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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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Abstract

Objective—The major challenges in using amniotic fluid (AF) cultivation techniques to diagnose microbial invasion of the amniotic cavity (MIAC) are: 1) several days are typically required to obtain results, and 2) many organisms implicated in the pathogenesis of human disease are difficult to culture. Here, we compare the performance of AF culture with a novel technique for the diagnosis of MIAC that can provide results within eight hours by combining broad-range real-time polymerase chain reaction with electrospray ionization mass spectrometry (PCR/ESI-MS) to identify and quantify genomic material from bacteria and viruses in AF.

Methods—AF samples obtained by transabdominal amniocentesis from 142 women with preterm labor (PTL) and intact membranes 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 ≥ 2.6 ng/mL], acute histologic chorioamnionitis, spontaneous preterm delivery, and perinatal mortality were examined according to the results of these two tests.

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Results—1) The prevalence of MIAC in patients with preterm labor and intact membranes was 7% using standard cultivation techniques and 12% using PCR/ESI-MS; 2) seven of ten patients with positive AF culture also had positive PCR/ESI-MS [≥17 genome equivalents per PCR reaction well (GE/well)] 3) patients with positive PCR/ESI-MS (≥17 GE/well) and negative AF cultures had significantly higher rates of intra-amniotic inflammation and histologic acute chorioamnionitis, shorter intervals to delivery [median (interquartile range-IQR)], and offspring at higher risk of perinatal mortality, than women with both tests negative [90% (9/10) vs. 32% (39/122); (p<0.001); 70% (7/10) vs. 35% (39/112); (p=0.04); 1 (IQR: <1 – 2) days vs. 25 (IQR: 5 – 51) days; (p=0.002); OR: 5.6; 95% CI: 1.4 – 22, respectively]; 5) there were no significant differences in these factors between patients with positive PCR/ESI-MS (≥17 GE/well) who had negative AF cultures compared to those with positive AF cultures; and 6) PCR/ESI-MS detected genomic material from viruses in two patients (1.4%).

Conclusion—1) Rapid diagnosis of intra-amniotic infection is possible using PCR/ESI-MS, which can provide results within 8 hours; 2) the combined use of biomarkers of inflammation and PCR/ESI-MS allows for the rapid identification of specific bacteria and viruses in women with preterm labor and intra-amniotic infection; and 3) this approach may allow for administration of timely and specific interventions to reduce morbidity attributed to infection-induced preterm birth.

Keywords

broad-range real-time polymerase chain reaction with electrospray ionization mass spectrometry; intra-amniotic inflammation; preterm delivery

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 underestimate since the diagnosis of MIAC has represented a challenge for decades.

Early investigation used transcervical catheters to evaluate the microbial state of the amniotic cavity;[44-49] however, this approach was abandoned because of the problem of contamination. Thereafter, identification of bacteria by cultivation techniques in amniotic fluid (AF) retrieved by amniocentesis 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 test results are needed to determine whether and which antimicrobial agents should be administered. Other decisions, such as the administration or discontinuation of tocolysis, timing of the delivery, etc., also depend on the availability of reliable information about the presence of microorganisms in the amniotic cavity. The application 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 microorganisms by identifying microbes which are difficult to culture, [34, 39, 42, 65, 89-102] and can provide information about anti-microbial 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 (PTL) and intact membranes, AF culture was compared to a new technique using a combination of broad-range real-time PCR with electrospray ionization mass spectrometry (PCR/ESI-MS) [*Ibis® technology*].[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 in several clinical settings[106, 114-134] and has the potential to revolutionize the detection of infectious agents in modern obstetrics. The study objectives were to: 1) compare the performance of AF culture and PCR/ESI-MS in identifying MIAC in patients with PTL and intact membranes; 2) assess the magnitude of intra-amniotic inflammation in patients with MIAC identified by PCR/ESI-MS; and 3) 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 biological 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 with intact membranes. Patients were included if they met the following criteria: 1) had a singleton gestation; 2) presented with PTL and intact membranes; and 3) had an amniocentesis (trans-abdominal amniocentesis) performed between 20 and 35 weeks of gestation with microbiological studies. Patients were excluded from the study if: 1) rupture of the chorioamniotic membranes occurred before AF collection; or 2) 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 and the use of biological 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) 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 stains of AF were also performed shortly after collection. AF not required for clinical assessment was centrifuged for 10 minutes 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 with PCR/ESI-MS (*Ibis® Technology* - Athogen, Carlsbad, CA). Briefly, DNA was extracted from 300 uL 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 on the bacterial artificial chromosome (BAC) spectrum 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] A total of 200 μL of extract was

used per sample. For viral detection, the nucleic acids were extracted from 300 uL of AF using a method that combines 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 reaction 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 Ibis assay for the detection of bacteria in blood is in average 100 CFU/mL (95% CI, 6 – 600 CFU/mL).[114] A comparison of detection limits between blood and amniotic fluid showed 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 amniotic fluid and plasma was performed by spiking known amounts of a DNA virus (HHV-5) and an RNA virus (Human enterovirus) into amniotic fluid and plasma. Detection limits in amniotic fluid were similar to plasma, ranging from ∼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). 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 minutes associated with cervical changes in patients with a gestational age between 20 and 36 6/7 weeks. Preterm delivery was defined as having occurred prior to the 37th week of gestation. Acute histological chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or chorioamniotic membranes.[139-141] Intra-amniotic inflammation was diagnosed when IL-6 AF concentration was ≥ 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 non-parametric correlation coefficients were calculated. The Kruskal-Wallis and Mann Whitney U tests were used to test for differences in arithmetic variable distributions. The X^2 or Fischer's exact test were 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) 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 histological acute 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 ≥ 2.6 ng/mL) among patients with detectable DNA from bacteria and/or virus using PCR/ESI-MS (Figure 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. 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, as well as the presence or absence of histologic acute 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].

Figures 3A and 3B 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 study group had positive AF culture and positive PCR/ESI-MS (≥17 GE/well) (n=7). The second study group included patients with positive PCR/ESI-MS (≥17 GE/well) who had negative AF cultures (n=10). The third and fourth study 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) vs. 87 (IQR: 11.3 - 264) ng/mL, p=0.43] (Figure 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 among: 1) women with positive AF cultures and negative PCR/ESI-MS results (n=3); and 2) patients (n=122) with negative AF cultures and negative PCR/ESI-MS [87 (IQR: 11.3 - 264) ng/mL vs. 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/ESI-MS) than in patients with positive AF culture and negative PCR/ESI-MS (p=0.009) (Figure 3A).

