ORIGINAL ARTICLE

A Novel Molecular Microbiologic Technique for the Rapid Diagnosis of Microbial Invasion of the Amniotic Cavity and Intra-Amniotic Infection in Preterm Labor with Intact Membranes

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Keywords
Broad-range real-time polymerase chain reaction, electrospray ionization mass spectrometry, inflammation, preterm delivery, time-of-flight

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Problem
The diagnosis of microbial invasion of the amniotic cavity (MIAC) has been traditionally performed using traditional cultivation techniques, which require growth of microorganisms in the laboratory. Shortcomings of culture methods include the time required (days) for identification of microorganisms, and that many microbes involved in the genesis of human diseases are difficult to culture. A novel technique combines broad-range real-time polymerase chain reaction with electrospray ionization time-of-flight mass spectrometry (PCR/ESI-MS) to identify and quantify genomic material from bacteria and viruses.

Method of study
AF samples obtained by transabdominal amniocentesis from 142 women with preterm labor and intact membranes (PTL) were analyzed using cultivation techniques (aerobic, anaerobic, and genital mycoplasmas) as well as PCR/ESI-MS. The prevalence and relative magnitude of intra-amniotic inflammation [AF interleukin 6 (IL-6) concentration $\geq$ 2.6 ng/mL], acute histologic chorioamnionitis, spontaneous preterm delivery, and perinatal mortality were examined.

Results
(i) The prevalence of MIAC in patients with PTL was 7% using standard cultivation techniques and 12% using PCR/ESI-MS; (ii) seven of ten patients with positive AF culture also had positive PCR/ESI-MS [$\geq$17 genome equivalents per PCR reaction well (GE/well)]; (iii) patients with positive PCR/ESI-MS ($\geq$17 GE/well) and negative AF cultures had significantly higher rates of intra-amniotic inflammation and acute histologic chorioamnionitis, a shorter interval to delivery [median (interquartile range-IQR)], and offspring at higher risk of perinatal mortality, than

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women with both tests negative [90% (9/10) versus 32% (39/122) OR: 5.6; 95% CI: 1.4–22; ($P < 0.001$); 70% (7/10) versus 35% (39/112); ($P = 0.04$); 1 (IQR: <1–2) days versus 25 (IQR: 5–51) days; ($P = 0.002$), respectively]; (iv) there were no significant differences in these outcomes between patients with positive PCR/ESI-MS (≥17 GE/well) who had negative AF cultures and those with positive AF cultures; and (v) PCR/ESI-MS detected genomic material from viruses in two patients (1.4%).

Conclusion

(i) Rapid diagnosis of intra-amniotic infection is possible using PCR/ESI-MS; (ii) the combined use of biomarkers of inflammation and PCR/ESI-MS allows for the identification of specific bacteria and viruses in women with preterm labor and intra-amniotic infection; and (iii) this approach may allow for administration of timely and specific interventions to reduce morbidity attributed to infection-induced preterm birth.

Introduction

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide.[1–6] Microbial invasion of the amniotic cavity (MIAC), determined with cultivation techniques, occurs in one of every four women who deliver preterm;[7–43] yet, this may be an underestimation because the diagnosis of MIAC represents a challenge.

At first, transcervical catheters were used to evaluate the microbial state of the amniotic cavity;[44–49] however, this approach was abandoned because of the problem of contamination. Subsequently, amniocenteses for the retrieval of amniotic fluid (AF) for culture became the 'gold standard' for the diagnosis of MIAC.[10,11,15,16,19,24,42,50–66] A major challenge using this approach is that culture results take several days to become available. This delay has led to the standard practice of initiating intravenous antimicrobial agents for patients with a positive Gram stain or indicators of inflammation prior to identifying the microorganism responsible for a suspected infection.[67–75] The latter assumes that inflammation is always due to infection, and this is not the case.[76–80]

Rapid tests are needed to determine whether and which antimicrobial agents should be administered. Other decisions, such as the administration or discontinuation of tocolysis or timing of the delivery, also depend on the availability of reliable information about the presence of microorganisms in the amniotic cavity. The use of polymerase chain reaction (PCR)-based techniques for the diagnosis of infectious diseases can provide results in time for rapid decision-making.[81–88] Moreover, these techniques can improve the detection of microbes difficult to culture,[34,39,42,65,89–102] as well as provide information about antimicrobial resistance and virulence factors.[85,103–105] It is unknown whether these techniques can be used at the point of care to inform treatment decisions in an obstetrical setting.

In this study of patients with spontaneous preterm labor and intact membranes (PTL), AF culture was compared to a new technique which combines broad-range real-time PCR with electrospray ionization time-of-flight mass spectrometry (PCR/ESI-MS).[106–114] This technique is capable of identifying the genus and species of microorganisms in AF within 8 hours and, therefore, in time for clinical decisions.[106,107,109–114] PCR/ESI-MS has been validated [106,114–134] and has the potential to revolutionize the detection of infectious agents in modern obstetrics. The study objectives were to: (i) compare the performance of AF culture and PCR/ESI-MS in identifying MIAC in patients with PTL; (ii) assess the magnitude of intra-amniotic inflammation in patients with MIAC identified by PCR/ESI-MS; and (iii)

examine the relationship between MIAC detected by PCR/ESI-MS and adverse pregnancy outcome.

Materials and methods

Study Population

A cohort study was conducted by searching the clinical database and bank of biologic samples of Wayne State University, the Detroit Medical Center, and the Perinatology Research Branch of the *Eunice Kennedy Shriver* National Institutes of Child Health and Human Development (NICHD) (Detroit, MI), to identify patients with a diagnosis of spontaneous PTL. Patients were included if they met the following criteria: (i) had a singleton gestation; (ii) presented with PTL; and (iii) had a transabdominal amniocentesis performed between 20 and 35 weeks of gestation with microbiologic studies. Patients were excluded from the study if: (i) rupture of the chorioamniotic membranes occurred before AF collection; or (ii) a chromosomal or structural fetal anomaly was present. Perinatal mortality was defined as the occurrence of fetal or neonatal death.

All patients provided written informed consent; the use of biologic specimens and clinical data for research purposes was approved by the Institutional Review Boards of NICHD and Wayne State University.

Sampling Procedures

Patients with preterm labor and intact membranes who underwent transabdominal ultrasound-guided amniocentesis for evaluating possible MIAC (within the standard of care at Hutzel Women's Hospital, Detroit, Michigan) were eligible for the study. AF was immediately transported in a capped sterile syringe to the clinical laboratory where it was cultured for aerobic and anaerobic bacteria, including genital mycoplasmas. Evaluation of white blood cell (WBC) count, glucose concentration, and Gram stain of AF were also performed shortly after collection. AF not required for clinical assessment was centrifuged for 10 min at 4°C shortly after the amniocentesis, and the supernatant was aliquoted and stored at −70°C until analysis. The presence of intra-amniotic inflammation was assessed by determination of AF interleukin-6 (IL-6) concentration by ELISA. AF IL-6 concentrations were determined for research purposes, and such results were not used in patient management.

Detection of Microorganisms with Cultivation and Molecular Methods

AF was analyzed using cultivation techniques (aerobic, anaerobic, and genital mycoplasmas) as well as PCR/ESI-MS (Ibis® Technology - Athogen, Carlsbad, CA, USA). Briefly, DNA was extracted from 300 μL of AF using a method that combines bead-beating cell lysis with a magnetic-bead-based extraction method.[135,136] The extracted DNA was amplified by the previously described broad bacteria and candida (BAC) detection assay according to the manufacturer's instructions. PCR/ESI-MS can identify 3400 bacteria and 40 Candida spp., which are represented in the platform's signature database.[112,114,137] For viral detection, the nucleic acids were extracted from 300 μL of AF using a method that combined chemical lysis with a magnetic-bead based extraction method. The extracted RNA/DNA was amplified on the broad viral assay according to the manufacturer's instructions. In the 8 wells, there were fourteen primer pairs used to detect the following viruses: *Herpes simplex virus 1* (*HHV-1*), *Herpes simplex virus 2* (*HHV-2*), *Varicella-zoster virus* (*HHV-3*), *Epstein-Barr virus* (*HHV-4*), *Cytomegalovirus* (*HHV-5*), *Kaposi's sarcoma-associated herpes virus* (*HHV-8*), human adenoviruses, human enteroviruses, *BK polyomavirus, JC polyomavirus,* and *Parvovirus B19*.[137]

