to the respective clinical histories and additional testing results. Acute inflammatory lesions of the placenta (acute chorioamnionitis and funisitis) were diagnosed according to established criteria, including staging and grading [136, 138, 139]. Severe acute placental inflammatory lesions are defined as stage 3 and/or grade 2 [136, 138].

Statistical analysis

The Kruskal–Wallis test, followed by the Mann-Whitney-Wilcoxon test for post-hoc analysis, was performed for the comparison of continuous variables among the subgroups. Categorical variables were compared by using the Chi-square test. To control the false discovery rate due to multiple comparisons, we used the Benjamini-Hochberg method for correction of nominal p-values. The analyses were conducted by using R language and environment for statistical computing (www.r-project.org). A heatmap, illustrating microbial burden, was generated by the ComplexHeatmap package in R, and the Euclidean distance was used for clustering after log-transformation of the data. Kaplan-Meier survival analysis and Cox proportional hazard regression models were used to compare the amniocentesis-to-delivery interval among the subgroups. Logistic regression was used to assess the association between microbial burden (above a given cut-off value) and the risk of preterm birth. The positive likelihood ratio [sensitivity/(1-specificity)] was determined

as a function of microbial burden. A p-value <0.05 was considered statistically significant.

Results

Characteristics of the study population

A total of 360 patients with preterm labor and intact membranes were included in this study. The demographic and clinical characteristics of the study population are displayed in Table 1. The median gestational age at amniocentesis was 28.7 weeks. The median gestational age at delivery was 33.6 weeks, and 14.4% (52/360) of the patients were delivered by cesarean section.

Table 1: Demographic and clinical characteristics of the study population.

Characteristics	Results (n=360)
Maternal age, years	23 (20–27)
Nulliparity	37.2% (134/360)
Smoking	23.6% (85/360)
Alcohol abuse	3.3% (12/359)
Pre-pregnancy body mass index, kg/m^2	24.4% (21.1–29.3)
Gestational age at amniocentesis, weeks	28.7 (24.9–32.0)
Gestational age at delivery, weeks	33.6 (28.4–36)
Cesarean delivery	14.4% (52/360)

Data are presented as median (interquartile range) or % (n/N).

The frequency of microorganisms in the amniotic fluid in the absence of inflammation

The frequency of microorganisms in amniotic fluid samples in the absence of intra-amniotic inflammation was 1.4% (5/360) by culture, 12.5% (45/360) by PCR/ESI-MS, and 13.3% (48/360) by using the combination of cultivation techniques and PCR/ESI-MS (either result – culture or PCR – was considered positive). The overall frequency of intra-amniotic inflammation in this cohort was 38% (136/360). In the presence of intra-amniotic inflammation, the frequency of microorganisms detected in amniotic fluid was 9.2% (33/360) by culture, 19.2% (69/360) by PCR/ESI-MS, and 19.4% (70/360) by using the combination of cultivation techniques and PCR/ESI-MS.

Based on the results of the amniotic fluid culture, PCR/ESI-MS, and amniotic fluid IL-6 concentration, patients were classified into four clinical subgroups: (1) 48.9% (176/360) did not have either intra-amniotic infection or intra-amniotic inflammation; (2) 13.3% (48/360) had microorganisms but without inflammation; (3) 18.3% (66/360) had sterile amniotic inflammation; and (4) 19.4% (70/360) had intra-amniotic infection.

Table 2 describes the results of the biomarkers of inflammation in amniotic fluid among the four subgroups. Most of the amniotic fluid samples (97%; 215/222) were collected before the administration of antibiotics, and there was no significant difference in the use of antibiotics before amniocentesis among the four subgroups. The

Table 2: Amniotic fluid analysis of four clinical subgroups according to results of amniotic fluid culture, PCR/ESI-MS, and amniotic fluid IL-6 concentrations in patients with preterm labor and intact membranes.

	No intra-amniotic inflammation/infection (n=176)	Microorganisms without intra-amniotic inflammation (n=48)	Sterile intra-amniotic inflammation (n=66)	Intra-amniotic infection (n=70)	p-Value
Gestational age at amniocentesis, weeks	30.1 (25.9–32.3)	29.3 (25.3–32.3)	27.6 (23.8–31.7)	26.4 (24.9–30.9)	0.02
Antibiotic use before amniocentesis	4.3% (4/94)	3.3% (1/30)	0% (0/43)	3.6% (2/55)	0.61
Positive amniotic fluid Gram stain	0% (0/174)	2.1% (1/48)	0% (0/65)	27.1% (19/70)	<0.001
Amniotic fluid WBC count, cells/mm^3	1 (0–7)	2 (0–8)	2 (0–10)	25 (2–430)[a,c,e]	<0.001
Amniotic fluid glucose, mg/dL	29 (23–33.5)	25 (20–31.5)	22 (18–27)[b]	13 (10–22)[a,c,e]	<0.001
Amniotic fluid interleukin-6, ng/mL	0.8 (0.4–1.2)	0.6 (0.3–1.2)	8.5 (4.4–17.5)[b,d]	75.4 (16.7–235.1)[a,c,e]	<0.001

Data are presented as median (interquartile range) or % (n/N). WBC, white blood cell; PCR/ESI-MS, polymerase chain reaction with electrospray ionization mass spectrometry. [a]p<0.05; No intra-amniotic inflammation/infection vs. Intra-amniotic infection. [b]p<0.05; No intra-amniotic inflammation/infection vs. Sterile intra-amniotic inflammation. [c]p<0.05; Microorganisms without inflammation vs. Intra-amniotic infection. [d]p<0.05; Microorganisms without inflammation vs. Sterile intra-amniotic inflammation. [e]p<0.05; Intra-amniotic infection vs. Sterile intra-amniotic inflammation.

