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Prevalence and Diversity of Microbes in the Amniotic Fluid, the Fetal Inflammatory Response, and Pregnancy Outcome in Women with Preterm Prelabor Rupture of Membranes

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Abstract

Problem—The role played by microbial invasion of the amniotic cavity (MIAC) in preterm prelabor rupture of membranes (pPROM) is inadequately characterized, in part because of reliance on cultivation-based methods.

Method of study—Amniotic fluid from 204 subjects with pPROM was analyzed with both cultivation and molecular methods in a retrospective cohort study. Broad-range and group-specific PCR assays targeted small subunit rDNA, or other gene sequences, from bacteria, fungi and archaea. Results were correlated with measurements of host inflammation, and pregnancy and perinatal outcomes.

Results—The prevalence of MIAC was 34% (70/204) by culture, 45% (92/204) by PCR, and 50% (101/204) by both methods combined. The number of bacterial species revealed by PCR (46 species-level phylotypes) was greater than that by culture (14 species) and included as-yet uncultivated taxa. Some taxa detected by PCR have been previously associated with the gastrointestinal tract (e.g., *Coprobacillus* sp.), the mouth (e.g., *Rothia dentocariosa*) or the vagina

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in the setting of bacterial vaginosis (e.g., *Atopobium vaginae*). The relative risk for histologic chorioamnionitis was 2.1 for a positive PCR (95% confidence interval [CI], 1.4–3.0), and 2.0 for a positive culture (95% CI, 1.4–2.7). Bacterial rDNA abundance exhibited a dose relationship with gestational age at delivery (R^2=0.26; P<0.01). A positive PCR was associated with lower mean birthweight, and with higher rates of respiratory distress syndrome and necrotizing enterocolitis (P<0.05 for each outcome).

Conclusion—MIAC in pPROM is more common than previously recognized and is associated in some cases with uncultivated taxa, some of which are typically associated with the gastrointestinal tract. The detection of MIAC by molecular methods has clinical significance.

Keywords

PPROM; Intra-amniotic infection; Intra-amniotic inflammation; preterm delivery; preterm birth; chorioamnionitis; FIRS; 16S; molecular microbiology; IL-6; cytokines

INTRODUCTION

Preterm prelabor rupture of membranes (preterm PROM) causes one-third of preterm births and contributes to significant perinatal morbidity and mortality.[1–6] Microbial invasion of the amniotic cavity (MIAC) is found in about 30% of preterm PROM cases,[2;7–26] and is associated with earlier gestational age at delivery.[2;25] However, the current understanding of MIAC derives largely from cultivation-dependent studies. Because many microbial species are not yet cultivated,[27] the role of MIAC in preterm PROM is likely under-recognized and incompletely characterized.

Molecular methods offer a sensitive, cultivation-independent approach for detecting microbes. In particular, broad-range PCR assays that target ribosomal DNA (rDNA) allow for detection and characterization of diverse microbial taxa, including unknown species.[28] These methods have been used to assess diversity within the human indigenous microbiota[29;30] and to characterize microbes associated with a wide range of clinical syndromes.[31;32]

Preterm parturition encompasses several distinct clinical phenotypes, including preterm PROM and preterm labor with intact membranes.[33] An association between MIAC and preterm parturition has been demonstrated in culture-based[34;35] and molecular[24;32;36–42] studies. However, the use of molecular techniques — and in particular, broad-range PCR — to investigate preterm parturition syndromes in a rigorous, systematic manner has been more limited.

We used a combination of culture and molecular methods to investigate MIAC in the setting of preterm PROM. Our objective was to determine the frequency, taxonomic diversity and relative abundance of microbes in amniotic fluid of women with preterm PROM, and to examine the relationship between MIAC, host inflammatory response as well as pregnancy/perinatal outcome. Our findings draw attention to the possible role of MIAC in preterm PROM and its association with adverse pregnancy outcome.

METHODS

Study population

A retrospective cohort study was conducted of patients with preterm PROM (defined below) who met the following inclusion criteria: 1) singleton gestation; 2) gestational age between 15 and 36.9 weeks; and 3) amniocentesis with microbiological studies of amniotic fluid. Patients were excluded from the study if: 1) delivery occurred elsewhere and/or clinical

metadata were unavailable; or 2) a major fetal chromosomal and/or congenital anomaly was present. All samples were collected in a single institution between December 1997 and March 2007.