After PCR amplification, 30-μL aliquots of each PCR product were desalted and analyzed via electrospray ionization time-of-flight mass spectrometer (ESI-MS) as previously described.[112,116] The presence of microorganisms was determined by signal processing and triangulation analysis of all base composition signatures obtained from each sample and compared to a database. Along with organism identification, the ESI-MS analysis includes a Q-score and level of detection (LOD). The Q-score, a rating between 0 (low) and 1 (high), represents a relative measure of the strength of the data supporting identification; only Q-scores ≥ 0.90 were reported for the BAC spectrum assay.[132] The LOD describes the amount of amplified DNA present in the sample: this is calculated with reference to an internal calibrant, as previously described[108] and is reported herein as genome equivalents per PCR well (GE/well). The bacterial/viral genome load per mL of AF (GE/mL) is equal to the GE/well multiplied by 133.33. The sensitivity (LOD) of the assay for the detection of bacteria in blood is, on average, 100 CFU/mL (95% CI, 6–600 CFU/mL).[114] A comparison of detection limits

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between blood and AF show that the assays have comparable detection limits (100 CFU/mL). The sensitivity (LOD) for the broad viral in plasma ranges from 400 copies/mL to 6600 copies/mL.[138] A comparison of detection limits between AF and plasma was performed by spiking known amounts of a DNA virus (HHV-5) and an RNA virus (human enterovirus) into AF and plasma. Detection limits in AF were similar to plasma, ranging from approximately 800 to 1600 copies/mL (depending upon the specific microorganism).

Determination of IL-6 in Amniotic Fluid

AF concentrations of IL-6 were determined to assess the magnitude of the intra-amniotic inflammatory response. We used a sensitive and specific enzyme immunoassay obtained from R&D Systems (Minneapolis, MN, USA). Briefly, the immunoassay utilized the quantitative sandwich enzyme immunoassay technique, and the concentrations were determined by interpolation from the standard curves. The inter- and intra-assay coefficients of variation for IL-6 were 8.7 and 4.6%, respectively. The detection limit of the IL-6 assay was 0.09 pg/mL.

Clinical Definitions

Preterm labor was diagnosed 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. Preterm delivery was defined as birth prior to the 37th week of gestation. Acute histologic chorioamnionitis was diagnosed based on the presence of neutrophils in the chorionic plate and/or chorioamniotic membranes.[139–141] Intra-amniotic inflammation was diagnosed when IL-6 AF concentration was $\geq$ 2.6 ng/mL.[41,142] MIAC was defined according to the results of AF culture and PCR/ESI-MS analysis.[99,101,143,144] Intra-amniotic infection was defined as a combination of MIAC with intra-amniotic inflammation.

Statistical Analysis

The Kolmogorov–Smirnov test and visual plot inspection were used to assess the normality of continuous data distributions. Spearman's nonparametric correlation coefficients were calculated. The Kruskal–Wallis and Mann–Whitney *U*-tests were used to test for differences in arithmetic variable distributions. The chi-square test or Fischer's exact test was used to test for differences in proportions, as appropriate. Quantile regression models were fit to test for median differences adjusting for potential confounders, including gestational age and cervical dilatation at amniocentesis. Kaplan–Meier survival curves were plotted, and the Mantel-Haenszel log-rank test was used to test for differences in time-to-spontaneous preterm delivery. Logistic and Cox proportional hazards regression models were fit to examine magnitudes of association. A cutoff for classifying PCR/ESI-MS results according to the microbial inoculum size (genome copies/well) in each sample was selected upon inspection of a receiver operating characteristic (ROC) curve for the identification of intra-amniotic inflammation among patients with detectable gene copies by PCR/ESI-MS. For all analyses, a two-tailed P value < 0.05 was considered significant. SPSS v.15.0 (SPSS, Chicago, IL, USA) was used to analyze the data.

Results

Characteristics of the Study Population

Baseline characteristics of the study population are displayed in Table I. The median [interquartile range (IQR)] gestational age at amniocentesis was 30 (IQR: 25–32) weeks. The median gestational age at delivery was 34 (IQR: 27–37) weeks. Extraplacental membranes were examined for 92% (131/142) of the study population. The prevalence of acute histologic chorioamnionitis was 41% (53/131).

The Prevalence of MIAC and Microbial Diversity

The prevalence of MIAC based on a positive AF culture was 7% (10/142). PCR/ESI-MS detected amplified DNA in 21% (30/142) of the AF samples (Table I). Upon inspection of a ROC curve for the identification of intra-amniotic inflammation (IL-6 $\geq$ 2.6 ng/mL) among patients with detectable DNA from bacteria and/or virus using PCR/ESI-MS (Fig. 1), results were classified according to microbial burden as positive or negative. Positive tests had 17 or more GE/well, whereas negative tests had fewer than 17 GE/well, or genomic material was undetected. Figure 2 shows a Venn diagram describing the combined results of AF culture and PCR/ESI-MS.

Table I Maternal Characteristics and Demographic Data of the Study Population

	Median (IQR) or % (*n*/*N*)
Maternal age (years)	23 (20–26.3)
Body mass index (kg/m^2)	24 (21–30)
Cervical dilatation at admission (cm)	3 (2.0–3.5)
AF glucose (mg/dL)	25 (19–31)
AF white blood cell count (cells/mm^3)	2 (0–12)
GA at amniocentesis (weeks)	30 (25–32)
GA at delivery (weeks)	34 (27–37)
Birth weight (grams)	2190 (930–2700)
AF culture positive	7 (10/142)
Detection of bacteria and/or virus genome by PCR/ESI-MS	21 (30/142)
Positive PCR-ESI-MS (GE/well ≥ 17)	12 (17/142)

AF, amniotic fluid; GA, gestational age; GE/well, genome equivalent per PCR; IQR, interquartile range; PCR, polymerase chain reaction; ESI-MS, electrospray ionization mass spectrometry.

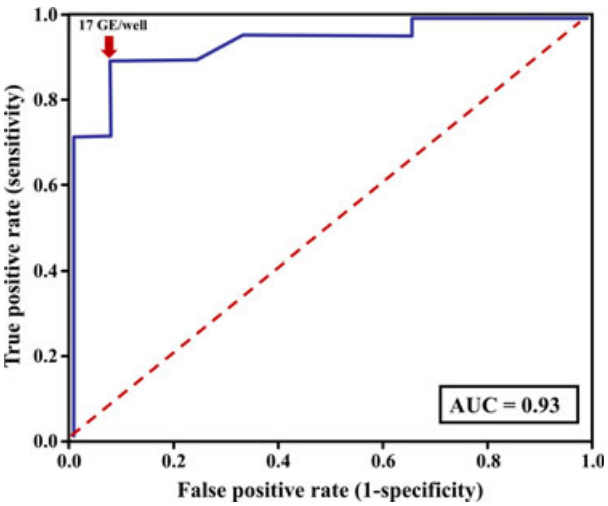

Fig. 1 Receiver operator characteristic curve analysis of PCR/ESI-MS genome equivalents per PCR well (GE/well) results for the identification of intra-amniotic inflammation (amniotic fluid IL-6 ≥ 2.6 ng/mL) among patients with detectable DNA of bacteria and/or viruses. A GE/well ≥17 had an area under the ROC curve of 0.93 (95% CI 0.83–1.0; $P < 0.001$).

Seven of the 17 patients with positive PCR/ESI-MS (>17 GE/well) had positive AF cultures.

For each patient with detectable genomic material by PCR/ESI-MS or positive AF cultures, the microorganisms identified, concentrations of inflammatory markers in amniotic fluid, gestational age at delivery, and the presence or absence of acute histologic chorioamnionitis, are shown in Table II. The most frequent microorganism identified by PCR/ESI-MS was *Ureaplasma parvum* (instead of *Ureaplasma urealyticum*, which was the most common microorganism identified by standard AF culture). *Fusobacterium nucleatum, Gardnella vaginalis, Mycoplasma hominis,* and *Acinetobacter junii* were only detected by PCR/ESI-MS. Two patients (1.4%) had a positive viral assay for *human enterovirus* using PCR/ESI-MS. One of these two patients also had genomic material consistent with *Acinetobacter junii*.

The Relationship Between the Presence and Burden of Microorganisms in the Amniotic Fluid and Intra-Amniotic Inflammation

The microbial inoculum size, expressed as GE/well, was significantly correlated with AF concentration of IL-6 and the AF WBC count [Spearman's rho (ρ) = 0.63, $P < 0.001$ and ρ = 0.67, $P < 0.0001$]. Exclusion of patients with positive AF cultures did not alter these findings [AF IL-6: ρ = 0.61 $P = 0.002$ and AF WBC: ρ = 0.51, $P = 0.01$].