Differences in the median AF WBC count among the four study groups were consistent with those of AF IL-6. There were no differences in the median WBC count among patients with positive PCR/ESI-MS (≥ 17 GE/well) who had positive (n=7) or negative (n=10) AF cultures [490 (IQR: 50-1920) vs. 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) (Figure 3B). Differences in the median AF IL-6 and WBC concentrations among the four study 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) by the combined 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. These patients accordingly had intra-amniotic infection (i.e., MIAC and intra-amniotic inflammation).

All but one participant with a positive PCR/ESI-MS (≥ 17 GE/well) and a negative AF cultures 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. Importantly, 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 (Figure 4A).

The relationship between detectable microorganisms in the amniotic fluid and acute histological chorioamnionitis

Figure 4B shows the prevalence of histologic acute chorioamnionitis among the four study 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 histologic acute chorioamnionitis. Histologic acute chorioamnionitis 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 histological chorioamnionitis than women with negative tests, adjusting for gestational age and cervical dilatation at amniocentesis [OR 5.1, 95%CI 1.01-26, respectively] (Table III). One of the three women with positive AF culture and negative PCR/ESI-MS had mild histologic acute chorioamnionitis (stage 1 subchorionitis/chorionitis) (Figure 4B).

The observations described in the previous paragraph, combined with the parameters assessing intra-amniotic inflammation, reveals 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. Similarly, the PPV of a positive PCR/ESI-MS (≥ 17 GE/well) and a positive AF culture for the identification of patients who delivered within 2 days of amniocentesis were 76.5% (13/17) and 50% (5/10), respectively (Table IV).

Presence and burden of microorganisms in amniotic fluid and the interval to spontaneous preterm delivery

Kaplan-Meier survival estimates, censoring subjects in whom labor was induced for maternal or fetal indications, show 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 vs. 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 (≥ 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) (Figure 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 compared to those 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 among the four study groups defined by the results of AF culture and PCR/ESI-MS (p=0.03) (Figure 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 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 perinatal death for offspring of women with positive PCR/ESI-MS according to the results of AF culture [positive culture 29% (2/7) vs. negative culture 40% (4/10); p=1.0].

Comment

Principal findings of the study—1) The prevalence of MIAC in patients with preterm labor and intact membranes was 7% using standard cultivation techniques and 12% using PCR/ESI-MS; 2) patients with positive PCR/ESI-MS (≥17 GE/well) and negative AF cultures had higher rates of intra-amniotic inflammation and histologic acute chorioamnionitis, shorter intervals to delivery, and their offspring were at greater risk of perinatal mortality than patients with negative results for both tests; 3) there were no differences in these factors when comparing patients with positive PCR/ESI-MS (≥17 GE/well) who had positive to those with negative AF cultures; and 4) genomic material from viruses in AF were detected in two patients [1.4% (2/142)]. Taken together, these findings strongly suggest that PCR/ESI-MS can be used as a rapid test to diagnose MIAC and guide patient management.

Microbial invasion of the amniotic cavity detected by cultivation and molecular microbiologic techniques

A key transition in human life is the emergence from a sterile to a non-sterile environment at the time of birth.[145] The amniotic fluid is sterile under normal circumstances.[43, 146-148] Upon conducting systematic investigations using cultivation and molecular techniques, we found that the overwhelming majority of women with a normal pregnancy outcome do not have evidence of bacteria [62, 147, 149, 150] or viruses in the amniotic cavity.[151-154] 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 in patients with PROM 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 or preterm PROM, the higher the frequency of MIAC.[20, 26, 41, 99, 101, 157-159] More importantly, in patients admitted with PROM, the frequency of MIAC increases from 39% (24/61) without labor, to 75% (36/48) with the onset of spontaneous preterm labor (p=0.0004).[53] This unique data was obtained before routine administration of antibiotics in patients with preterm PROM, and is the basis for the clinical dogma that the onset of labor in these women is frequently due to subclinical intra-amniotic infection.

Using cultivation techniques, MIAC has been identified in several obstetrical disorders with the prevalence ranging from 10–12% in patients with preterm labor and intact membranes,[9-16, 20-27, 30-37, 61] to as high as 51% in pregnant women diagnosed with acute cervical insufficiency (Table V).[160-163] These cultivation-based studies provide strong evidence for the involvement of microorganisms in multiple pregnancy complications. Yet, cultivation techniques represent a minimum estimate of the prevalence of MIAC.[39, 99, 156, 164, 165]

The advantages of molecular techniques for the identification of microorganisms

Molecular testing for the detection of microorganisms is considered to be the "diagnostic tool for the new millennium".[85, 166, 167] In the U.S. alone, 5 million cases of infectious disease-related illness are reported annually, and many are considered to be undiagnosed.[168] Accordingly, the application of these tools to acute care settings is a major emphasis.

In the context of pregnancy, 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] Often, these conditions are unknown, rendering standard techniques inadequate in detecting many organisms implicated in human diseases.[170-174] For example, *Treponema pallidum*, the organism responsible for syphilis, has remained difficult to culture for decades.[175, 176] In addition, cultivation methods are frequently unreliable (and 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 inform clinical decisions (e.g. antibiotic administration). This is why physicians have adopted the standard approach of beginning intravenous antibiotic therapy without knowing the etiologic agent responsible for a suspected infection.[67-75]

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 relatively low cost.[85] These methods were used to identify agents responsible for multiple conditions previously considered as being 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 is longer than when using emerging techniques, such as PCR/ESI-MS.

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 fundamental premise that 16S rRNA gene is evolutionarily conserved exclusively in bacterial species.[189, 190] The detection of this gene in a sterile biological fluid (such as amniotic fluid) accordingly indicates the presence of bacteria. This approach has been used by many investigators to identify bacteria in amniotic fluid using species-specific [34, 39, 42, 65, 89, 92, 93, 95-97, 100, 191-196] or broad-range PCR.[90-92, 99, 101, 143, 144, 197-200] Most of the studies have shown that broad-range PCR assays and specific assays are superior to culture in the detection of microorganisms in the amniotic fluid.[90-92, 99, 101, 143, 144, 197-200] 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.