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

Definitions

Membrane rupture was diagnosed by: i) pooling of amniotic fluid in the vagina; ii) a positive nitrazine test; and, iii) a positive ferning test.[2] Clinical chorioamnionitis was diagnosed according to criteria previously proposed by Gibbs et al.[43] Histologic chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or chorioamniotic membranes. [42];[44] Acute funisitis was diagnosed by the presence of neutrophils in the wall of the umbilical vessels and/or Wharton's jelly using criteria previously described.[45]

Sampling procedures

Patients with preterm PROM were offered amniocentesis to assess the microbial status of the amniotic cavity, and/or fetal lung maturity. Amniocentesis is part of the standard of care of patients with preterm PROM at the participating institution. Amniotic fluid was immediately transported in a capped sterile syringe to the clinical laboratory where it was cultured for aerobic and anaerobic bacteria, and for genital mycoplasmas (Mycotrim® GU Triphasic Culture System, Irvine Scientific, Santa Ana, CA, USA), as described.[32] White blood cell (WBC) count[46] and Gram stain[47] of amniotic fluid were also performed shortly after collection using methods previously described. Shortly after the amniocentesis, amniotic fluid not required for clinical assessment was centrifuged at 1300 × *g* for 10 minutes at 4°C, and the supernatant was aliquoted into gamma-irradiated nonpyrogenic DNase/RNase-free cryovials (Corning, Acton, MA, USA), and immediately frozen at −70°C. Amniotic fluid interleukin-6 (IL-6) concentrations were determined using a specific and sensitive immunoassay which had been validated for amniotic fluid. IL-6 determinations were performed after all patients were delivered and were not used in clinical management.

Genomic DNA extraction

After a storage interval of 1 to 9 years, amniotic fluid that was not required for clinical purposes (≈120 μl from each sample) was shipped on dry ice to Stanford, CA, where genomic DNA was extracted as described,[32] with the exception that the lysozyme preparation was replaced with a recombinant form (Epicentre Biotechnologies, Madison, WI, USA). Extracted DNA was eluted into a final volume of 100 μl of QIAamp® AE buffer and stored at −20°C or colder until thawing for molecular analyses. Strategies to prevent, detect and neutralize potential contamination were implemented at critical steps,[48] according to a previously described protocol that included mock extraction blanks to monitor potential contamination (at least one mock per 17 processed samples).[32]

Qualitative analysis by end-point PCR

DNA extracts from each amniotic fluid sample were analyzed by broad-range end-point PCR using broad-range bacterial 16S rDNA primers, and by group-specific end-point PCR using primers specific for six taxonomic groups (Table 1). PCRs were performed as described,[32] with the exception that each reaction contained 2 μl of prepared DNA template

and was carried out in a Veriti thermal cycler (Applied Biosystems). Ten microliters from each PCR well was electophoresed through a 1.5% (wt/vol) Tris-acetate-EDTA-agarose gel containing GelGreen nucleic acid stain (Biotium, Inc, Hayward, CA, USA). Amplicons producing visible bands upon scanning with a Typhoon 9410 variable mode imager (Amersham Biosciences, Piscataway, NJ, USA) were purified and, if from broad-range PCR, cloned as described.[32] Sequencing of amplicons from group-specific PCRs, and of positive recombinants from broad-range PCRs (up to 10 clones per reaction) was performed as described.[32]

Sequence alignment and phylogenetic analysis

Forward and reverse sequence reads were assembled into contigs as described.[32] Assembled sequences from group-specific PCR were queried against NCBI's GenBank database using a basic local alignment search tool (BLAST) algorithm[49] to confirm specificity. Assembled sequences from broad-range end-point PCR were aligned using the Greengenes NAST aligner[50] and imported into the Greengenes version of the Arb software package,[51,52] where they were compared to a database of over 200,000 small subunit rRNA sequences. Alignments were manually inspected and edited based on the original chromatograms and inserted into the Greengenes phylogeny according to a maximum parsimony algorithm. Sequences with no close relative in the Greengenes database were queried against NCBI's GenBank database using a BLAST algorithm[49] to determine approximate phylogenetic affiliation, and their closest neighbors were added to the alignment. Sequences without close neighbors were screened using RDP's 'Chimera Check' program (http://35.8.164.52/cgis/chimera.cgi?su=SSU). After removal of chimeric, vector, human, and poor-quality sequences from the alignment, a neighbor-joining tree was generated based on Felsenstein correction and 682 unambiguous filter positions. Phylotypes were defined using a 99% sequence similarity threshold, which approximates species-level classification.