Figure 3a,b display AF concentrations of IL-6 and AF WBC count, respectively, among four study groups according to results of PCR/ESI-MS and AF cultures. The first group had a positive AF culture and a positive PCR/ESI-MS (≥17 GE/well) ($n = 7$). The second group included patients with positive PCR/ESI-MS (≥17 GE/well) who had negative AF cultures ($n = 10$). The third and fourth groups included patients with negative PCR/ESI-MS (<17 GE/well) who had negative ($n = 122$) or positive ($n = 3$) AF cultures, respectively.

Among patients with positive PCR/ESI-MS (≥17 GE/well), there was no significant difference in the median AF IL-6 concentration between those with ($n = 7$) and without ($n = 10$) positive AF cultures [185.4 (IQR: 30–275.4) versus 87 (IQR: 11.3–264) ng/mL, $P = 0.43$] (Fig. 3a). The median AF IL-6 concentration was significantly higher in patients with positive PCR/ESI-MS results (≥ 17 GE/well) and negative AF cultures than in: (i) women with positive AF cultures and negative PCR/ESI-MS results ($n = 3$); and (ii) patients with negative AF cultures and negative PCR/ESI-MS ($n = 122$) [87 (IQR: 11.3–264) ng/mL versus 0.2 (IQR: 0.2–0.3), and 1.1 (IQR: 0.7– 5) ng/mL, respectively, $P < 0.001$]. However, the median AF IL-6 concentration was significantly higher in patients without MIAC (by AF culture and PCR/

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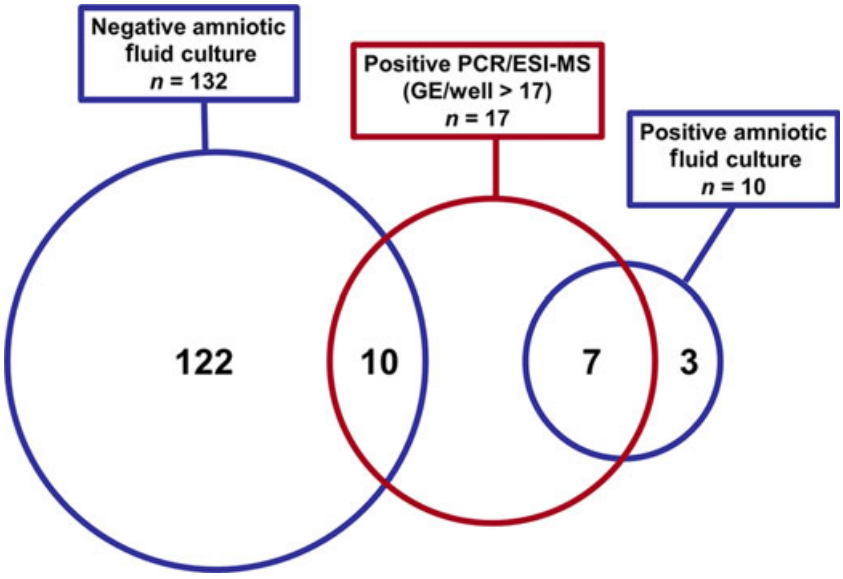

Fig. 2 Bacteria and viruses detected in amniotic fluid of patients with preterm labor using standard cultivation techniques versus PCR/ESI-MS. Amniotic fluid culture includes routine cultivation techniques for bacteria (aerobes, anaerobes, and genital mycoplasmas). PCR/ESI-MS refers to broad-range polymerase chain reaction (PCR) and electrospray ionization time-of-flight mass spectrometry (ESI-MS).

ESI-MS) than in patients with positive AF culture and negative PCR/ESI-MS ($P = 0.009$) (Fig. 3a).

Differences in the median AF WBC count between the four groups were consistent with those of AF IL-6. There were no differences in the median WBC count between patients with positive PCR/ESI-MS (≥ 17 GE/well) who had positive ($n = 7$) or negative ($n = 10$) AF cultures [490 (IQR: 50–1920) versus 185 (IQR: 0.7–1043) cell/mm^3, $P = 0.28$]. The median AF WBC count among these two study groups was significantly greater than the median AF WBC count of the two study groups with negative PCR/ESI-MS (<17 GE/well), irrespective of the AF culture results ($P < 0.0001$) (Fig. 3b). Differences in the median AF IL-6 and WBC concentrations between the four groups were consistent both in direction and statistical significance when fitting a quantile regression model to adjust for potential confounders, including gestational age and cervical dilatation at amniocentesis [data not shown].

Figure 4a shows the prevalence of intra-amniotic inflammation (IL-6 ≥ 2.6 ng/mL) according to the results of AF culture and PCR/ESI-MS. Intra-amniotic inflammation was diagnosed in 70% ($n = 7/10$) and 94% ($n = 16/17$) of patients with positive AF culture and a positive PCR/ESI-MS (≥17 GE/well) results, respectively. Accordingly, these patients had intra-amniotic infection (i.e. MIAC and intra-amniotic inflammation).

All but one patient with a positive PCR/ESI-MS (≥ 17 GE/well) and a negative AF culture had intra-amniotic inflammation [90%, (9/10)]. These patients were significantly more likely to have intra-amniotic inflammation than women with negative tests, adjusting for gestational age and cervical dilatation at amniocentesis [odds ratio (OR) 12.2, 95% confidence interval (CI) 1.5–97] (Table III). None of the three women with positive AF cultures and negative PCR/ESI-MS had intra-amniotic inflammation; 32% (39/122) of the patients with negative results for both tests (AF culture and PCR/ESI-MS), which are considered 'sterile' samples, had intra-amniotic inflammation (Fig. 4a).

The Relationship Between Detectable Microorganisms in the Amniotic Fluid and Acute Histologic Chorioamnionitis

Figure 4b shows the prevalence of acute histologic chorioamnionitis among the four groups, according to the results of PCR/ESI-MS and AF culture. Each of the seven patients with positive AF cultures and positive PCR/ESI-MS results (GE/well ≥ 17) had acute histologic chorioamnionitis. This lesion was diagnosed in 70% (7/10) of patients with a positive PCR/ESI-MS (≥ 17 GE/well) and negative AF cultures. These patients were significantly more likely to have acute histologic chorioamnionitis than women with negative tests, adjusting for gestational age and cervical dilatation at amniocentesis [OR 5.1,

Table II Amniotic Fluid Inflammatory Profile, Delivery Information, Placenta Pathology, and Perinatal Mortality in Patients with Microbial Invasion of

		Amniotic Fluid Culture and PCR/ESI-MS Results					Amniotic fluid (AF) test results	
Patient number	Group	AF culture	PCR/ESI-MS bacteria	GE/well bacteria	PCR/ESI-MS viruses	GE/well viruses	AF-IL6 (ng/mL)	Intra-amniotic inflammation
1	Positive AF Culture and positive PCR/ESI-MS (n = 7)	*Ureaplasma spp*	*Ureaplasma parvum*	575	Not detected	0	29.8	Yes
2		*Candida albicans*	*Candida albicans*	215	Not detected	0	201.3	Yes
3		*Ureaplasma spp*	*Ureaplasma parvum* *Fusobacterium nucleatum*	129 6	Not detected	0	275.5	Yes
4		*Ureaplasma spp*	*Ureaplasma parvum*	1795	Not detected	0	86	Yes
5		*Streptococcus*	*Gardnerella vaginalis* *Mycoplasma hominis*	69 83	Not detected	0	185.5	Yes
6		*Ureaplasma urealyticum*	*Ureaplasma urealyticum*	1484	Not detected	0	9.9	Yes
7		*Bacteroides*	*Fusobacterium nucleatum*	20	Not detected	0	517.8	Yes
8	Negative AF culture and GE/Well ≥ 17 (n = 10)	Negative	Not detected	0	*Human Enterovirus*	>1000	6.0	Yes
9		Negative	*Fusobacterium nucleatum*	27	Not detected	0	46.8	Yes
10		Negative	*Ureaplasma urealyticum*	768	Not detected	0	265.5	Yes
11		Negative	*Acinetobacter junii*	68	*Human Enterovirus*	24	77.7	Yes
12		Negative	*Acinetobacter junii*	41	Not detected	0	0.28	No
13		Negative	*Sneathia species*	280	Not detected	0	290.7	Yes
14		Negative	*Pseudomonas entomophila/putida*	23	Not detected	0	13.1	Yes
15		Negative	*Ureaplasma parvum*	571	Not detected	0	118.5	Yes
16		Negative	*Candida albicans*	57	Not detected	0	96.3	Yes
17		Negative	Sneathia species	152	Not detected	0	263.3	Yes
18	Negative AF culture and GE/Well < 17 (n = 13)	Negative	*Aeromonas caviae*	3	Not detected	0	0.6	No
19		Negative	*Acinetobacter junii*	14	Not detected	0	1.6	No
20		Negative	*Moraxella osloensis*	3	Not detected	0	1.0	No
21		Negative	*Staphylococcus aureus*	5	Not detected	0	0.6	No
22		Negative	*Staphylococcus aureus*	7	Not detected	0	1.6	No
23		Negative	*Acidovorax sp.*	11	Not detected	0	0.4	No
24		Negative	*Streptococcus species*	3	Not detected	0	1.0	No
25		Negative	*Lactobacillus acidophilus/crispatus*	5	Not detected	0	2.3	No
26		Negative	*Ureaplasma parvum*	8	Not detected	0	0.2	No
27		Negative	*Gardnerella vaginalis*	5	Not detected	0	16.5	Yes
28		Negative	*Ureaplasma parvum*	4	Not detected	0	0.2	No
29		Negative	Staphylococcus aureus	8	Not detected	0	51.8	Yes
30		Negative	*Pantoea dispersa*	3	Not detected	0	0.9	No
31	Positive AF culture and Negative PCR/ESI-MS (n = 3)	*Ureaplasma urealyticum*	Not detected	0	Not detected	0	0.2	No
32		*Ureaplasma urealyticum*	Not detected	0	Not detected	0	0.4	No
33		Staphylococcus aureus	Not detected	0	Not detected	0	0.2	No