A fundamental 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] This is a key feature since failure to identify pathogens in time to inform clinical decisions is a major impediment to the treatment of infectious diseases. The PCR/ESI-MS system is also able to

quantify the amount of genomic material present in a biological sample.[108, 109] Genome quantification is useful in clinical circumstances including the selection of antimicrobial agents and the monitoring of therapeutic benefit. This can also be used in selecting the route of delivery (vaginal vs. cesarean delivery) in cases of HIV infection.[201-207] Other examples in which this approach has been valuable include visceral *leishmaniasis*, [208-210] infectious mononucleosis,[211-213] and mortality risk assessment in patients with pneumococcal pneumonia.[214, 215]

PCR/ESI-MS technology also 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 rapid detection of intra-amniotic infection

The identification of bacteria in amniotic fluid is a pathological finding, which we have defined as "microbial invasion of the amniotic cavity".[18, 24, 40, 57, 59, 60, 62, 143, 144, 160, 165, 216-220] 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, 217, 218, 220-236] Thus, intra-amniotic infection refers to the combination of MIAC with intra-amniotic inflammation.[40, 41, 43, 162, 165, 219, 235, 237-239] 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). In contrast, 70% (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 twofold greater prevalence of intra-amniotic infection (5% vs. 11%).

Viral infection in patients with preterm labor

The role of viral infection in spontaneous preterm labor has not been examined rigorously. Previous studies using PCR-based methods have shown conflicting results describing the prevalence of viral invasion into the amniotic cavity (VIAC), ranging from non-detectable, to as high as 6.4%.[151-153, 240-242] In this study, we used PCR/ESI-MS to detect multiple organisms in the same sample, (viruses and bacteria), as co-infection may occur.[109] However, only two patients (1.4%) had positive results for *Enterovirus*. Recently, Gervasi et al., using specific PCR assays for a panel of viruses reported that the prevalence of VIAC during the second trimester was 2.2%.[154] In that study, Gervasi et al. collected amniotic fluid samples from asymptomatic patients during the second trimester.[154] 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., suggested that the isolation of viruses in the amniotic cavity was not associated with adverse maternal or perinatal outcome.[153]

Recent studies suggest that systemic viral infections may predispose pregnant mice to the effect of microbial products such as endotoxin.[243] Specifically, pregnant mice infected with a *herpesvirus* were more likely to have spontaneous preterm labor and deliver following endotoxin administration than those who were not exposed to the virus.[243, 244] Further studies are required to determine if 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.[245-248] The same may be the case in the lower genital tract.[249] 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 cannot be ignored.

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, histologic acute 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 amniotic fluid cultures. The prevalence of intra-amniotic inflammation and histologic acute 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 amniotic fluid 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 amniotic fluid 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 amniotic fluid 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.[250] In previous studies, we identified patients with positive amniotic fluid cultures who had negative PCR using broad primers and specific assays.[94, 99, 101, 194] These results were attributed to possible DNA degradation,[251] low inoculum size,[252] etc. The observations herein suggest that false negative PCR/ESI-MS results are an extremely rare phenomenon. None of the three patients with positive amniotic fluid cultures and negative PCR/ESI-MS results in this study had intra-amniotic inflammation, only one had mild histologic acute chorionitis/subchorionitis, and all three delivered after 35 weeks of gestation without major complications. It is accordingly doubtful that positive amniotic fluid 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 amniotic fluid culture. The microbial burden in this case was low, (11 GE/well), the amniotic fluid Gram stain was negative, there was no evidence of intra-amniotic infection (by amniotic fluid IL-6, glucose, WBC count, or histologic acute chorioamnionitis); yet, the patient delivered before the 28th week of gestation. *Acidovorax sp*. has been identified as a contaminant in other studies [253-255] and thus, it is unlikely that PCR/ESI-MS identified 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 negative PCR/ESI-MS results using fresh samples collected in a clinical setting.

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.[256-266] However, these efforts have had limited success.[263, 265, 267-269] This is partly attributable to the syndromic nature of preterm labor,[164, 165, 219, 270-272] 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 [273-285] and chronic lung disease.[41, 286-301] However, with the exception of asymptomatic bacteriuria,[302-306] prophylactic antibiotic treatment has not been successful in the prevention of preterm birth,[307-331] and some have claimed that it may increase the rate of preterm delivery.[332]

The ineffectiveness of antibiotics has been attributed to the inclusion in randomized clinical trials of patients who do not evidence of infection, and therefore cannot benefit from antimicrobial therapy,[309, 314-320, 322-324, 326-331, 333] the choice and timing of antimicrobial agents,[334] the presence of biofilm [335-340] 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 amniotic fluid.[334, 341-343] Moreover, Grigsby et al. have shown that in an animal model of intra-amniotic infection, generated by the inoculation of *Ureaplasma parvum*, the administration of specific maternal antibiotic therapy (Azithromycin) can eradicate *Ureaplasma parvum* from the amniotic fluid and fetal lungs, prolonging pregnancy.[344] Eradication of MIAC with parenteral antibiotic has also been documented in patients with a short cervix as well as in patients with preterm labor.[345-349] 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.[350]

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,[309, 345, 346, 351, 352] such studies may be difficult to conduct under the current framework of regulations protecting human subjects from research risks, since 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 the amniotic fluid of patients with PTL and intact membranes, as well as the first direct comparison of this technology with standard cultivation techniques. These results provide evidence of the importance of the number of

genome copies of bacteria during intra-amniotic infection. Future studies are required to assess the performance of PCR/ESI-MS in freshly collected samples.

Conclusion

1) Rapid diagnosis of intra-amniotic infection is possible using PCR/ESI-MS, which can provide results within 8 hours; 2) the combined use of biomarkers of inflammation and PCR/ESI-MS allows for the rapid identification of specific bacteria in cases of preterm labor and intra-amniotic inflammation; and 3) this approach may allow for administration of timely and specific interventions to reduce morbidity attributed to infection-induced preterm birth.