Quantitative analysis by real-time PCR

Extracted DNA from each sample was analyzed by means of two real-time PCR assays, each of which was designed to specifically amplify and quantify 16S rDNA of domain *Bacteria* or domain *Archaea* (Table 1[53–59]). Reactions were carried out as described[32] with the exception that amplifications were carried out in a StepOnePlus real-time PCR system (Applied Biosystems), and absolute rDNA abundance was estimated from the standard curves using StepOne software version 2.0 (Applied Biosystems).

Outcome Measures

In order to assess the clinical significance of MIAC detected with our approach, outcome variables from four broad categories were measured: 1) intra-amniotic inflammation at presentation (including amniotic fluid WBC count[46] and IL-6 concentration[60]); 2) histopathologic inflammation of the placenta and chorioamniotic membranes after delivery; 3) pregnancy outcomes (including gestational age at delivery, and amniocentesis-to-delivery interval); and, 4) perinatal outcomes (including respiratory distress syndrome, pneumonia, necrotizing enterocolitis, intraventricular hemorrhage ≥grade III, sepsis and bronchopulmonary dysplasia), which were diagnosed according to previously described criteria,[21] as well as birthweight, admission to the neonatal intensive care unit and perinatal death.

Statistical analysis

Statistical analyses were performed using 'R' (open source, www.r-project.org) version 2.4.1, including the 'Epi' and 'Survival' packages. Differences in the mean between two groups were computed using Student's t-test and assuming unequal variances. Differences in

the median between two groups were computed using the Mann Whitney *U* test, and the Kruskal-Wallis analysis of variance test for more than two groups. Differences in proportions were computed using Fisher's exact test when samples were independent. Correlation coefficients for continuous outcomes were estimated by least squares linear regression modeling. Time-to-event outcomes were modeled by Kaplan-Meier survival methods and differences between survival curves were evaluated by means of the Mantel-Haenszel log-rank test. Prior to survival analysis, influential outliers, as defined by a dfbetas residual >3 standard deviations, were excluded[61] (n=3). In addition, patients who delivered preterm for maternal or fetal indications (except clinical chorioamnionitis) were included in the analysis with a censored time that was equal to the amniocentesis-to-delivery interval. For all analyses, a two-tailed *P* value <0.05 was considered significant.

RESULTS

Table 2 presents baseline characteristics of the 230 enrolled subjects. For 26 subjects, results were unavailable from at least one of the three culture assays used in the study (i.e., for aerobes, anaerobes or genital mycoplasmas); therefore, the remaining 204 subjects were used for comparisons of cultivation and molecular methods.

Culture methods underestimate microbial prevalence and diversity

The rate of MIAC in preterm PROM was 34% (70/204) based on cultivation, 45% (92/204) based on PCR, and 50% (101/204) based on the combined results of both methods (Figure 1). PCR was positive in 87% (61/70) of culture-positive subjects, and culture was positive in 66% (61/92) of PCR-positive subjects. Thus, findings from culture alone — which is the conventional diagnostic approach — underestimated MIAC prevalence by at least 30% (31/101).

Figure 2 presents the bacterial taxa encountered in this study. The species richness revealed by PCR (n=44) was three times that found by culture (n=14). In addition, the types of bacterial taxa revealed by molecular methods were notable for three reasons. First, some taxa, including a *Coprobacillus* species, an uncultured *Bacteroides* species (clone PP209-b04), an uncultured *Clostridiaceae* bacterium (clone PP209-b07), and another uncultured *Clostridiaceae* bacterium (clone PP209-b10) appear to be commensals of the gastrointestinal tract (see Discussion). Second, one phylotype is previously-uncharacterized: clone PP254-b02 (<94% sequence similarity to its nearest database relative) clustered near the phylum TM7, a deeply-branching taxonomic group from which no members have been cultivated to purity. Third, to our knowledge, a number of additional bacterial taxa have never been detected in amniotic fluid, including species associated recently with bacterial vaginosis (e.g., *Atopobium vaginae*), with the oral cavity (e.g., *Rothia dentocariosa*), or with rare infections of the urogenital tract (e.g., *Myroides* sp.). A total of 34 cases (34%) were found to be polymicrobial; 12 of these were found to be polymicrobial by culture alone, and 22 were found to be polymicrobial by PCR alone.