GA, gestational age; AF, amniotic fluid; WBC, white blood cells; IL-6, interleukin-6; PCR, polymerase chain reaction; ESI-MS, electrospray
Severity of acute histologic chorioamnionitis: Stage 1 – Early: Acute Subchorionitis/Chorionitis; Stage 2 – Intermediate: Acute Chorioamnionitis;

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the Amniotic Cavity According to Amniotic Fluid Cultures versus GE/Well Results Using PCR/ESI-MS

			Delivery information			Histologic Placenta Lesions		
AF Gram stain	AF Glucose	AF WBC	GA at amniocentesis (weeks)	GA at delivery (weeks)	Interval-amniocentesis-to delivery (days)	Acute histologic chorioamnionitis	Severity of histologic chorioamnionitis	Perinatal mortality
Neg	10	490	32.4	32.6	1	Yes	2	No
Neg	10	2160	26.3	26.4	1	Yes	3	No
Pos	ND	50	24.9	26.0	8	NI	NI	Yes
Neg	10	500	32.9	33.0	1	Yes	2	No
Neg	10	1920	31.7	31.9	1	Yes	3	No
Pos	20	10	32.1	32.7	4	Yes	2	No
Neg	10	295	22.6	22.6	0	Yes	3	Yes
Neg	21	0	21.6	21.7	1	No	No	Yes
Neg	24	1	25	25.7	5	Yes	4	Yes
Neg	11	357	20.4	20.4	0	Yes	3	Yes
Neg	20	0	24.6	24.7	1	Yes	1	No
Neg	30	2	29.1	39.9	75	No	No	No
Pos	10	440	26	26.0	0	Yes	3	No
Neg	17	13	33.9	33.9	0	No	No	No
Neg	10	2750	26.7	27.0	2	Yes	3	No
Pos	10	1292	32.4	32.6	1	Yes	2	No
Pos	10	960	23.6	23.6	0	Yes	4	Yes
Neg	ND	10	30.1	30.1	0	No	No	No
Neg	30	3	32.1	33.6	10	No	No	No
Neg	29	8	32.4	39.9	52	No	No	No
Neg	30	0	33.3	37.6	30	No	No	No
Neg	10	0	34.1	34.9	5	No	No	No
Neg	29	20	27	27.3	2	No	No	No
Neg	10	0	34.1	36.9	19	Yes	2	No
Neg	25	1	28.1	39.7	81	Yes	2	No
Neg	31	0	22.3	25.3	21	Yes	3	No
Neg	52	0	28.4	35.4	49	No	No	No
Neg	22	2	26.4	27.0	4	No	No	No
Neg	25	5	29.7	33.9	29	No	No	No
Neg	54	2	27.4	28.3	6	No	No	No
Neg	24	0	34.6	35.1	4	Yes	1	No
Neg	19	2	29.1	39.3	71	No	No	No
Neg	20	225	31	36.9	41	No	No	No

ionization mass spectrometry; GE/well, genome equivalent per PCR; NI, not information.
Stage 3 – Necrotizing Chorioamnionitis; Stage 4 – Subacute Chorioamnionitis.

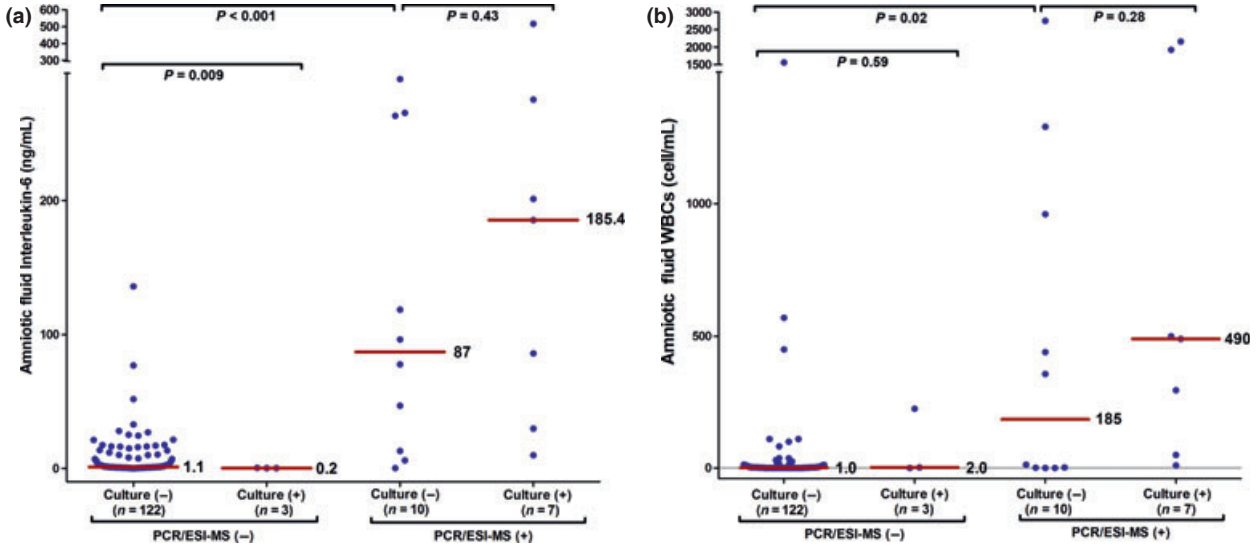

Fig. 3 Markers of infection in amniotic fluid according to the results of standard AF culture and PCR/ESI-MS. Amniotic fluid concentrations of interleukin-6 (Panel a) and white blood cell (WBCs) count (Panel b) as a function of PCR/ESI-MS and AF culture results. There was no significant difference in the median AF IL-6 concentration and WBC count between patients with a negative AF culture and a positive PCR/ESI-MS (GE/well ≥17) and those with a positive AF culture and positive PCR/ESI-MS (P = 0.43 and 0.28, respectively). Among patients with a negative AF culture, those with a positive PCR/ESI-MS had a significantly higher median AF IL-6 concentration and WBC count than patients with both tests negative (P < 0.001 and P = 0.02, respectively). The median AF IL-6 concentration in patients with a positive AF culture and negative PCR/ESI-MS (GE/well < 17) was always < 2.6 ng/mL.

95% CI 1.01–26, respectively] (Table III). One of the three women with a positive AF culture and a negative PCR/ESI-MS had mild acute histologic chorioamnionitis (stage 1 subchorionitis/chorionitis) (Fig. 4b).

The observations described in the previous paragraph, combined with the parameters assessing intra-amniotic inflammation, reveal that a positive AF culture among patients with negative PCR/ESI-MS results likely reflect either early-phase microbial invasion, in which an inflammatory response is not detectable, or contamination of the specimen (false-positive culture).