Table 3: Microbiology of amniotic fluid in samples with positive cultures and/or PCR/ESI-MS according to the presence or absence of intra-amniotic inflammation.

Microorganisms without intra-amniotic inflammation					Microorganisms with intra-amniotic inflammation (intra-amniotic infection)				
Name of microorganisms	No. of culture-positive samples	No. of PCR-positive samples	No. of both-positive samples	Microbial burden (GE/well)[a]	Name of microorganisms	No. of culture-positive samples	No. of PCR-positive samples	No. of both-positive samples	Microbial burden (GE/well)[a]
Bacteria and fungi	5	55	0	17 (10–38)	**Bacteria and fungi**	37	82	18	80 (20–178)
Propionibacterium acnes	1	16	0	18 (14–31)	*Ureaplasma parvum*	0	8	0	573 (114–1,245)
Acinetobacter junii	0	14	0	23 (18–27)	*Haemophilus influenzae*	2	2	2	158; 818
Staphylococcus aureus	1	5	0	7 (5–8)	*Mycoplasma hominis*	3	5	2	446 (247–451)
Streptococcus viridans	0	2	0	3; 11	*Streptococcus pneumonia*	0	1	0	286
Acidovorax temperans	0	2	0	29; 54	*Ureaplasma urealyticum*	5	2	0	11; 272
Actinomyces odontolyticus	0	1	0	3	*Staphylococcus aureus*	1	2	1	1; 201
Aeromonas caviae	0	1	0	3	*Gardnerella vaginalis*	3	2	0	69; 171
Moraxella osloensis	0	1	0	3	*Sneathia* spp.	3	12	3	168 (117–180)
Pantoea dispersa	0	1	0	3	*Streptococcus agalactiae*	1	3	1	101; 142; 153
Streptococcus spp.	0	1	0	3	*Escherichia coli*	1	1	1	149
Lactobacillus acidophilus	0	1	0	5	*Candida albicans*	4	4	4	136 (45–337)
Streptococcus infantis	0	1	0	6	*Fusobacterium nucleatum*	4	12	4	108 (32–120)
Haemophilus influenzae	0	1	0	9	*Rhodococcus jostii*	0	1	0	58
Clostridium septicum	0	1	0	12	*Pseudomonas mendocina*	0	1	0	51
Corynebacterium tuberculostearicum	0	1	0	15	*Peptostreptococcus anaerobius*	1	1	0	50
Candida tropicalis	0	1	0	35	*Kytococcus schroeteri*	0	1	0	48
Acinetobacter baumannii	0	1	0	35	*Acinetobacter junii*	0	5	0	43 (15–61)
Staphylococcus arlettae	0	1	0	43	*Streptococcus* spp.	1	1	0	36
Stenotrophomonas maltophilia	0	1	0	45	*Streptobacillus moniliformis*	0	1	0	29
Pseudomonas fluorescens	0	1	0	47	*Bacteroides fragilis*	0	1	0	21
Streptococcus agalactiae	0	1	0	65	*Corynebacterium accolens*	0	1	0	19
Corynebacterium spp.	1	0	0	–	*Propionibacterium acnes*	0	13	0	14 (12–20)
Ureaplasma urealyticum	2	0	0	–	*Acinetobacter lwoffii*	0	1	0	14
Viruses	0	19	0	17 (7–100)	*Pseudomonas entomophila*	0	1	0	10
Human parvovirus B19	0	9	0	6 (4–10)	*Bacteroides ureolyticus*	3	0	0	
Human enterovirus	0	1	0	17	*Mobiluncus* spp.	2	0	0	–
Human herpesvirus 2 (HHV-2)	0	2	0	90; 152	*Clostridium sporogenes*	1	0	0	–
Human herpesvirus 5 (HHV-5)	0	7	0	109 (48–187)	*Staphylococcus capitis*	1	0	0	–
					Streptococcus anginosus	1	0	0	–
					Viruses	0	19	0	24 (15–78)
					Human enterovirus	0	2	0	24; 1,000
					Human herpesvirus 2 (HHV-2)	0	3	0	15; 67; 88
					Human herpesvirus 5 (HHV-5)	0	6	0	34 (20–111)
					Human parvovirus B19	0	7	0	15 (10–23)
					Roseolovirus	0	1	0	20

PCR/ESI-MS, polymerase chain reaction with electrospray ionization mass spectrometry. [a]Data are presented as median (interquartile range) or each value.