Five bacterial species were detected in the study population by culture but not by PCR. One was recovered from more than one subject (*Propionibacterium* sp. (n=2)), and the remaining four species were found in one subject each (*Actinomyces* sp., *Mobiluncus mulieris*, *Peptostreptococcus* sp., and a Gram-positive bacillus that was not further identified). However, in all but one instance (one case with *Propionibacterium* sp.), the infection was polymicrobial and the PCR was positive, but for a different bacterial species. It is therefore unclear whether the failure of PCR to detect these five taxa reflected bias in DNA amplification, the limited number of clones sequenced (n=10), or other factors (see Discussion).

For 26 additional subjects, results from one or more culture assays were unavailable. Although excluded from outcomes analyses (below), these subjects provided an opportunity to investigate further the molecular microbial diversity of MIAC. PCR was positive in 54% (14/26) of subjects and revealed 16 phylotypes. The most common taxa were *Ureaplasma* spp. (5 subjects; 23 clones), *Sneathia sanguinegens* (3; 17) and *Fusobacterium nucleatum* (2; 7). One sample contained several taxa, each found once, that are typical commensals of the gastrointestinal tract: *Bacteroides xylanisolvens* (>99% similarity), *Eubacterium halii* (100%), *Faecalibacterium* sp. (98.8%), and *Finegoldia magna* (>99%) (Figure 3).

Candida species were under-recognized by culture, and were associated with pregnancies with an intrauterine device (IUD)

Results from fungal culture methods were available for 221 subjects. The overall rate of MIAC due to *Candida* species — as detected by PCR or culture — was 57% higher than the rate detected by culture methods alone (5% [11/221] vs. 3.2% [7/221], respectively). PCR for *Candida* was positive in 82% (9/11) of all detected cases, and culture was positive in 64% (7/11). MIAC due to *Candida* occurred significantly more frequently in subjects with an IUD than in subjects without an IUD (28% [5/18] vs. 3% [6/203], respectively; $P<0.01$). By contrast, the rate of MIAC due to bacteria was equivalent in women with and without an IUD (56% [10/18] vs. 45% [92/203], respectively; $P=0.5$).

Association of MIAC with host inflammation

The median amniotic fluid WBC count at presentation was higher in women who had PCR-positive results only, than in those who had PCR-negative and culture-negative results (median [cells/ml]: 15 vs. 5; $P<0.01$) (Figure 4A). Similarly, the median amniotic fluid IL6 concentration was higher in patients who had PCR-positive results of amniotic fluid, than in those who were negative by both PCR and culture; however, this difference did not reach statistical significance (median [ng/ml]: 1.38 vs. 1.0; $P=0.16$) (Figure 4B). For both AF WBCs and IL6, there was no difference between the groups that were positive by each method alone; also, levels of each marker of inflammation were higher in the group of patients that was positive by both methods (PCR and culture) combined, than in the other clinical groups (see Figure 4).

Table 3 presents the relative risk for histologic inflammation of maternal and fetal membranes at delivery. The analysis included 146 subjects for whom histologic data were available. The relative risk for histologic chorioamnionitis was 2.1 for a positive PCR (95% confidence interval [CI], 1.4 – 3.0), and 2.0 for a positive culture (95% CI, 1.4 – 2.7). The relative risk for funisitis was 2.2 for a positive PCR (95% CI, 1.3 – 3.9), and 2.8 for a positive culture (95% CI, 1.7 – 4.7).