The Presence of Microorganisms in the Amniotic Fluid by Molecular Techniques and the Risk of Spontaneous Preterm Delivery

Table IV presents the positive predictive values (PPV) of AF culture and positive PCR/ESI-MS (≥ 17 GE/well) for the identification of patients who delivered spontaneously prior to 37 and 32 weeks, respectively. The PPV of a positive AF culture for spontaneous delivery before 37 and 32 weeks was 90 and 66.7%, respectively. In contrast, the PPV of a positive PCR/ESI-MS (≥ 17 GE/well) was 94.1 and 91.7% for the same outcomes.

Presence and Burden of Microorganisms in Amniotic Fluid and the Interval to Spontaneous Preterm Delivery

Kaplan–Meier survival estimates, censoring patients in whom labor was induced for maternal or fetal indications, showed that the amniocentesis-to-delivery interval was significantly shorter in patients with positive PCR/ESI-MS results (≥ 17 GE/well) and negative AF culture than in patients with negative results for both tests [1 (IQR: <1–2) days versus 25 (IQR: 5–51) days; P = 0.002]. There were no significant differences in the median amniocentesis-to-delivery interval between patients who had positive PCR/ESI-MS results (≥ 17 GE/well) with negative AF cultures [1 (IQR: <1–2) days] and those patients with positive findings for both tests [1 (IQR: 1–8) days] (P = 0.6) (Fig. 5).

Figure 6 shows a forest plot of the hazard ratios (HR) describing the relative risks of spontaneous preterm delivery (sPTD) for patients with positive PCR/ESI-MS (≥ 17 GE/well) and/or AF cultures

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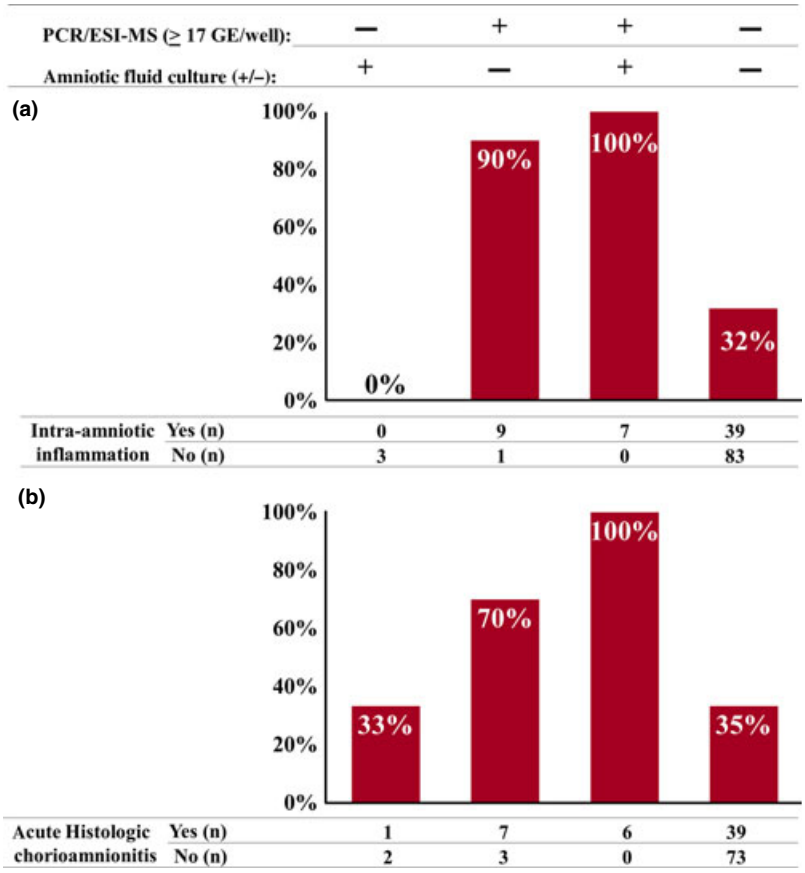

Fig. 4 Prevalence of intra-amniotic inflammation (Panel a) and acute histologic chorioamnionitis (Panel b) according to the results of PCR/ESI-MS and amniotic fluid cultures. Intra-amniotic inflammation was diagnosed in 70% (n = 7/10) and 94% (n = 16/17) of patients with a positive AF culture and positive PCR/ESI-MS (≥17 GE/well) results, respectively. Patients with positive PCR/ESI-MS (GE/well ≥ 17) and negative AF culture had a 70% prevalence of acute histologic chorioamnionitis of 70% (7/10). In contrast, the prevalence of acute histologic chorioamnionitis in patients with negative AF culture and negative PCR/ESI-MS (GE/well < 17) was 35% (39/112).

compared to with negative tests, adjusted for gestational age at amniocentesis and cervical dilatation. The risks (i.e., hazard) of sPTD at <37 weeks and, separately, <32 weeks were significantly greater among patients with positive PCR/ESI-MS results (≥ 17 GE/well), both with and without positive AF cultures, when compared to women with negative tests [sPTD <37: AF+, HR 3.9 (95% CI 1.7–9.1) and AF-, HR 3.8 (95% CI 1.8–8.1); sPTD <32: AF+, HR 3.3 (95% CI 1.1–10.1) and AF-, HR 5.2 (95% CI 2–13.4)].

Detection of Microbial Invasion of the Amniotic Cavity Using Cultivation and Molecular Techniques and the Risk of Perinatal Death

The prevalence of perinatal death (fetal and neonatal death) differed significantly between the four study groups defined by the results of AF culture and PCR/ESI-MS (P = 0.03) (Fig. 7). Twenty percent of perinatal death (4/19) occurred in the 7% (10/142) of women with positive PCR/ESI-MS and negative AF culture results. Offspring of these women were at a fivefold greater risk of perinatal mortality than those of women with negative AF culture and negative PCR/ESI-MS results (OR 5.6; 95% CI 1.4–22). There was no difference in the rate of perinatal death for the offspring of women with positive PCR/ESI-MS according to the results of AF culture [positive culture 29% (2/7) versus negative culture 40% (4/10); P = 1.0].

Comment

Principal findings of the study were as follows: (i) the prevalence of MIAC in patients with preterm labor and intact membranes was 7% using standard cultivation techniques and 12% using PCR/ESI-MS; (ii) patients with positive PCR/ESI-MS (≥17 GE/well) and negative AF cultures had higher rates of intra-amniotic inflammation and acute histologic chorioamnionitis, a shorter interval to delivery, and their offspring were at greater risk of perinatal mortality than patients with negative results for both

Table III Magnitudes of Association Among Combined Test Results and Intra-Amniotic Inflammation and acute Histologic Chorioamnionitis

	Intra-amniotic inflammation			Acute Histologic chorioamnionitis		
	OR	95% CI		OR	95% CI	
Unadjusted						
PCR/ESI-MS (+) – AF (+)	31.7	1.5	691	25.8	1.1	591
PCR/ESI-MS (+) – AF (–)	13.4	2.1	85	4.3	1.1	17
PCR/ESI-MS (–) – AF (+)	0.3	0.01	9	1.2	0.1	13
PCR/ESI-MS (–) – AF (–)	1	Reference		1	Reference	
Adjusted[a]						
PCR/ESI-MS (+) – AF (+)	47.8	1.8	>999	32.6	1.3	788
PCR/ESI-MS (+) – AF (–)	12.2	1.5	97	5.1	1.01	26
PCR/ESI-MS (–) – AF (+)	0.9	0.02	35	1.7	0.2	20
PCR/ESI-MS (–) – AF (–)	1	Reference		1	Reference	

AF, amniotic fluid; GE/well, genome equivalent per well; PCR, polymerase chain reaction; ESI-MS, electrospray ionization mass spectrometry.
[a]Adjusted for cervical dilation and gestational age at amniocentesis; Firth's penalized maximum likelihood estimation was performed where necessary to resolve separation issues.

tests; (iii) there were no differences in these factors when comparing patients with positive PCR/ESI-MS (≥17 GE/well) who had positive AF cultures to those with negative AF cultures; and iv) genomic material from viruses was detected in two patients [1.4% (2/142)]. Altogether, these findings indicate that PCR/ESI-MS can be used as a rapid test to diagnose MIAC.