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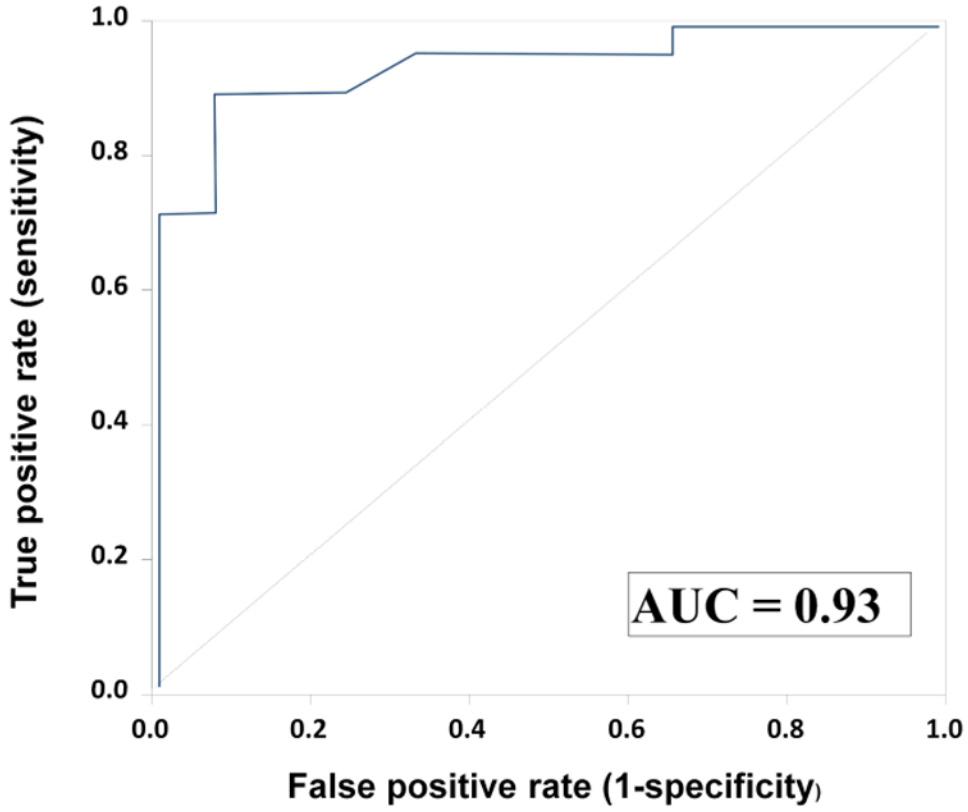


Figure 1.

Receiver operator characteristic curve analysis of PCR/ESI-MS genome equivalents per PCR reaction 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. Among patients with detectable DNA of bacteria or viruses using PCR/ESI-MS, a GE/well ≥17 had an area under the ROC curve of 0.93 (95% CI 0.83 – 1.0; $p<0.001$), a positive predictive value of 94.1% (16/17), and a negative predictive value of 84.6% (11/13) for the identification of intra-amniotic inflammation.

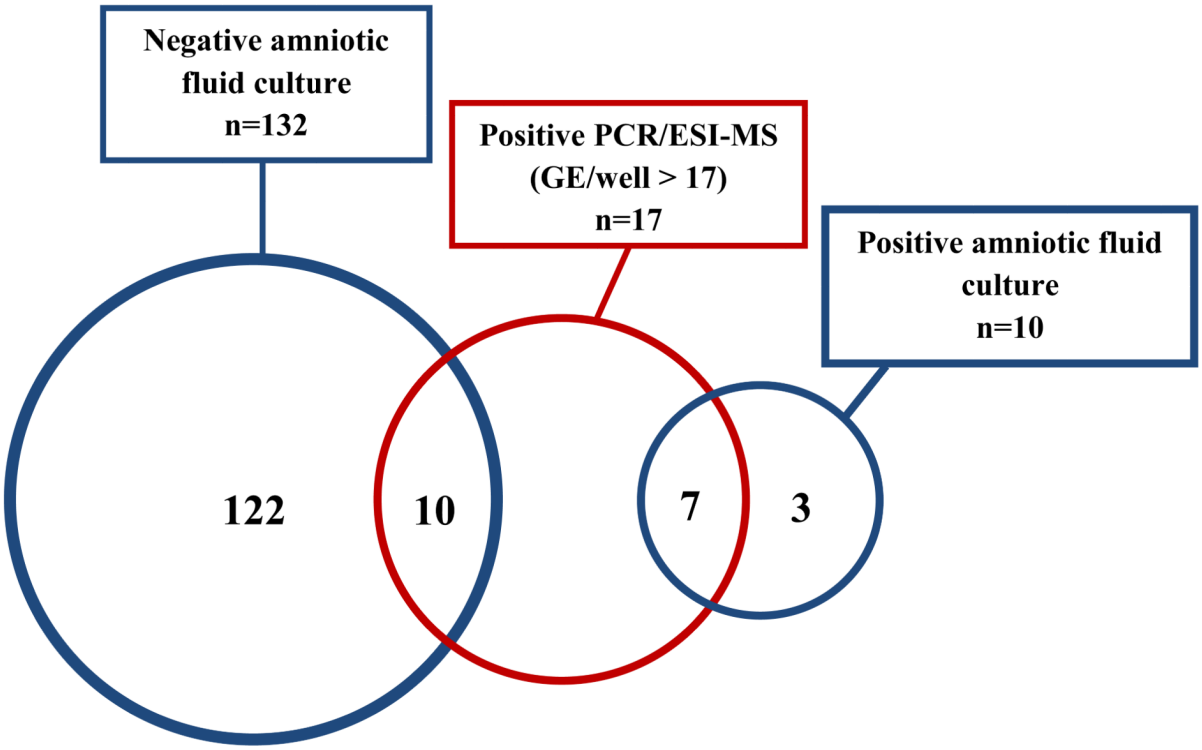


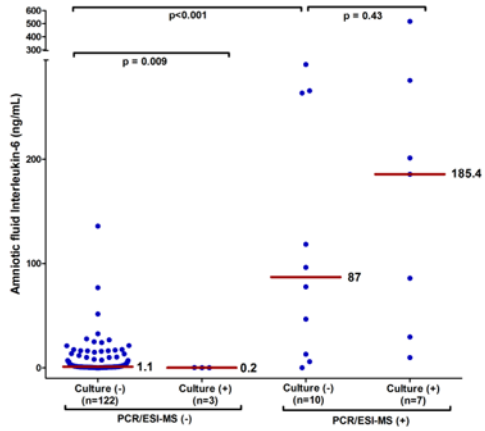
Figure 2.