Associations of microbial DNA in amniotic fluid with adverse pregnancy outcomes, and with perinatal morbidity and mortality

Bacterial 16S rRNA gene copy number exhibited a statistically significant correlation with early gestational age at presentation ($R^2=0.17$; $P<0.01$) (Figure 5, Panel A), and with early gestational age at delivery ($R^2=0.26$; $P<0.01$) (Figure 5, Panel B). Subjects with a negative amniotic fluid culture but positive PCR delivered at an earlier median gestational age than did subjects with a negative amniotic fluid culture and negative PCR (31.9 vs. 33.4 weeks, respectively, $P=0.02$) (Table 4). There was no significant difference in the median gestational age at delivery between subjects with a negative amniotic fluid culture but positive PCR and subjects with a positive culture (31.9 vs. 29.6 weeks, respectively, $P=0.08$).

Furthermore, subjects with a negative amniotic fluid culture but positive PCR had a significantly shorter amniocentesis-to-delivery interval than did subjects with a negative amniotic fluid culture and negative PCR (P=0.01) (Figure 6). In contrast, there was no difference in the amniocentesis-to-delivery intervals of subjects with a negative amniotic fluid culture but positive PCR and those with a positive culture (regardless of PCR result) (P=0.8).

A negative amniotic fluid culture but positive PCR was associated with a lower mean birthweight, and with higher rates of respiratory distress syndrome and necrotizing enterocolitis, as compared with the finding of a negative amniotic fluid culture and negative PCR (P<0.05 for each outcome) (Table 5).

DISCUSSION

Principal findings of the study

By including molecular methods in our approach we found that MIAC in preterm PROM: 1) affected half of all subjects, yet was frequently undetected by culture-based methods; 2) was caused by diverse microbes, including taxa that are as-yet uncultivated, uncharacterized, or not previously found in amniotic fluid; 3) was associated in some women with microbial species that may have originated from the gastrointestinal tract; 4) was more frequently associated with *Candida* species than previous believed, especially in the presence of an IUD; 5) exhibited temporal and dose-response associations (based on microbial DNA presence or abundance) with early delivery, suggesting a causal relationship; 6) was associated with a fetal inflammatory response as reflected by the amniotic fluid white blood cell count; and 7) was associated with adverse perinatal outcomes.

Microbial invasion of the amniotic cavity detected by cultivation methods vs. molecular methods

Culture is the standard approach for the identification of MIAC and was positive in one-third of subjects in this study. This frequency is similar to the average frequency reported by others. [2] However, when molecular methods were included, MIAC was found in one-half of subjects. Interestingly, the rate at which culture underestimated MIAC in the present study (31% [31/101]) was comparable to that found by our group in a recent study of preterm labor with intact membranes, using a similar molecular approach (36% [9/25]).[32] In addition to facilitating a more accurate estimation of microbial prevalence, the molecular findings from this study advance our understanding of microbial diversity associated with MIAC.

Microbial diversity in preterm PROM

The conventional view is that microorganisms from the lower genital tract ascend into the amniotic cavity. This can occur in patients with either intact, or ruptured membranes. However, our data are the first, to our knowledge, to implicate the gastrointestinal tract as a potential source of the microbes that invade the amniotic cavity. This identification of source is based on reviewing: i) the Human Oral Microbiome Database[62] (to help ascertain the possibility of a given sequence having an oral source); ii) systematic studies of human indigenous microbiota; iii) reports identified by relevant PubMed queries; and, iv) GenBank metadata of close sequence neighbors (to review the anatomic source reported by the submitting investigator).

Although the concept of microbial hematogenous dissemination from the gastrointestinal tract to the amniotic cavity is relatively unexplored, it is consistent with accepted or emerging paradigms of pathogenesis. For example, it has long been established that the gastrointestinal tract can serve as a portal for pathogens to enter the bloodstream and cause

distant infection (e.g., endocarditis caused by *Streptococcus bovis* in the setting of colonic neoplasia). And more recent data suggest that some microbes invade the amniotic cavity from the bloodstream after dissemination from remote sites (e.g., the mouth[63–65]). It therefore stands to reason that residents of the gastrointestinal microbiota may be capable of entering and transiting the bloodstream to cause MIAC. Among women undergoing cesarean delivery, bacteremia was found to be common in women who were in labor or had membrane rupture, especially if these occurred preterm.[66] Our data suggest the need for studies that define more clearly the time-course and sources of bacteremia in this setting.