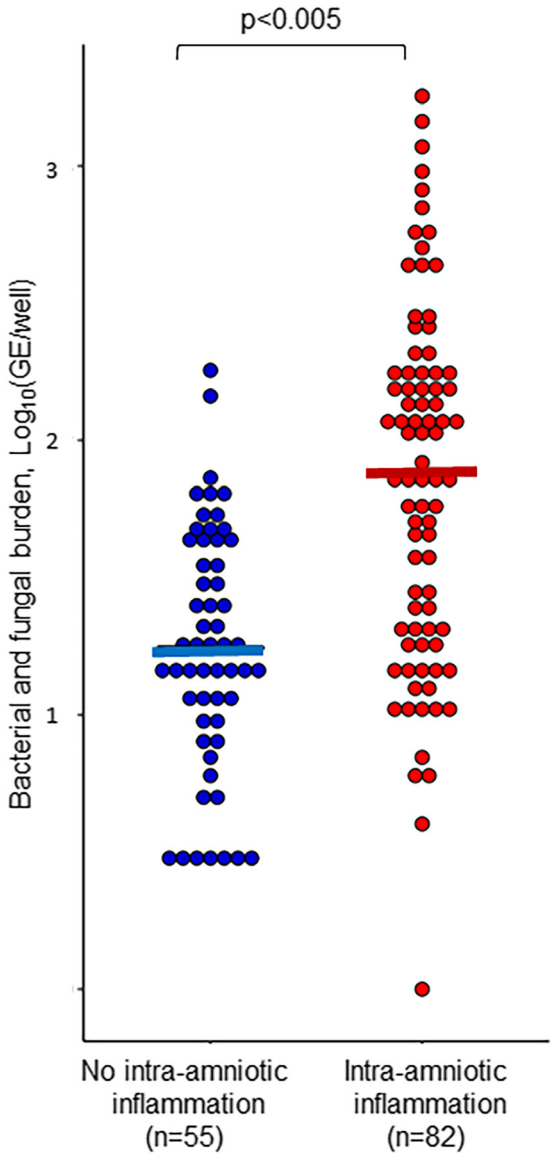

Figure 1: Microbial burden in amniotic fluid between patients with intra-amniotic inflammation and those without intra-amniotic inflammation [median (IQR), 1.2 (1.0–1.6) vs. 1.9 (1.3–2.3) $\log_{10}$ (GE/well), p<0.005]. The presence of intra-amniotic inflammation was defined as an elevated IL-6 (cut-off > 2.6 ng/mL).

distribution of the amniotic fluid WBC count and the concentrations of amniotic fluid IL-6 and amniotic fluid glucose varied significantly among the four subgroups (Kruskal–Wallis, p<0.001 for all).

Microorganisms reported in amniotic fluid samples in the absence of intra-amniotic inflammation

Forty-eight amniotic fluid samples reported by the laboratory had microorganisms detected by culture or PCR but no intra-amniotic inflammation: 35.4% (17/48) had two or more microorganisms and, overall, a total of 79 microorganisms (bacteria, n=59; fungus, n=1; and viruses, n=19) were identified (Table 3). *Propionibacterium acnes* (n=17) was the most frequent microorganism reported, followed by *Acinetobacter junii* (n=14). *P. acnes* was isolated by culture in only one patient [6% (1/17)] and, in the rest of the patients, positive results were attributable to PCR analysis [94% (16/17)]. All samples positive for *A. junii* were detected by PCR/ESI-MS (i.e. this organism was not detected by culture).

Among the 70 patients with intra-amniotic infection (positive for microorganisms and intra-amniotic inflammation), 47.1% (33/70) had two or more microorganisms and, overall, a total of 120 microorganisms (bacteria, n=97; fungus, n=4; and viruses, n=19) were identified (Table 3). Amniotic fluid samples from patients with intra-amniotic infection were dominated by *Sneathia* spp., *Fusobacterium nucleatum*, *Ureaplasma parvum*, *Mycoplasma hominis*, *Gardnerella vaginalis*, and *Candida albicans*, which were not found in patients without intra-amniotic inflammation.

Microbial burden was low in amniotic fluid samples with microorganisms in the absence of intra-amniotic inflammation

The microbial burden was defined as the number of microorganisms in amniotic fluid and estimated by the number of gene copies per PCR well reaction (GE/well). Bacterial and fungal burdens were lower in amniotic fluid samples in the absence of intra-amniotic inflammation than in those with intra-amniotic inflammation [median (interquartile range (IQR), 17 (10–38) vs. 80 (20–178) GE/well, p<0.005] (Figure 1). However, there was no significant difference in viral burden between those with or without inflammation [median (IQR), 17 (7–100) vs. 24 (15–78) GE/well, p=0.34].

Figure 2 is a heatmap illustrating microbial burden in the amniotic fluid as a function of the color (blue – low; red – high) according to the presence or absence of intra-amniotic inflammation. Amniotic fluid samples from patients with intra-amniotic infection were dominated by *U. parvum*, *M. hominis*, *Sneathia* spp., *C. albicans*, and *F. nucleatum* and exhibited high microbial burden [median (IQR), GE/well; *U. parvum*: 573 (114–1,245), *M. hominis*: 446 (247–451), *Sneathia* spp.: 168 (117–180), *C. albicans*: 136 (45–337), and *F. nucleatum*: 108 (32–120)]. Most patients with these microorganisms in amniotic fluid delivered within 7 days after amniocentesis [*U. parvum*: 75% (6/8),