Microbial Invasion of the Amniotic Cavity

A key transition in human life is the emergence from a sterile to a non-sterile environment at the time of birth.[145] The AF is sterile under normal circumstances as demonstrated by studies with cultivation and molecular methods.[43,62,146–154] In contrast, 25–40% of patients with preterm labor and intact membranes who deliver preterm have evidence of MIAC,[7–43,155,156] whereas 50–75% of women with pre-labor rupture of membranes (PROM) have MIAC.[25,53,101,156]

The prevalence of MIAC is a function of the obstetrical circumstances that prompted investigation of the microbial state in the amniotic cavity. For example, the earlier the gestational age at presentation with preterm labor with intact membranes or preterm PROM, the higher the frequency of MIAC.[20,26,41,99,101,157–159] In PROM, the frequency of MIAC increases from 39% (24/61) in patients not in labor to 75% (36/48) with the onset of spontaneous preterm labor ($P = 0.0004$).[53] These data were obtained before routine administration of antibiotics to patients with preterm PROM and are the basis for the clinical view that the onset of labor in these women is frequently related to subclinical intra-amniotic infection.

The prevalence of MIAC ranges from 10–12% in patients with preterm labor and intact membranes[9–16,20–27,30–37,61] to 51% in pregnant women diagnosed with acute cervical insufficiency (Table V).[160–163] These cultivation-based studies provided strong evidence for the involvement of microorganisms in pregnancy complications. Yet, cultivation techniques can only provide a minimum estimate of the prevalence of MIAC.[39,99,156,164165]

Molecular Techniques for the Identification of Microorganisms

Molecular detection of microorganisms has been proposed to be a 'diagnostic tool for the new millennium'.[85,166,167] In the United States 5 million cases of infectious disease-related illness are reported annually, and many more are considered to be undiagnosed.[168] Accordingly, the application of molecular testing to acute care settings is of major interest.

Identification and culture of bacteria from AF has been considered the 'gold standard' for the diagnosis of MIAC.[10,11,15,16,19,24,42,50–66] However, successful cultivation of an organism requires knowledge of the conditions necessary to support growth of specific microorganisms.[169] These conditions are often

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Table IV Positive Predictive Value (PPV) of GE/Well Reported by PCR/ESI-MS and AF Cultures for the Identification of Adverse Pregnancy Outcomes

	sPTD < 37 weeks	sPTD < 32 weeks	Delivery within 7 days of amniocentesis	Delivery within 2 days of amniocentesis
Prevalence	79.6% (113/142)	52.6% (50/95)	37.3% (53/142)	21.1% (30/142)
PPV of positive culture	90% (9/10)	66.7% (4/6)	70% (7/10)	50% (5/10)
PPV of PCR/ESI/MS (GE/well ≥ 17)	94.1% (16/17)	91.7% (11/12)	88.2% (15/17)	76.5% (13/17)

AF, amniotic fluid; PCR, polymerase chain reaction; ESI-MS, electrospray ionization mass spectrometry; GE/well, genome equivalent per PCR.

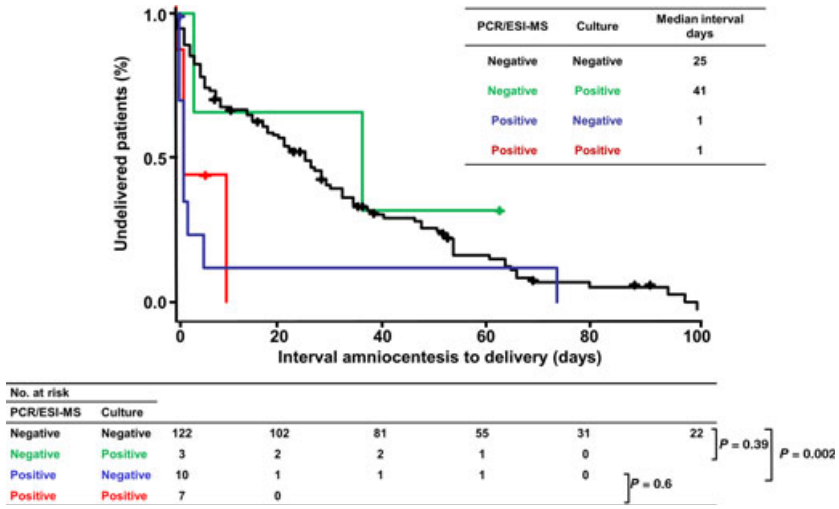

Fig. 5 Kaplan–Meier survival analysis of amniocentesis-to-delivery interval (days) according to amniotic fluid cultures and PCR/ESI-MS results. Patients in whom labor was induced were censored and are represented by crosses. Among patients with negative amniotic fluid cultures, the amniocentesis-to-delivery interval was significantly shorter in patients with a positive PCR/ESI-MS than in those with a negative PCR/ESI-MS result [1 (IQR: < 1–2) days versus 25 (IQR: 5–51) days; $P = 0.002$]. There were no significant differences in the median amniocentesis-to-delivery interval between patients who had a positive PCR/ESI-MS with a negative AF culture [1 (IQR: < 1–2) days] and patients with both tests positive [1 (IQR: 1–8) days] ($P = 0.6$).

unknown, rendering standard techniques inadequate in detecting many organisms implicated in human diseases.[170–174] For example, *Treponema pallidum* has remained difficult to culture for decades.[175,176] In addition, cultivation methods are prone to false-negative results when antibiotics are administered before the biological fluid is obtained for culture.[67–75] Moreover, the results generally take days and are often not available in time to make informed clinical decisions (e.g., antibiotic administration).

The realization that only a small fraction of the microbial world is readily culturable in clinical laboratories[177–181] prompted the development of PCR-based diagnostic techniques for infectious diseases.[83–88] The advantages of molecular techniques are that they allow broad-spectrum microbial detection, evaluation of emerging novel infections, assessment of antimicrobial resistance profiling,[85,103,105] virulence factors,[85,104] and their relatively low cost.[85] These methods have been used to identify agents responsible for conditions previously considered to be of unknown etiology (e.g., *Tropheryma whipplei*, the agent of Whipple's disease,[182,183] *Mycobacterium genavense*, a cause of disseminated infections in AIDS patients,[184] *Ehrlichia chaffeensis*, an agent of human tick-borne monocytic ehrlichiosis,[185] and *Bartonella henselae*, the agent of Trench fever).[186–188] However, implementation of PCR techniques in clinical practice has not been easy. Bacteria, viruses, and fungi must be investigated using separate assays, and the time required to obtain results has been lengthy.

Broad-Range PCR with ESI-MS for the Rapid Detection of Microorganisms

Broad-range PCR methods for the detection of bacteria are based on the premise that the 16S rRNA gene is evolutionarily conserved in bacterial species.[189,190] The detection of this gene in a sterile biologic fluid (such as AF) indicates the presence of bacteria. This approach has been used by many investigators to identify bacteria in AF using species-specific[34,39,42,65,89,92–97,100,191–195] or broad-range PCR.[90–92,99,101,143,144,196–199] Most of the studies

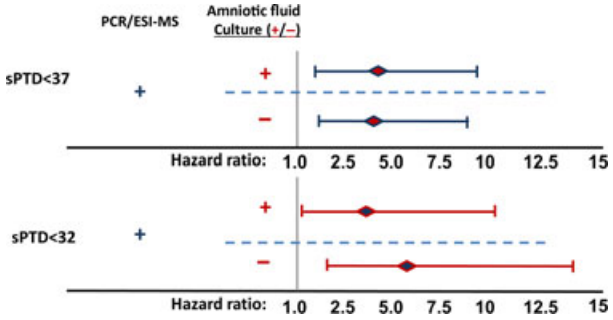

Fig. 6 Forest plot of hazard ratios for spontaneous preterm delivery (<37, <32 weeks) by PCR/ESI-MS and amniotic fluid culture results compared with patients with negative tests. The risks of spontaneous preterm delivery (sPTD) <37 and, separately, <32 weeks were significantly greater among patients with positive PCR/ESI-MS (GE/well ≥17), both with and without positive AF cultures, when compared to women with negative tests.

have shown that broad-range PCR assays and specific assays are superior to culture in the detection of microorganisms in the AF.[90–92,99,101,143,144,196–199] Yet, until recently, these techniques have remained research procedures because the time required to obtain results has precluded their use as a point-of-care test.