One taxon detected by PCR, but not culture, represented a novel species based on sequence divergence from database relatives. This sequence type (clone PP254-b02) clustered near TM7, a candidate phylum from which no representatives have yet been propagated to purity in culture.[67] TM7 members have been detected in the oral cavity,[68] including in association with periodontitis,[57] and in the vagina in the setting of bacterial vaginosis.[31] Our data expand the human habitats and clinical syndromes with which TM7-like taxa are associated.

Other taxa — in addition to those discussed above — were also detected in amniotic fluid for the first time. These taxa comprised three categories. The first has been associated with bacterial vaginosis in recent molecular studies,[31;69] and includes *Atopobium* sp., *Dialister* sp., and *Peptoniphilus* sp. Bacterial vaginosis is associated with a perturbed microbial ecosystem and an increased risk of preterm birth.[70] Our data support the hypothesis that members of the vaginal microbiota associated with bacterial vaginosis invade the amniotic cavity. The second category includes taxa known to be associated with the oral cavity. *Filifactor alocis* has been linked to primary endodontic infections,[71] and *Rothia dentocariosa* has been associated not only with odontogenic abscesses but also with intrauterine fetal death[72] and neonatal septicemia.[73] Our identification of these species expands the census of oral-associated taxa previously found in amniotic fluid[32;63–65] and strengthens the link between the oral microbiota, MIAC and preterm birth. The third category includes rare pathogens whose role in human disease appears to be incompletely characterized, such as *Myroides* (formerly *Flavobacterium*) spp.,[74] which have been implicated in cases of endocarditis,[74] urogenital disease,[75] and ventriculitis and bacteremia in a six-week old premature infant.[76]

We did not detect members of the domain *Archaea*, although some members have been found in the vagina of some women with bacterial vaginosis in a small study[77] Our data regarding the absence of *Archaea* in amniotic fluid mirror findings of a study of women in preterm labor.[32] This suggests that *Archaea* invade the amniotic cavity either very rarely or never, or that their abundance or sequence diversity is not detected by our assays.

Our data on *Candida* species and MIAC in preterm PROM corroborate findings from a recent large study that demonstrated a significantly higher rate of intra-amniotic *Candida* infection in pregnancies associated with an IUD.[78] Although a link between *Candida* infection and IUDs was described decades ago,[79] *Candida* biofilm formation on IUDs was reported only recently.[80] Our findings suggest a need to re-think the clinical management of pregnancies with preterm PROM associated with an IUD.

The host response to MIAC

To determine the clinical relevance of a positive molecular assay result, we compared the findings from PCR with those from culture in relation to several clinical outcomes. With respect to host inflammation (e.g., amniotic fluid WBC count and IL-6 concentration at presentation, histologic chorioamnionitis and funisitis at delivery), earlier gestational age at delivery, and perinatal morbidity (e.g., respiratory distress syndrome, necrotizing enterocolitis), the strength of association was equivalent for both PCR and culture. In

addition, PCR results exhibited both temporal (e.g., shortened amniocentesis-to-delivery interval) and dose-response relationships (e.g., gestational age at delivery as a function of bacterial rDNA abundance), in support of a possible causal link with clinical outcomes.[32,81–83]

Microbial footprints detected by molecular techniques were associated with a fetal inflammatory response[21,84–86] as gauged by the number of WBCs in the amniotic cavity (which are of fetal origin[87]). This observation is consistent with previous observations that fetal inflammation, as assessed by fetal plasma IL-6 concentration or by histologic inflammation of fetal membranes, is associated with a shorter interval-to-delivery, a higher neonatal morbidity, and evidence of multi-systemic involvement (adrenal,[88] central nervous system[89], thymus,[90–93] lung,[94–96] etc.). Studies that used a primate model of intra-uterine infection and included measurements of amniotic fluid cytokines have reported similar findings.[97–99] However, regulation of cytokines in amniotic fluid appears to be complex. Not only are some cytokines constitutively produced by non-inflammatory amnion cells (e.g., fibroblasts and epithelial cells),[100,101] but cytokine release by fetal membranes in response to bacteria appears to be heterogeneous.[102] In addition, little is known regarding the time-course of cytokine release in the human amniotic cavity, or the effect of factors such as duration of infection, inoculum size, and the number or virulence of microbial species present. Further studies are warranted.