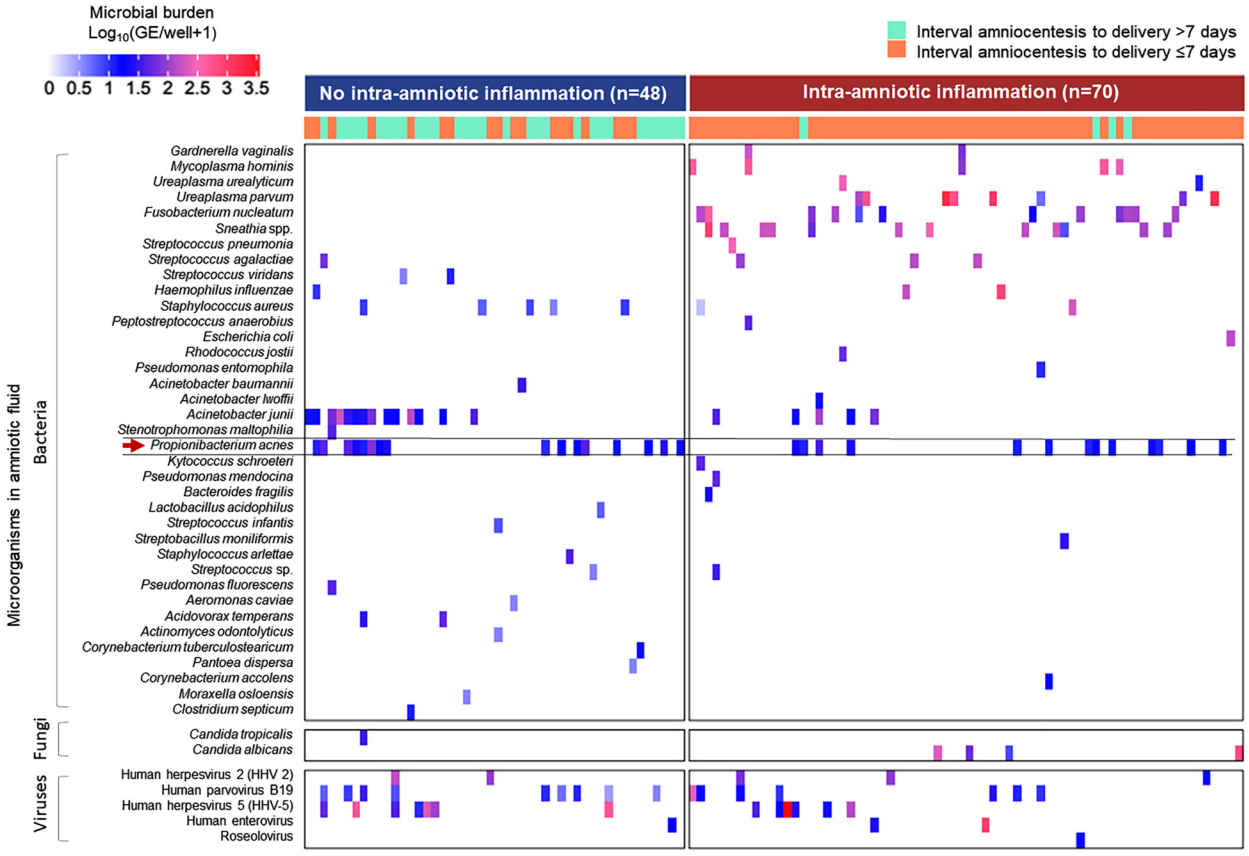

Figure 2: Heatmap showing microbial taxa and microbial load in the amniotic fluid, [$\log_{10}$ (GE/well + 1)], according to PCR-based methods. High microbial burden is represented with color gradation which increases toward red, while low microbial burden tends toward blue, as shown in the color-key. The arrow indicates *Propionibacterium acnes*.

M. hominis: 100% (6/6), *Sneathia* spp.: 100% (12/12), *C. albicans*: 100% (4/4), *F. nucleatum*: 83% (10/12)] (Figure 2).

By contrast, *P. acnes* and *A. junii* were abundant in the samples of amniotic fluid without intra-amniotic inflammation and exhibited a low microbial burden [median (IQR), GE/well; *P. acnes*: 18 (14–31) and *A. junii*: 23 (18–27)]. *P. acnes* was also detected in the amniotic fluid samples with inflammation but consistently had a low microbial burden [median (IQR), 14 (12–20) GE/well] (Figure 2).

Pregnancy outcomes, acute placental inflammatory lesions, and neonatal outcomes

Table 4 shows the perinatal outcomes of the four subgroups: (1) patients reported by the laboratory to have microorganisms in the amniotic fluid but not intra-amniotic inflammation had similar outcomes as patients without either microorganisms or intra-amniotic inflammation [spontaneous preterm birth <34 weeks of gestation (p=1), severe acute histologic chorioamnionitis (p=0.76), severe funisitis (necrotizing funisitis and/or intense umbilical vasculitis) (p=1), and composite neonatal morbidity (p=0.8)]; 2) patients with intra-amniotic infection, overall, had worse perinatal outcomes than those with microorganisms in the absence of inflammation [spontaneous preterm birth <34 weeks of gestation: 90% (63/70) vs. 31.2% (15/48), p<0.001; severe acute histologic chorioamnionitis: 55.7% (34/61) vs. 10.3% (4/39), p<0.001; and composite neonatal morbidity: 86.6% (58/67) vs. 33.3% (16/48), p<0.001] or those with sterile intra-amniotic inflammation [severe acute histologic chorioamnionitis: 55.7% (34/61) vs. 13.3% (8/60), p<0.001; funisitis [68.7% (46/67) vs. 27% (17/63), p<0.001]; and 3) patients with sterile intra-amniotic inflammation had worse perinatal outcomes than those with microorganisms in the absence of inflammation [spontaneous preterm birth <34 weeks of gestation: 83.3% (55/66) vs. 31.2% (15/48); composite neonatal morbidity: 76.6% (49/64) vs. 33.3% (16/48), p<0.001].