Bacteria and viruses detected in amniotic fluid of patients with preterm labor using standard cultivation techniques vs. 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 Mass Spectrometry (ESI-MS).

Microorganisms detected in the amniotic fluid of patients with preterm labor and intact membranes using standard cultivation techniques versus PCR/ESI-MS.

PCR: polymerase chain reaction; ESI-MS: electrospray ionization mass spectrometry; GE/well: genome equivalent per PCR reaction.

A.



B.

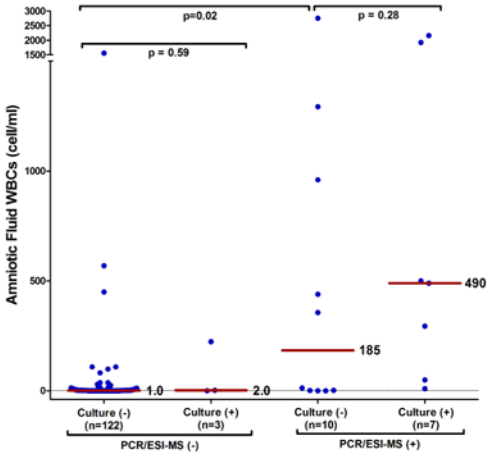


Figure 3.

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 cells (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 negative AF culture and a positive PCR/ESI-MS (GE/well ≥17) and those patients with positive AF culture and positive PCR/ESI-MS (p= 0.43 and 0.28, respectively). Among patients with negative AF culture, those with a positive PCR/ESI-MS had a significantly higher median AF IL-6 concentration and WBC count than those patients with both tests negative (p<0.001 and p=0.02, respectively). The median AF IL-6 concentration in patients with positive AF culture and negative PCR/ESI-MS (GE/well < 17) was always < 2.6 ng/mL.

Amniotic fluid IL-6 concentration and white blood cell count according to the results of standard AF cultures and PCR/ESI-MS.

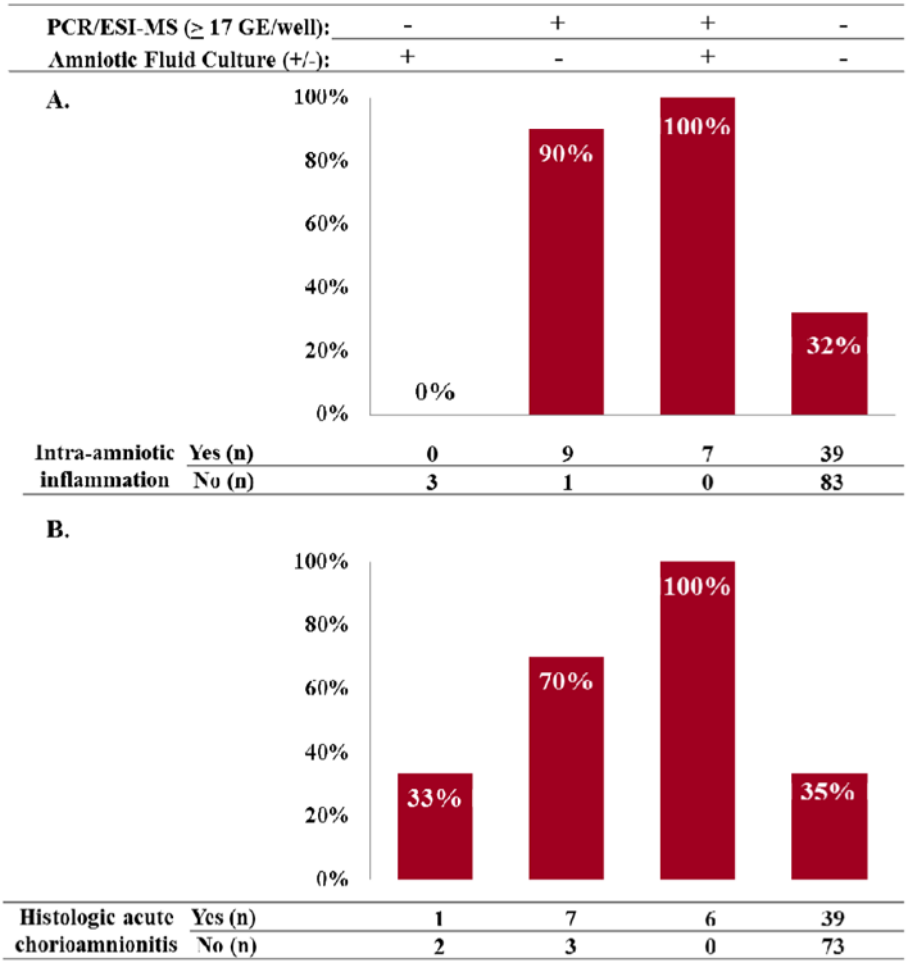


Figure 4.

Prevalence of intra-amniotic inflammation (Panel A) and histologic acute 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 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 have a prevalence of histological acute chorioamnionitis of 70% (7/10). In contrast, the prevalence of histological acute chorioamnionitis in patients with negative AF culture and negative PCR/ESI-MS (GE/well < 17) was 35% (39/112).

Prevalence of intra-amniotic inflammation and histologic acute chorioamnionitis by PCR/ESI-MS and amniotic fluid culture results

PCR: polymerase chain reaction; ESI-MS: electrospray ionization mass spectrometry.