Strengths and limitations of the study

The current study is one of the largest studies reported to date of patients with preterm PROM, presenting to a single institution, with an interest in the role of infection in preterm birth. We used state-of-the-art molecular techniques and we assessed the inflammatory response, as well as pregnancy and neonatal outcome. The limitations include the high rate of oligohydramnios in patients with preterm PROM which limited the sample volumes available for research assays (average of ≈120 μl); specifically, the equivalent of only ≈2 μl of amniotic fluid was used in each PCR assay (by contrast, 150 μl was used for aerobic and anaerobic cultures, and 250 μl for mycoplasma cultures). Second, although culture was performed immediately after sample collection, DNA extraction and molecular analyses were performed 1 to 9 years later, increasing the likelihood of DNA degradation.[103] In fact, the median time interval to DNA extraction for the group that was positive by culture but negative by PCR was 88 months [range 65–113], as compared to 74 months [18–115] for the group that was positive by PCR regardless of culture (P=0.03; Figure 7). Third, our molecular approach may have failed to detect phylotypes present in polymicrobial samples at low relative abundance because we sequenced only a limited number of clones per sample. Fourth, molecular findings may have been affected by PCR inhibition,[104] or by biases in DNA extraction,[104] PCR amplification efficiency,[104] or PCR primer specificity.[105] Last, we did not target viruses or non-fungal eukaryotic microbes. It is possible that some of these limitations (e.g., sample volumes) may have diminished the yield from PCR disproportionately; thus, our data are likely to underestimate the true microbial prevalence and diversity in preterm PROM. Further studies should include exploration of the role of viruses.

Implications of the findings

Randomized clinical trials of antibiotic administration to women with preterm PROM[106–114] indicate that antimicrobial agents can prolong pregnancy, and reduce the rate of proven neonatal sepsis and of clinical chorioamnionitis. However, recent observations indicate that the standard treatment recommended by professional societies does not eradicate or prevent MIAC in patients with preterm PROM.[115]

Knowledge of the microbial species involved in MIAC may be important to inform prognosis and therapy. First, inflammatory cytokines have been implicated in the pathogenesis of preterm birth;[17;116–140] however, fetal membrane cytokine response profiles are highly variable and may reflect stereotypic responses to divergent bacterial species.[102;141;142] Second, biofilm formation — a process known to diminish antibiotic efficacy — requires the expression of diverse genes[143] that may vary between microbial species. Indeed, recent observations suggest that biofilm formation occurs in MIAC[144] but the prevalence and nature of this association with respect to various microbial taxa remains largely unexplored. Third, polymicrobial infection may promote pathogen synergy as a result of the particular microbes involved,[145;146] or may enable horizontal transfer of antibiotic resistance genes between co-located species.[147;148] Our data indicate that most polymicrobial MIAC cases are misclassified by cultivation methods as either monomicrobial or free of microbial invasion altogether. Fourth, other poorly characterized factors, including the role of microbial products,[46;149–152] amniotic fluid "sludge"[153–155] and the poor transplacental passage of certain antibiotics such as erythromycin,[156;157] may have implications for pathogenesis or therapy of MIAC that vary with respect to the microbial species present. A deeper understanding of the microbial diversity of MIAC in preterm PROM and improved risk stratification may be necessary to develop more effective clinical strategies.

Our findings argue for additional detailed molecular studies of MIAC in the preterm parturition syndromes, and may inform the rational design of prophylactic and therapeutic interventions in patients with preterm PROM or who are otherwise at risk for preterm delivery (e.g. an elevated IL-6 or MMP-8 in amniotic fluid at the time of midtrimester amniocentesis[135]).

Conclusions

1) Molecular methods enable the detection of MIAC cases that are undiagnosed by cultivation techniques. 2) Greater microbial diversity is found in MIAC by sequence-based methods than by cultivation techniques. 3) Culture-negative cases of MIAC are associated with fetal inflammation, as assessed by amniotic fluid white blood cell count. 4) MIAC detected by molecular methods carries clinical significance, even in the setting of a negative culture; pregnancy and neonatal outcomes were similar whether MIAC was detected by molecular methods alone, or by cultivation methods.