Table 4: Perinatal outcomes among the four subgroups of patients according to results of amniotic fluid culture, PCR/ESI-MS, and amniotic fluid IL-6 concentrations in patients with preterm labor and intact membranes.

	No intra-amniotic inflammation/infection (n=176)	Microorganisms without intra-amniotic inflammation (n=48)	Sterile intra-amniotic inflammation (n=66)	Intra-amniotic infection (n=70)	p-Value
Pregnancy outcomes					
Gestational age at delivery, weeks	35.3 (33.4–37.3)	34.9 (31.7–36.5)	29.9 (25.1–33.0)[c,e]	27.2 (25.2–31.9)[b,d]	<0.001
Preterm birth < 37 weeks	72.2% (127/176)	83.3% (40/48)	97% (64/66)[c,e]	97% (64/66)[b,d]	<0.001
Preterm birth < 34 weeks	31.2% (55/176)	37.5% (18/48)	84.8% (56/66)[c,e]	84.8% (56/66)[b,d]	<0.001
Spontaneous preterm birth < 37 weeks	66.5% (117/176)	70.8% (34/48)	95.5% (63/66)[c,e]	95.7% (67/70)[b,d]	<0.001
Spontaneous preterm birth < 34 weeks	30.1% (53/176)	31.2% (15/48)	83.3% (55/66)[c,e]	90% (63/70)[b,d]	<0.001
Interval amniocentesis to delivery, days	37.1 (19–63)	29.4 (12–57)	3.9 (1.4–10.4)[c,e,f]	1.4 (0.7–5)[b,d]	<0.001
Interval amniocentesis to delivery < 7 days	12.5% (22/176)	14.6% (7/48)	65.2% (43/66)[c,e]	82.9% (58/70)[b,d]	<0.001
Cesarean delivery	12.5% (22/176)	18.8% (9/48)	16.7% (11/66)	14.3% (10/70)	0.67
Fetal death	1.8% (3/164)	0% (0/40)	3.4% (2/59)	4.3% (3/70)	0.48
Acute placental inflammation	34.2% (55/161)	34.1% (14/41)	60.3% (38/63)[c,e]	79.1% (53/67)[b,d]	<0.001
Acute chorioamnionitis	33.5% (54/161)	31.8% (13/41)	60.3% (38/63)[c,e]	79.1% (53/67)[b,d]	<0.001
Severe lesions	6.5% (10/155)	10.3% (4/39)	13.3% (8/60)[c,f]	55.7% (34/61)[b,d]	<0.001
Stage 3: Necrotizing chorioamnionitis	4.3% (7/161)	7.3% (3/41)	9.5% (6/63)[f]	49.3% (33/67)[b,d]	<0.001
Grade 2: Subchorionic microabscess	3.9% (6/153)	5.4% (2/37)	6.8% (4/59)[f]	31.5% (17/54)[b,d]	<0.001
Funisitis	23.6% (38/161)	24.4% (10/41)	27% (17/63)[f]	68.7% (46/67)[b,d]	<0.001
Severe lesions	1.6% (2/127)	4.2% (1/24)	4.4% (2/45)	19.5% (8/41)[b]	<0.001
Stage 3: Necrotizing funisitis	0.6% (1/161)	2.4% (1/41)	1.6% (1/63)	10.4% (7/67)[b]	0.001
Grade 2: Intense umbilical vasculitis	0.8% (1/126)	0% (0/23)	4.4% (2/45)	7.7% (3/39)	0.08
Neonatal outcomes					
Birth weight, g	2,376 (1965–2,935)	2,142.5(1,640–2,554)[a]	1,222.5(743–1979)[c,e]	1,060 (815–1,660)[b,d]	<0.001
Male sex neonate	51.1% (89/174)	50% (24/48)	505 (33/66)	45.7% (32/70)	0.90
Neonatal death	3.1% (5/159)	0% (0/38)	16.4% (9/55)[c]	13.1% (8/61)[b,d]	<0.001
5-min Apgar score<7	8.1% (14/172)	8.5% (4/47)	31.8% (21/66)[c,e]	37.7% (26/69)[b,d]	<0.001
Composite neonatal morbidity[g]	30.1% (52/173)	33.3% (16/48)	76.6% (49/64)[c,e]	86.6% (58/67)[b,d]	<0.001

Data are presented as median (interquartile range) or % (n/N) PCR/ESI-MS; polymerase chain reaction with electrospray ionization mass spectrometry. [a]p<0.05; no intra-amniotic inflammation/infection vs. microorganisms without inflammation. [b]p<0.05; No intra-amniotic inflammation/infection vs. Intra-amniotic infection. [c]p<0.05; no intra-amniotic inflammation/infection vs. sterile intra-amniotic inflammation. [d]p<0.05; microorganisms without inflammation vs. Intra-amniotic infection. [e]p<0.05; microorganisms without inflammation vs. Sterile intra-amniotic inflammation. [f]p<0.05; intra-amniotic infection vs. sterile intra-amniotic inflammation. [g]Composite neonatal morbidity includes more than one of the following: 5-min Apgar score<7, bronchopulmonary dysplasia, respiratory distress syndrome, requirement for ventilation, necrotizing enterocolitis, intraventricular hemorrhage, retinopathy of prematurity, neonatal bacteremia, or neonatal intensive care unit admission.