An advantage of the PCR/ESI-MS technology over conventional PCR methods using broad primers is that mass spectrometry allows the rapid identification of the organism.[109,111] PCR/ESI-MS results can be obtained within 8 hours.[106,112,113] The PCR/ESI-MS system is able to quantify the amount of genomic material present in a biologic sample.[108,109] Genome quantification is useful in clinical circumstances to monitor therapeutic effects. This can also be used in selecting the route of delivery (vaginal versus cesarean) in cases of HIV infection.[200–206] Other examples in which this approach has been valuable include the diagnosis/identification of visceral *leishmaniasis,*[207–209] infectious mononucleosis,[210–212] and pneumococcal pneumonia.[213,214]

PCR/ESI-MS technology uses the concept of 'triangulation' to distinguish among microorganisms. This term refers to taking measurements from multiple loci distributed across the microbial genome.[108,109] Subsequently, ESI-MS is used to rapidly determine the precise mass-to-charge ratio of the fragments amplified based upon their nucleotide composition to create a signature that allows for the identification of a large number of microorganisms[109,112] at the genus and species levels.[112,116,120–122,131]

Broad-Range PCR with ESI-MS for the Detection of Microorganisms in Amniotic Fluid Intra-Amniotic Infection

The identification of bacteria in AF is a pathological finding ('microbial invasion of the amniotic cavity').[18,24,40,57,59,60,62,143,144,160,165,215–219] The distinction between such invasion and infection is predicated on the host response to the invading microorganism(s).[23,42,43,60,149,156,157,165,216,217,219–235] Thus, intra-amniotic infection refers to the combination of MIAC with intra-amniotic inflammation.[40,41,43,162,165,218,234,236–238] In this study, MIAC was diagnosed by PCR/ESI-MS in 12% (n = 17/142) of the study population, and 94% (n = 16/17) of these patients exhibited a host inflammatory response consistent with intra-amniotic infection (AF IL-6 ≥ 2.6 ng/mL). Seventy percent (n = 7/10) of patients with positive amniotic fluid cultures had such a host response, although each of the seven cases with both tests positive met the criteria for intra-amniotic infection. Accordingly, use of PCR/ESI-MS resulted in a two-fold greater prevalence of intra-amniotic infection (5% vs 11%).

Detection of Viruses in the Amniotic Cavity

The role of viral infection in spontaneous preterm labor has not been rigorously examined. Previous studies using PCR-based methods have shown conflicting results describing the prevalence of viral invasion of the amniotic cavity (VIAC), ranging from non-detectable to as high as 6.4%.[151–153,239–241] In this study, we used PCR/ESI-MS to detect multiple organisms in the same sample (viruses and bacteria).[109] However, only two patients (1.4%) had positive results for *Enterovirus*. Gervasi et al.,[154] using specific PCR assays for a panel of viruses, reported that the prevalence of VIAC during the second trimester was 2.2%. In that study, AF samples were obtained from asymptomatic patients during the second trimester. The presence of viral nucleic acid in AF was not associated with detectable inflammation as reflected by AF WBC count, glucose and IL-6 concentrations between patients with and without VIAC.[154] Similarly, Baschat et al.[153] suggested that the isolation of viruses in the amniotic cavity was not associated with adverse maternal or perinatal outcome.

Viral infection may predispose to microbial products. Recent studies suggest that systemic viral infections may predispose pregnant mice to the

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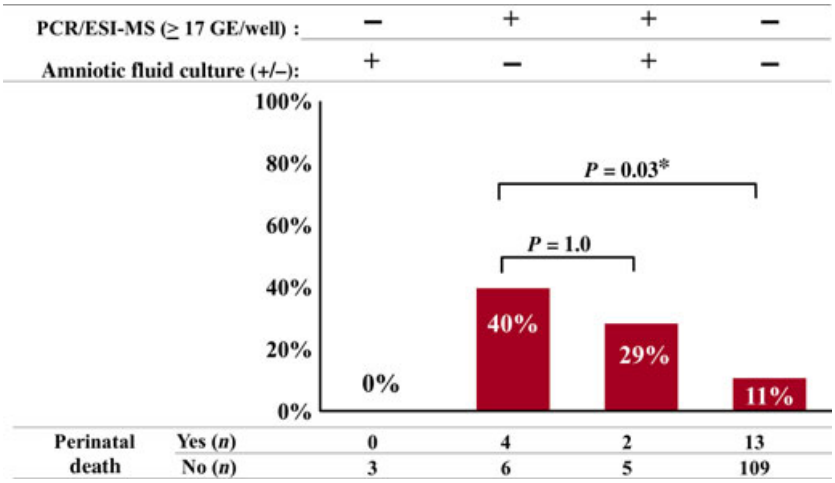

Fig. 7 Prevalence of perinatal death (fetal and neonatal deaths) by the combined results of amniotic fluid culture and PCR/ESI-MS. Patients with positive PCR/ESI-MS (≥17 GE/well) and negative AF cultures had significantly higher rates of perinatal mortality than women with both tests negative [40% (4/10) versus 11% (13/122); (P = 0.03)]. There was no difference in the rate of perinatal death for offspring of women with positive PCR/ESI-MS according to the results of AF culture [positive culture 29% (2/7) versus negative culture 40% (4/10); P = 1.0].

effect of microbial products such as endotoxin.[242] Specifically, pregnant mice infected with a *herpesvirus* were more likely to have spontaneous preterm labor and deliver following endotoxin administration than those not exposed to the virus.[242,243] Further work is required to determine whether this is a mechanism of disease operative in humans. It is well known that viral infections of the respiratory tract predispose to bacterial infections and pneumonia.[244–247] The same may be the case in the lower genital tract.[248] However, if the viral infections are not systemic (and associated with a symptomatic state), but are localized to the uterine cervix or other parts of the genital tract the identification of subclinical infection would pose an important challenge. Yet, given the high prevalence and diversity of viruses, as well as the severity of the consequences of viral predisposition to bacterial infections, this possibility deserves further study.

True-Positive, False-Negative, and False-Positive PCR/ESI-MS Results

A challenge when interpreting the results of molecular microbiologic techniques is differentiating true- from false-positive results. PCR methods can amplify extremely small quantities of microbial DNA such that contamination of a specimen may appear as a positive result.

In this study, we used the host response to microbial invasion (e.g., intra-amniotic inflammation, acute histologic chorioamnionitis, and the onset of spontaneous preterm delivery) to assess the clinical significance of microbial detection by PCR/ESI-MS. We found that the microbial burden (expressed as gene copies/well) was significantly correlated with each marker of intra-amniotic inflammation, even when excluding patients with positive AF cultures. The prevalence of intra-amniotic inflammation and acute histologic chorioamnionitis in patients with negative cultures and positive PCR/ESI-MS was 90% and 70%, respectively. Patients with positive PCR/ESI-MS results and negative AF cultures also had a significantly shorter interval to spontaneous preterm delivery than women with negative tests. Moreover, neonates born to mothers with positive PCR/ESI-MS and negative AF cultures were at five-fold greater risk of perinatal mortality than those born to mothers with negative tests. Together, these findings strongly support the conclusion that a positive PCR/ESI-MS (≥17 GE/well) result represents a true-positive finding, even when AF cultures are negative.

Bacterial cultures are considered a sensitive test for the identification of microorganisms when the conditions for supporting growth of that particular microorganism are optimal. Even one organism can form a colony in culture under ideal circumstances; yet, one organism may be insufficient for detection using PCR methods.[249] In previous studies, we identified patients with positive AF cultures who had negative PCR using broad primers and specific assays.[94,99,101,193] These results were attributed to possible DNA degradation,[250] low inoculum size,[251] etc. The observations made in the current study suggest that false-negative PCR/ESI-MS results are an extremely rare phenomenon. None of the three

Table V Microbial Invasion of the Amniotic Cavity (MIAC) in Obstetric Disorders as Determined by Amniotic Fluid Studies Obtained by Transabdominal Amniocentesis Using Cultivation Techniques