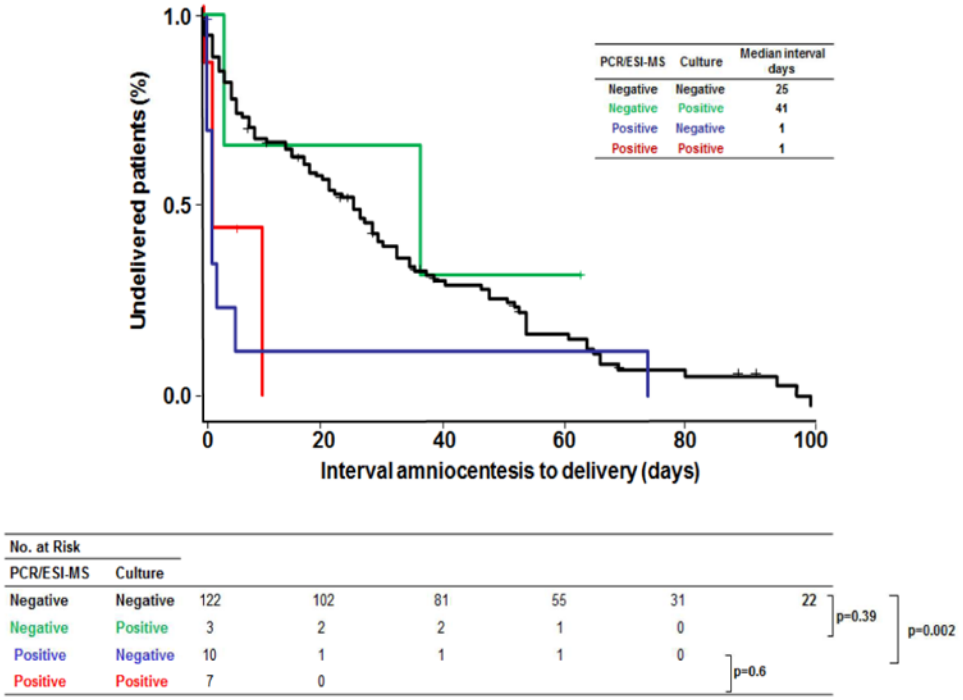


Figure 5.

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 AF 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 results [1 (IQR: < 1 – 2) days vs. 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 those patients with both tests positive [1 (IQR: 1 – 8) days] (p=0.6).

Kaplan-Meier survival analysis of amniocentesis-to-delivery interval (days) according to amniotic fluid cultures and PCR/ESI-MS results.

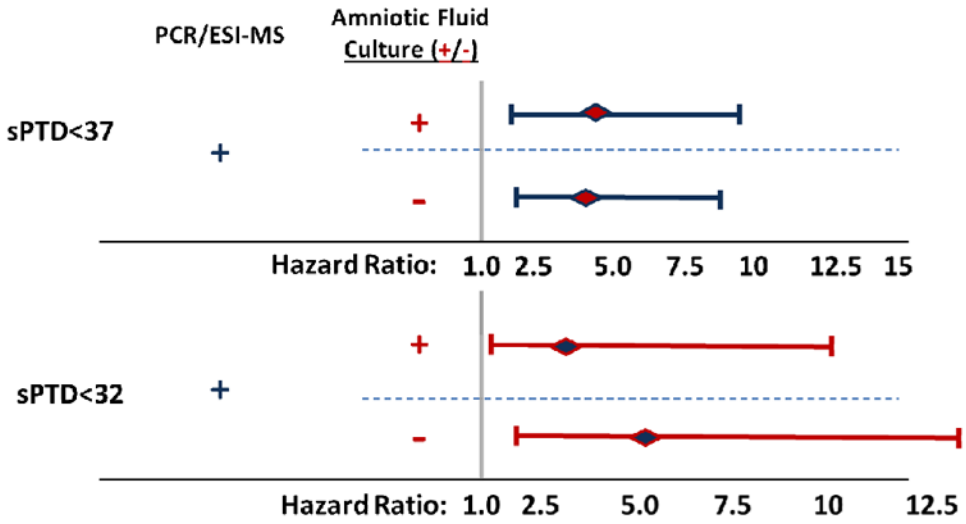


Figure 6.

Forest plot of hazard ratios for spontaneous preterm delivery (<37, <32 weeks) by PCR/ESI-MS and amniotic fluid culture results compared to 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.

Forest plot of hazard ratios for spontaneous preterm delivery (<37, <32 weeks) by PCR/ESI-MS and amniotic fluid culture findings compared to patients with negative tests.

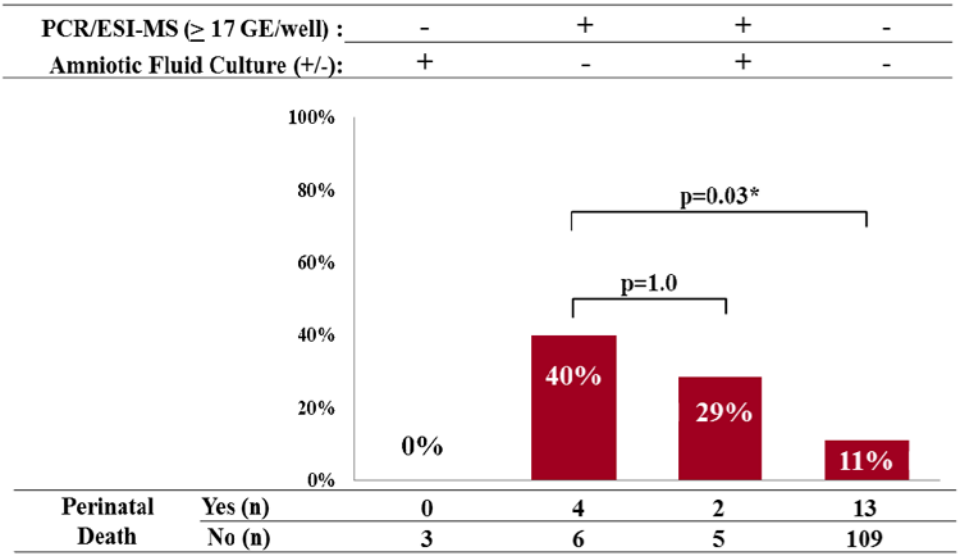


Figure 7.

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) vs. 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) vs. negative culture 40% (4/10); p=1.0]. Prevalence of perinatal death (fetal and neonatal deaths) by the combined results of amniotic fluid culture and PCR/ESI-MSI.

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)
Birthweight (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 reaction; IQR: interquartile range; PCR: polymerase chain reaction; ESI-MS: electrospray ionization mass spectrometry.

Table II

Amniotic fluid inflammatory profile, delivery information, placenta pathology and perinatal mortality in patients with microbial invasion of the amniotic cavity according to amniotic fluid cultures vs. GE/well results using PCR/ESI-MS.