Patients with bacteria in amniotic fluid but without intra-amniotic inflammation have a similar interval-to-delivery to those without either bacteria or intra-amniotic inflammation

Figure 3 displays the amniocentesis-to-delivery interval according to the presence or absence of microorganisms in amniotic fluid and intra-amniotic inflammation. Patients with microorganisms without intra-amniotic inflammation had a significantly longer amniocentesis-to-delivery interval than those with intra-amniotic inflammation regardless of the presence of microorganisms [median (IQR), 29.4 (12–57) vs. 1.4 (0.7–5) days, p<0.001] or the absence of microorganisms [median (IQR), 29.4 (12–57) vs. 3.9 (1.4–10.4) days, p<0.001]. There were no significant differences in the amniocentesis-to-delivery interval between patients with microorganisms in the absence of inflammation and those with amniotic fluid negative for microorganisms (p=0.31).

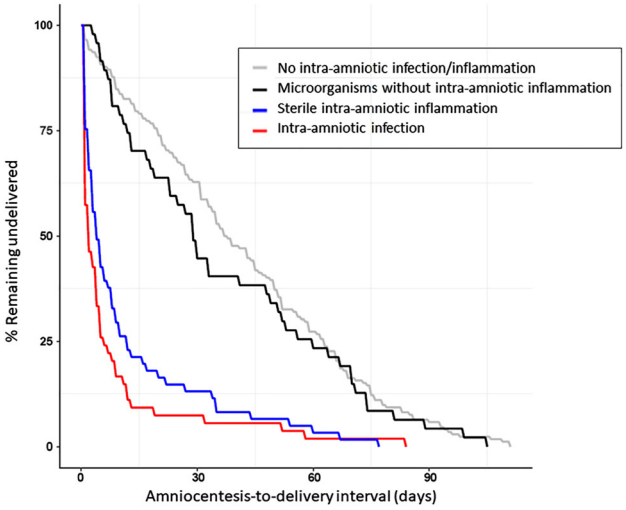

Figure 3: Kaplan-Meier survival curves of amniocentesis-to-delivery interval (days) among four subgroups of patients according to the presence or absence of microorganisms in amniotic fluid and intra-amniotic inflammation.
Patients with bacteria in the amniotic fluid without intra-amniotic inflammation have a similar interval-to-delivery as those without bacteria or intra-amniotic inflammation (p=0.31).

Discussion

Principal findings of the study

(1) The frequency of microorganisms in amniotic fluid without intra-amniotic inflammation was 1.4% (5/360) by culture techniques, 12.5% (45/360) by PCR/ESI-MS, and 13.3% (48/360) by the combination of cultivation and PCR/ESI-MS; (2) the most frequently identified microorganisms in the 48 amniotic fluid specimens were *P. acnes*, followed by *A. junii*; 3) patients whose amniotic fluid samples had microorganisms but no indication of inflammation had a similar perinatal outcome to those in whom microorganisms were not found in the amniotic fluid [amniocentesis-to-delivery interval (p=0.31), frequency of spontaneous preterm birth before 34 weeks of gestation (p=0.83), acute placental inflammatory lesions (p=1), and composite neonatal morbidity (p=0.8)]; and 4) the microbial load of samples with bacteria reported by the laboratory in the absence of intra-amniotic inflammation was low. Therefore, we conclude that the presence of microorganisms in the absence of intra-amniotic inflammation most likely represents contamination at the time of specimen collection or laboratory processing and that it is a benign condition.

What is the difference? Intra-amniotic infection vs. colonization vs. contamination

When the culture of amniotic fluid obtained by amniocentesis is positive, the result could indicate one of three possibilities: infection, colonization, or contamination. **Intra-amniotic infection** is defined as the presence of microorganisms in the amniotic fluid, retrieved by amniocentesis, regardless of the presence or absence of clinical evidence of infection (e.g. fever, uterine tenderness, malodorous vaginal discharge, etc.) [1, 45], while **colonization of the amniotic cavity** refers to the presence of microorganisms in the amniotic fluid without any pathologic consequences [1, 45]. Microbes can colonize on a body surface, such as the skin, mouth, intestines, or airway, without causing disease in the person [140, 141]. However, amniotic fluid is normally sterile [37–42, 44, 142, 143], as is cerebrospinal fluid, or pleural or pericardial fluid. Thus, we have not used the term "colonization" of the amniotic cavity.

Given that the host immune response to intra-amniotic infection develops over time [66, 144–146], microorganisms may be able to **colonize the amniotic cavity** for a period of time without pathological consequences [147–150]. For example, an experiment based on a primate model showed that the number of *U. parvum* in amniotic fluid peaked at 3 days and uterine contractions peaked at 6–8 days after inoculation [146]. This finding suggests that microorganisms may exist in the amniotic cavity without clinical symptoms in the early phase of infection.