		Prevalence of MIAC	
Obstetric disorders	Authors	%	(*n*/*N*)
Spontaneous labor at term with intact membranes	Romero et al.[62]	18.8	(17/90)
	Gomez et al.[63]	15.6	(14/90)
	Yoon et al.[149]	6.3	(12/190)
Preterm labor with intact membranes	Miller et al.[9]	20	(15)
	Bobbit et al.[11]	25	(8/31)
	Wallace and Herrick[10]	12	(3/25)
	Wahbeh et al.[12]	21.2	(7/33)
	Hameed et al.[13]	10.8	(4/37)
	Weible and Randall[352]	3	(1/35)
	Leigh et al.[15]	12	(7/59)
	Gravett et al.[14]	24	(13/54)
	Duff and Kopelman[353]	4	(1/24)
	Romero et al.[16]	9.8	(4/41)
	Romero et al.[20]	9.1	(24/264)
	Skoll et al.[21]	5.5	(7/127)
	Romero et al.[20]	9.1	(24/64)
	Romero et al.[23]	13.8	(15/109)
	Romero et al.[22]	13.7	(23/168)
	Romero et al.[24]	12.8	(25/195)
	Harger et al.[354]	0	(0/38)
	Gauthier et al.[25]	15.9	(18/113)
	Coultrip et al.[27]	11.2	(12/107)
	Watts et al.[26]	19	(20/105)
	Romero et al.[61]	9.2	(11/120)
	Coultrip et al.[355]	13	(12/89)
	Yoon et al.[356]	10.8	(11/102)
	Markenson et al.[196]	9.3	(5/54)
	Gomez et al.[32]	10.7	(11/103)
	Hussey et al.[31]	12.6	(16/127)
	Kara et al.[357]	33.8	(25/74)
	Oyarzun et al.[358]	12	(6/50)
	Rizzo et al.[33]	12.5	(18/144)
	Greci et al.[359]	8.7	(9/103)
	Yoon et al.[30]	11.6	(21/181)
	Elimian et al.[35]	11.5	(12/104)
	Gonzalez/Bosquet et al.[36]	11.5	(13/113)
	Locksmith et al.[37]	13.6	(6/44)
	Ovalle et al.[38]	23.8	(15/63)
	Yoon et al.[41]	10	(21/209)
Pre-labor premature rupture of membranes without labor	Garite et al.[50]	30	(9/30)
	Garite and Freeman[360]	23.3	(20/86)
	Cotton et al.[52]	17	(7/41)
	Zlatnik et al.[361]	31	(9/29)
	Broekhuizen et al.[66]	28.3	(15/53)
	Vintzileos et al.[362]	22.2	(12/54)
	Felnstein et al.[363]	20	(12/50)
	Romero et al.[53]	25.6	(41/160)
	Gauthier et al.[25]	53.8	(49/91)
	Coultrip et al.[27]	41.4	(12/29)
	Gauthier and Meyer[364]	47.9	(56/117)

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Table V (Continued)

Obstetric disorders	Authors	Prevalence of MIAC %	(*n*/*N*)
	Romero et al.[60]	38.2	(42/110)
	Font et al.[365]	56.8	(21/37)
	Averbuch et al.[366]	35.6	(32/90)
	Carroll et al.[367]	30.9	(30/97)
	Gomez et al.[32]	57.7	(30/52)
	Hussey et al.[31]	15.4	(4/26)
Pre-labor premature rupture of membranes in labor	Romero et al.[53]	75	(36/48)
Spontaneous rupture of membranes at term	Romero et al.[59]	34.3	(11/32)
Sonographic short cervix	Gomez et al.[368]	7	(28/401)
	Hassan et al.[349]	9	(5/57)
	Vaisbuch et al.[369]	4.3	(2/47)
Cervical insufficiency	Romero et al.[160]	51.5	(17/33)
	Mays et al.[161]	39	(7/18)
	Lee et al.[162]	8	(4/52)
	Bujold et al.[163]	47	(7/15)
Twin gestations with preterm labor and intact membranes	Romero et al.[370]	11.9	(5/42)
	Mazor et al.[371]	12	(9/74)
	Yoon et al.[372]	35	(7/20)
Meconium stained amniotic fluid in preterm gestations	Romero et al.[215]	33	(10/30)
Meconium stained amniotic fluid in term gestations	Romero et al.[373]	19.6	(16/66)
Placenta previa	Madan et al.[374]	5.7	(2/35)
Idiopathic vaginal bleeding	Gomez et al.[375]	14	(16/114)
Pregnancy with intra-uterine device	Kim et al.[376]	45.9	(45/98)

patients with positive AF cultures and negative PCR/ESI-MS results in this study had intra-amniotic inflammation, only one had mild acute histologic chorionitis/subchorionitis, and all three delivered after 35 weeks of gestation without major complications. It is doubtful that positive AF culture with negative PCR/ESI-MS results constitutes a true-positive culture.

Acidovorax sp. was identified by PCR/ESI-MS in one patient who had a negative AF culture. The microbial burden in this case was low (11 GE/well), the AF Gram stain was negative, and there was no evidence of intra-amniotic inflammation (by AF IL-6, glucose, WBC count, or acute histologic chorioamnionitis); yet, the patient delivered before the 28th week of gestation. *Acidovorax sp*. has been identified as a contaminant in other studies,[252–254] and thus, it is unlikely that PCR/ESI-MS identified a true infection, responsible for the process that led to preterm delivery. Preterm labor, in this case, is most likely attributable to a mechanism other than intra-amniotic infection. Further studies are necessary to investigate the frequency of false-positive and false-negative PCR/ESI-MS results using fresh samples collected in a clinical setting.

Potential Clinical Value of the Rapid Detection of MIAC in Preterm Labor

Therapy to reduce the rate of preterm birth in patients with symptoms of preterm labor has focused almost exclusively on tocolytic agents.[255–265] However, these efforts have had limited success.[262,264,266–268] This is partly attributable to the syndromic nature of preterm labor,[164,165,218,269–271] which may require an etiology-based approach to diagnosis, treatment, and prevention.

MIAC and subclinical intra-amniotic inflammation are implicated in at least one-third of the cases of spontaneous preterm labor and delivery.[7–43,155,156] Furthermore, intra-amniotic infection has been recognized as predisposing to cerebral palsy[272–284] and chronic lung disease.[41,285–300] However, with the exception of asymptomatic bacteriuria,[301–305] prophylactic antibiotic treatment has not been successful in the prevention of preterm birth,[306–330] and

some have claimed that it may increase the rate of preterm delivery.[331]

The lack of efficacy of antibiotics has been attributed to the inclusion in randomized clinical trials of patients who do not have evidence of infection and therefore cannot benefit from antimicrobial therapy,[308,313–319,321–323,325–330,332] the choice and timing of antimicrobial agents,[333] the presence of biofilms[334–339] in which bacteria are refractory to antimicrobial treatment, etc. Benefits of antimicrobial treatment could include eradication of intra-amniotic infection in early phases, down-regulation of the inflammatory cascade that leads to preterm labor and delivery, prevention of an exaggerated inflammatory response syndrome that may cause fetal injury, and even congenital neonatal sepsis.

Animal models of intra-amniotic infection have shown that early administration of antibiotics at or within 12 hours after inoculation of bacteria in AF reduces the bacterial burden in blood, the peritoneum, the uterus, and AF.[333,340–342] Moreover, Grigsby et al. have shown in a primate model of intra-amniotic infection generated by the inoculation of *Ureaplasma parvum*, that the administration of azithromycin can eradicate *Ureaplasma parvum* from the AF and fetal lungs, prolonging pregnancy.[343] Eradication of MIAC with parenteral antibiotics has also been documented in patients with a short cervix as well as in patients with preterm labor.[344–348] For example, Hassan et al. reported that in patients with short cervix and positive AF culture for *Ureaplasma urealyticum*, AF cultures were sterile following 7 days of intravenous azithromycin, and three of the four cases delivered at term.[349]

The rapid, high-throughput technique used in this study is a promising option that facilitates broader and faster identification of MIAC in patients with preterm labor and intact membranes and is applicable to other complications of pregnancy. Accordingly, this technique may render standard cultivation methods of limited value, as it would allow targeting of specific interventions for at-risk patients, including hospitalization, delivery, or transfer to high-level facilities with specialized neonatal care. Future studies are required to clarify whether timely administration of specific antibiotic therapy for intra-amniotic infection could improve perinatal outcomes. Yet, as noted previously by our group,[308,344,345,350,351] such studies may be difficult to conduct under the current framework of regulations protecting human subjects from research risks, because withholding antimicrobial therapy from immune-compromised patients (the human fetus) would most likely be ethically unacceptable.

Strengths and Limitations

This is the first report of the use of PCR/ESI-MS in AF, as well as the first direct comparison of this technology with standard cultivation techniques. The results provide evidence that microbial burden has clinical implications. Future work is are required to assess the performance of PCR/ESI-MS in freshly collected samples.

Conclusion

(i) Rapid diagnosis of MIAC is possible using PCR/ESI-MS, which can provide results within 8 hours; (ii) the combined use of biomarkers of inflammation and PCR/ESI-MS allows for the diagnosis of intra-amniotic inflammation and infection; and (iii) this approach may result in the implementation of timely and specific interventions to reduce morbidity attributed to infection-induced preterm birth.

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