		Amniotic Fluid Culture and PCR/ESI-MS Results						Amniotic fluid (AF) test results				Delivery information			Histologic Placenta Lesions		
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**	**AF Gram stain**	**AF Glucose**	**AF WBC**	**GA at amniocentesis (weeks)**	**GA at delivery (weeks)**	**Interval-amniocentesis-to delivery (days)**	**Acute histological chorioamnionitis**	**Severity of histological chorioamnionitis**	**Perinatal mortality**
1	**Positive AF Culture and positive PCR/ESI-MS (n=7)**	*Ureaplasma spp*	*Ureaplasma parvum*	575	No detected	0	29.8	Yes	Neg	10	490	32.4	32.6	1	Yes	2	No
2		*Candida albicans*	*Candida albicans*	215	No detected	0	201.3	Yes	Neg	10	2160	26.3	26.4	1	Yes	3	No
3		*Ureaplasma spp*	*Ureaplasma parvum Fusobacterium nucleatum*	1296	No detected	0	275.5	Yes	Pos	ND	50	24.9	26.0	8	NI	NI	Yes
4		*Ureaplasma spp*	*Ureaplasma parvum*	1795	No detected	0	86	Yes	Neg	10	500	32.9	33.0	1	Yes	2	No
5		*Streptococcus*	*Gardnerella vaginalis Mycoplasma hominis*	6983	No detected	0	185.5	Yes	Neg	10	1920	31.7	31.9	1	Yes	3	No
6		*Ureaplasma urealyticum*	*Ureaplasma urealyticum*	1484	No detected	0	9.9	Yes	Pos	20	10	32.1	32.7	4	Yes	2	No
7		*Bacteroides*	*Fusobacterium nucleatum*	20	No detected	0	517.8	Yes	Neg	10	295	22.6	22.6	0	Yes	3	Yes
8	**Negative AF culture and GE/Well ≥ 17 (n=10)**	Negative	No detected	0	*Human Enterovirus*	>1000	6.0	Yes	Neg	21	0	21.6	21.7	1	No	No	Yes
9		Negative	*Fusobacterium nucleatum*	27	No detected	0	46.8	Yes	Neg	24	1	25	25.7	5	Yes	4	Yes
10		Negative	*Ureaplasma urealyticum*	768	No detected	0	265.5	Yes	Neg	11	357	20.4	20.4	0	Yes	3	Yes
11		Negative	*Acinetobacter junii*	68	*Human Enterovirus*	24	77.7	Yes	Neg	20	0	24.6	24.7	1	Yes	1	No
12		Negative	*Acinetobacter junii*	41	No detected	0	0.28	No	Neg	30	2	29.1	39.9	75	No	No	No
13		Negative	*Sneathia species*	280	No detected	0	290.7	Yes	Pos	10	440	26	26.0	0	Yes	3	No
14		Negative	*Pseudomonas entomophila/putida*	23	No detected	0	13.1	Yes	Neg	17	13	33.9	33.9	0	No	No	No
15		Negative	*Ureaplasma parvum*	571	No detected	0	118.5	Yes	Neg	10	2750	26.7	27.0	2	Yes	3	No
16		Negative	*Candida albicans*	57	No detected	0	96.3	Yes	Pos	10	1292	32.4	32.6	1	Yes	2	No
17		Negative	Sneathia species	152	No detected	0	263.3	Yes	Pos	10	960	23.6	23.6	0	Yes	4	Yes
18	**Negative AF culture and GE/Well < 17 (n=13)**	Negative	*Aeromonas caviae*	3	No detected	0	0.6	No	Neg	ND	10	30.1	30.1	0	No	No	No
19		Negative	*Acinetobacter junii*	14	No detected	0	1.6	No	Neg	30	3	32.1	33.6	10	No	No	No
20		Negative	*Moraxella osloensis*	3	No detected	0	1.0	No	Neg	29	8	32.4	39.9	52	No	No	No
21		Negative	*Staphylococcus aureus*	5	No detected	0	0.6	No	Neg	30	0	33.3	37.6	30	No	No	No
22		Negative	*Staphylococcus aureus*	7	No detected	0	1.6	No	Neg	10	0	34.1	34.9	5	No	No	No
23		Negative	*Acidovorax sp.*	11	No detected	0	0.4	No	Neg	29	20	27	27.3	2	No	No	No
24		Negative	*Streptococcus species*	3	No detected	0	1.0	No	Neg	10	0	34.1	36.9	19	Yes	2	No
25		Negative	*Lactobacillus acidophilus/crispatus*	5	No detected	0	2.3	No	Neg	25	1	28.1	39.7	81	Yes	2	No
26		Negative	*Ureaplasma parvum*	8	No detected	0	0.2	No	Neg	31	0	22.3	25.3	21	Yes	3	No
27		Negative	*Gardnerella vaginalis*	5	No detected	0	16.5	Yes	Neg	52	0	28.4	35.4	49	No	No	No
28		Negative	*Ureaplasma parvum*	4	No detected	0	0.2	No	Neg	22	2	26.4	27.0	4	No	No	No
29		Negative	Staphylococcus aureus	8	No detected	0	51.8	Yes	Neg	25	5	29.7	33.9	29	No	No	No

		Amniotic Fluid Culture and PCR/ESI-MS Results					Amniotic fluid (AF) test results				Delivery information			Histologic Placenta Lesions		
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**	**AF Gram stain**	**AF Glucose**	**AF WBC**	**GA at amniocentesis (weeks)**	**GA at delivery (weeks)**	**Interval-amniocentesis-to delivery (days)**	**Acute histological chorioamnionitis**	**Severity of histological chorioamnionitis**	**Perinatal mortality**
30		Negative	*Pantoea dispersa*	3	No detected	0	0.9	No	Neg	54	2	27.4	28.3	6	No	No	No
31		*Ureaplasma urealyticum*	No detected	0	No detected	0	0.2	No	Neg	24	0	34.6	35.1	4	Yes	1	No
32	**Positive AF culture and Negative PCR/ ESI-MS (n=3)**	*Ureaplasma urealyticum*	No detected	0	No detected	0	0.4	No	Neg	19	2	29.1	39.3	71	No	No	No
33		Staphylococcus aureus	No detected	0	No detected	0	0.2	No	Neg	20	225	31	36.9	41	No	No	No

GA= gestational age; AF = amniotic fluid; WBC = white blood cells; IL-6 = Interleukin-6; PCR = polymerase chain reaction; ESI-MS = electrospray ionization mass spectrometry; GE/well: genome equivalent per PCR reaction; NI = not information

Severity of Acute histological chorioamnionitis:
Stage 1 - Early: Acute Subchorionitis/Chorionitis
Stage 2 - Intermediate: Acute Choriamnionitis
Stage 3 - Necrotizing Chorioamnionitis
Stage 4 - Subacute Chorioamnionitis

Table III

Magnitudes of association among combined test results and intra-amniotic inflammation and histologic acute chorioamnionitis

	Intra-amniotic inflammation			Histologic acute 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*						
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.