Moreover, clinical manifestations of microbial invasion of the amniotic cavity vary according to the virulence of the microorganisms [21, 65, 89, 116, 151–157], microbial burden [11, 20, 26, 64, 121, 158–160], and time frame of the acute inflammatory response [66, 146, 161].

Contamination occurs when microorganisms from an outside source are introduced into a sample [162]. For example, microorganisms normally present on the skin can gain access to amniotic fluid during amniocentesis or during the procedures required to prepare a specimen [20, 163, 164]. Indeed, contamination can originate from many sources including the laboratory environments [165, 166], plastic consumables [167], nucleic acid extraction kits [168–173], laboratory reagents [174–180], and cross-contamination from other samples [181, 182]. It is now well accepted that laboratory reagents and nucleic acid extraction kits harbor low levels of bacterial DNA [170, 183] similar to that found in soil or water samples [170, 172]. DNA contamination of reagents is unavoidable, given the ubiquity of microorganisms and the fact that many reagents are products of microbial processes and engineering [184].

Which bacteria are typical contaminants in amniotic fluid?

P. acnes was the most common microorganism isolated from amniotic fluid in the absence of intra-amniotic inflammation, and its presence should be considered suggestive of contamination rather than true infection. Several arguments support this view: (1) *P. acnes*, a commensal bacterium in the human skin microbiome [185, 186], is a common contaminant detected in cultures of blood and cerebrospinal fluid [187, 188]. Such skin bacteria can gain access to the amniotic fluid during an amniocentesis or through the procedures required to prepare the specimen [20, 163, 164]; (2) *Propionibacterium* spp. are reported as common contaminants present in the DNA extraction kits and other laboratory reagents [170, 171, 189–191]; in the current study, nearly all detection of *P. acnes* (97%; 31/32) came through the PCR method; and (3) all amniotic fluid samples positive with *P. acnes* yielded a consistently low bacterial burden regardless of the presence or absence of intra-amniotic inflammation.

Acidovorax temperans, Pantoea dispersa, Staphylococcus arlettae, and *Stenotrophomonas maltophilia* were detected in amniotic fluid samples with the absence of intra-amniotic inflammation. These microorganisms have rarely been reported to cause human infection and are found in laboratory environments [170, 189], reagents [170, 189], nebulizers [192], water dispensers [193], hemodialysis fluids [194], and intravenous fluids [194]. Therefore, in amniotic fluid, these microorganisms are likely to be contaminants.

A. junii was the second most common species of microorganism isolated from samples with the absence of intra-amniotic inflammation. *Acinetobacter* spp. are found in water and soil environments [195] and have previously been identified as contaminants [189, 196] in biology grade water [170], PCR reagents [170, 190], DNA extraction kits [170, 190], and air samples collected from a patient's room [197]. In the current study, any time *Acinetobacter* spp. (*Acinetobacter baumannii, A. junii,* and *Acinetobacter lwoffii*) were found, the microbial burden was low, which is also a characteristic of contamination. Although *Acinetobacter* spp. have been reported as potential pathogens causing nosocomial sepsis [198–200], preterm delivery [201, 202], acute chorioamnionitis, and a fetal inflammatory response [201, 202], questions always arise about whether these organisms are contaminants and whether the condition occurs as a sterile intra-amniotic inflammatory process or as organisms that escape detection by conventional methods. It is important to remember that, even in recent times, some bacteria have been difficult to identify for decades, including *Borrelia burgdorferi*, the organism responsible for Lyme disease [203], and *Helicobacter pylori* [204].

Bacteria likely to be pathogens in amniotic fluid

Microorganisms implicated as "true pathogens" in intra-amniotic infection include *Ureaplasma parvum*, *Mycoplasma hominis*, *Sneathia* spp., *Candida albicans, Fusobacterium nucleatum, Staphylococcus aureus, Gardnerella vaginalis, Haemophilus influenzae,* and *Streptococcus agalactiae* [20, 89, 116, 121, 151, 152, 158, 163, 205–213]. In the current study, these taxa were abundant in the amniotic fluid samples with intra-amniotic inflammation, as demonstrated in Figure 2, and their presence was associated with adverse pregnancy outcomes, including spontaneous preterm delivery, severe acute chorioamnionitis or funisitis, and a short interval-to-delivery.

Abundance of microorganisms to differentiate infection from contamination

Quantification of bacterial growth has been used to distinguish between contamination and true infection in

the clinical setting. For example, the difference between asymptomatic bacteriuria and contamination of a urine specimen is based on the number of colony-forming units in a urine specimen obtained via clean-catch. The presence of bacteria is considered to be clinically significant if there are more than 10^5 colony-forming units (CFU)/mL, and a lower number is thought to reflect contamination when the urine travels from the bladder to the container through the urethra, which normally contains bacteria [214].

In the past, quantitative cultivation-based microbiology methods have been used to assess the microbial burden in amniotic fluid. We have observed that patients with a higher microbial burden are more likely to have a positive Gram stain in amniotic fluid [4, 126, 215] or to present with preterm labor leading to preterm delivery [14].

Given that bacteria grow exponentially in amniotic fluid over time [66, 144–146], amniotic fluid samples with a true infection will have a much higher microbial burden in later stages of gestation than those that have been contaminated during collection and/or processing.

A low microbial burden in amniotic fluid assessed by PCR methods has been attributed to background DNA contamination in the extraction kit [42, 160], whereas a high microbial burden has been observed in patients who have intra-amniotic infection [160] with a strong intra-amniotic inflammatory response [11, 20, 26, 121, 160]. We reported that patients exhibiting a microbial burden higher than 17 GE/well had a higher frequency of intra-amniotic inflammation, acute histologic chorioamnionitis, and perinatal morbidity than those with a lower microbial burden, assessed by PCR [11].

In the current study, pathogenic bacteria in the amniotic fluid exhibited a high microbial burden [i.e. median (IQR), GE/well; *U. parvum*: 573 (114–1,245), *M. hominis*: 446 (247–451), *Sneathia* spp.: 168 (117–180), *C. albicans*: 136 (45–337), and *F. nucleatum*: 108 (32–120)], whereas bacteria considered as contaminants in amniotic fluid showed a lower microbial load [i.e. median (IQR), GE/well; *P. acne*: 17 (13–24) GE/well] regardless of the presence or absence of intra-amniotic inflammation. Moreover, the risk of preterm birth begins to increase exponentially when the microbial burden in amniotic fluid exceeds 17 GE/well (Figure 4). Therefore, we believe that a microbial burden, assessed with molecular microbiologic techniques, can assist in determining whether a positive result reflects contamination rather than a true infection.

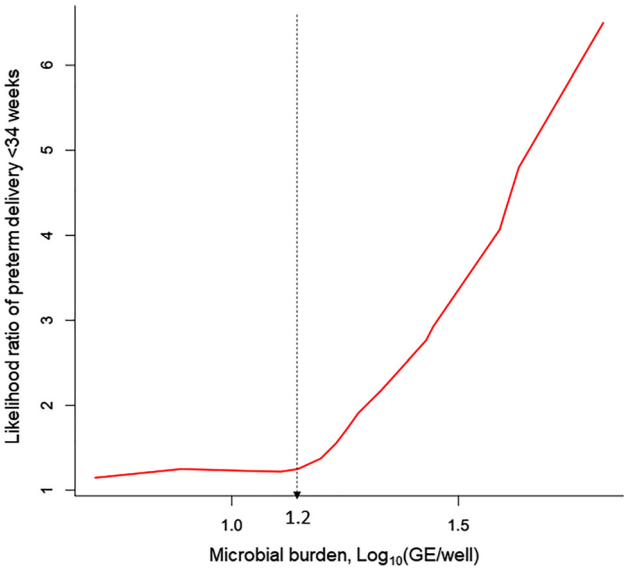

Figure 4: Positive likelihood ratio for preterm birth before 34 weeks of gestation as a function of microbial burden [$\log_{10}$ (GE/well)]. The risk of preterm birth before 34 weeks begins to increase exponentially when the microbial burden (bacterial and fungal) exceeds 1.2 $\log_{10}$ (GE/well). Viral invasion of the amniotic cavity was excluded from this analysis. In patients with several microorganisms, the calculation was based on the microorganism with the highest microbial burden.

Clinical significance of microorganisms in amniotic fluid without intra-amniotic inflammation: contamination or early infection?

Patients with microorganisms in amniotic fluid specimens (detected by either culture or molecular microbiologic techniques) without intra-amniotic inflammation have similar pregnancy outcomes to patients who did not have bacteria or inflammation. Therefore, we propose that finding bacteria in amniotic fluid in samples without intra-amniotic inflammation represents contamination. Consequently, clinical decisions, such as inducing labor or withholding treatment, given the suspicion of intra-amniotic infection does not seem to be justifiable.

Strengths and limitations

The major strengths of this study are emphasized as follows: (1) both cultivation and molecular microbiologic techniques were used to identify microorganisms in the samples of amniotic fluid collected by transabdominal amniocentesis from the amniotic cavity; therefore, the

diagnosis of microbial invasion was based on the use of state-of-the-art methodologies; (2) the assessment of intra-amniotic inflammation using the concentration of IL-6 in amniotic fluid; (3) the blinding of pathologists to obstetrical diagnoses and perinatal outcomes; and (4) the use of standardized protocols for placental examination.

The study also comprises the following limitations: (1) the duration of storage of the samples may have led to a degradation of the IL-6 concentration in amniotic fluid, which, in turn, may have yielded a lower concentration of the analytes as compared to the use of freshly collected and processed samples of amniotic fluid [216, 217]; (2) the lack of use of molecular markers to identify the presence of microorganisms in the extra-chorionic membranes, chorionic plate, and umbilical cord resulted in a lack of morphologic evidence of the location of microorganisms at different sites in the samples; and (3) the lack of use of metagenomics, strain culture, and/or strain-directed sequencing, which may be utilized to make the distinction between contamination and colonization.

Conclusions

The isolation of microorganisms or the detection of microbial nucleic acids from a sample of amniotic fluid, by cultivation and/or microbiologic molecular techniques in a clinical laboratory setting in the absence of intra-amniotic inflammation is a benign condition. Such a result most likely represents contamination of the specimen during the collection procedure or laboratory processing rather than early colonization or infection.

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Competing interests: Authors state no conflict of interest.

Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: The use of biological specimens as well as clinical and ultrasound data for research purposes was approved by the Human Investigation Committee of Wayne State University.

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