*Note: adjusted for cervical dilation and gestational age at amniocentesis; Firth's penalized maximum likelihood estimation was performed where necessary to resolve separation issues.

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 reaction.

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

Obstetric disorders	Authors	Prevalence of MIAC		
		%	(n/N)	
Spontaneous labor at term with intact membranes	Romero et al. (1993)	18.8	(17/90)	62
	Gomez et al. (1994)	15.6	(14/90)	63
	Yoon et al. (2003)	6.3	(12/190)	149
Preterm labor with intact membranes	Miller et al. (1980)	20	(15)	9
	Bobbit et al. (1981)	25	(8/31)	11
	Wallace and Herrick (1981)	12	(3/25)	10
	Wahbeh et al. (1984)	21.2	(7/33)	12
	Hameed et al. (1984)	10.8	(4/37)	13
	Weible and Randall (1985)	3	(1/35)	353
	Leigh et al. (1986)	12	(7/59)	15
	Gravett et al. (1986)	24	(13/54)	14
	Duff and Kopelman (1987)	4	(1/24)	354
	Romero et al (1988)	9.8	(4/41)	16
	Romero et al. (1989)	9.1	(24/264)	20
	Skoll et al. (1989)	5.5	(7/127)	21
	Romero et al. (1989)	9.1	(24/64)	20
	Romero et al. (1990)	13.8	(15/109)	23
	Romero et al. (1990)	13.7	(23/168)	22
	Romero et al. (1991)	12.8	(25/195)	24
	Harger et al. (1991)	0	(0/38)	355
	Gauthier et al. (1991)	15.9	(18/113)	25
	Coultrip et al. (1992)	11.2	(12/107)	27
	Watts et al. (1992)	19	(20/105)	26
	Romero et al. (1993)	9.2	(11/120)	61
	Coultrip et al. (1994)	13	(12/89)	356
	Yoon et al. (1996)	10.8	(11/102)	357
	Markenson et al (1997)	9.3	(5/54)	197
	Gomez et al. (1998)	10.7	(11/103)	32
	Hussey et al. (1998)	12.6	(16/127)	31
	Kara et al. (1998)	33.8	(25/74)	358
	Oyarzun et al. (1998)	12	(6/50)	359
	Rizzo et al. (1998)	12.5	(18/144)	33
	Greci et al. (1998)	8.7	(9/103)	360
	Yoon et al. (1998)	11.6	(21/181)	30

Obstetric disorders	Authors	Prevalence of MIAC		
		%	(n/N)	
	Elimian et al. (1998)	11.5	(12/104)	35
	Gonzalez/Bosquet et al (1999)	11.5	(13/113)	36
	Locksmith et al. (1999)	13.6	(6/44)	37
	Ovalle et al. (2000)	23.8	(15/63)	38
	Yoon et al. (2001)	10	(21/209)	41
Prelabor premature rupture of membranes without labor	Garite et al. (1979)	30	(9/30)	50
	Garite and Freeman (1982)	23.3	(20/86)	361
	Cotton et al. (1984)	17	(7/41)	52
	Zlatnik et al. (1984)	31	(9/29)	362
	Broekhuizen et al. (1985)	28.3	(15/53)	66
	Vintzileos et al. (1986)	22.2	(12/54)	363
	Felnstein et al. (1986)	20	(12/50)	364
	Romero et al. (1988)	25.6	(41/160)	53
	Gauthier et al. (1991)	53.8	(49/91)	25
	Coultrip et al. (1992)	41.4	(12/29)	27
	Gauthier and Meyer (1992)	47.9	(56/117)	365
	Romero et al. (1993)	38.2	(42/110)	60
	Font et al. (1995)	56.8	(21/37)	366
	Averbuch et al. (1995)	35.6	(32/90)	367
	Carroll et al. (1996)	30.9	(30/97)	368
	Gomez et al. (1998)	57.7	(30/52)	32
	Hussey et al. (1998)	15.4	(4/26)	31
Prelabor premature rupture of membranes in labor	Romero et al. (1988)	75	(36/48)	53
Spontaneous rupture of membranes at term	Romero et al. (1992)	34.3	(11/32)	59
Sonographic short cervix	Gomez et al. (2005)	7	(28/401)	369
	Hassan et al. (2006)	9	(5/57)	350
	Vaisbuch et al. (2010)	4.3	(2/47)	370
Cervical insufficiency	Romero et al. (1992)	51.5	(17/33)	160
	Mays et al. (2000)	39	(7/18)	161
	Lee et al. (2008)	8	(4/52)	162
	Bujold et al. (2008)	47	(7/15)	163
Twin gestations with preterm labor and intact membranes	Romero et al. (1990)	11.9	(5/42)	371
	Mazor et al. (1996)	12	(9/74)	372
	Yoon et al. (1997)	35	(7/20)	373
Meconium stained amniotic fluid in preterm gestations	Romero et al. (1991)	33	(10/30)	216

Obstetric disorders	Authors	Prevalence of MIAC		
		%	(n/N)	
Meconium stained amniotic fluid in term gestations	Romero et al. (2013)	19.6	(16/66)	[374]
Placenta previa	Madan et al. (2010)	5.7	(2/35)	[375]
Idiopathic vaginal bleeding	Gomez et al. (2005)	14	(16/114)	[376]
Pregnancy with intra-uterine device	Kim et al (2010)	45.9	(45/98)	[377]