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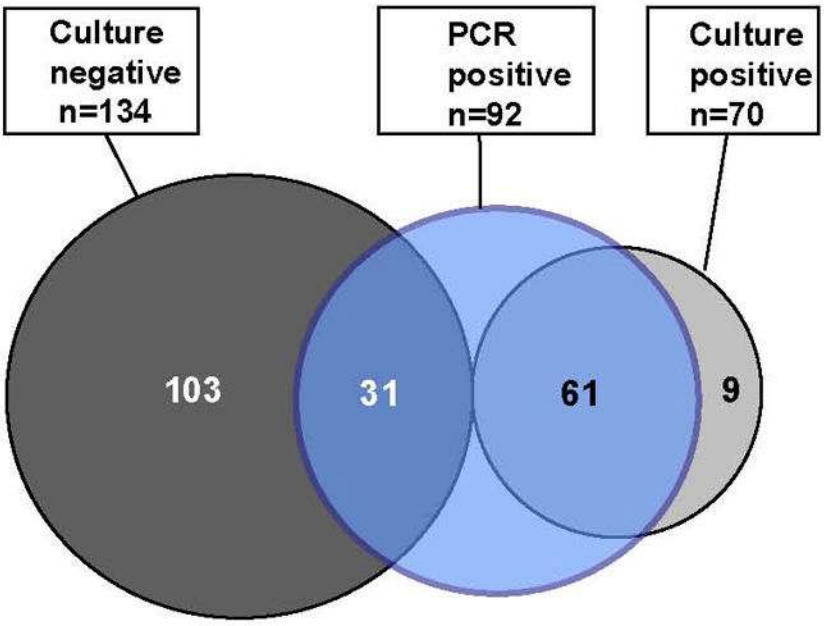

Figure 1. Distribution of subjects (n=204) according to results of PCR and culture of amniotic fluid

Data are from amniocentesis at presentation of 204 subjects for whom results from all culture assays were available. Culture refers to the aggregate results from routine cultivation methods for bacteria (aerobes, anaerobes and genital mycoplasmas) and for fungi. PCR refers to the aggregate results from end-point or real-time PCR targeting domain *Bacteria*, domain *Archaea*, genus *Candida*, and five specific bacterial groups (see Methods). Circle areas are not to scale.

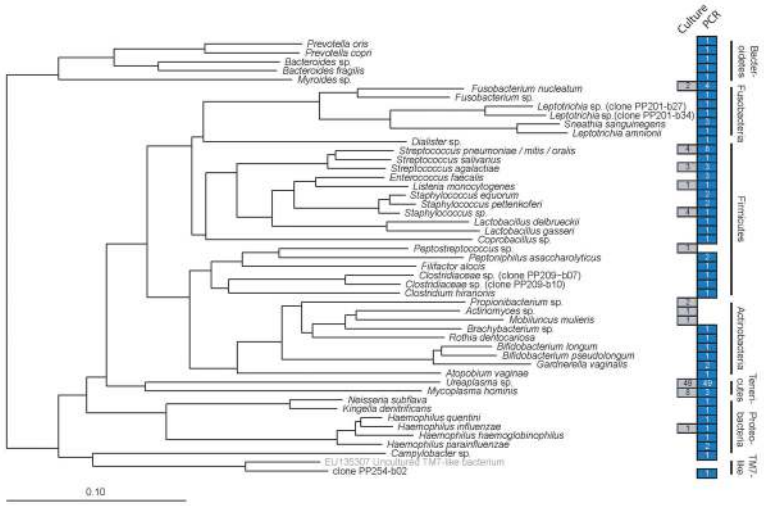

Figure 2. Bacterial taxa detected by PCR or culture

Phylogeny of the bacterial taxa identified in this study, based on a neighbor-joining algorithm with Felsenstein correction and a 682-column filter. The scale bar represents evolutionary distance (10 substitutions per 100 nucleotides). The taxon in brackets and gray type (uncultured TM7-like bacterium) is a public database sequence included for reference and was not detected in this study. Colored boxes indicate the number of subjects who were positive for a given taxon by culture (gray) or PCR (blue) (some samples were polymicrobial). Because culture isolates were not sequenced, each is represented by a GenBank sequence that corresponds to the taxonomic resolution to which culture isolates were phenotypically identified. Two culture isolates are not represented because they were not characterized to a sufficiently narrow taxonomic resolution to allow tree placement (viridans group streptococcus, and gram positive bacillus, each detected in a separate subject). *Candida* was the lone fungal genus detected in the study population (not shown in this figure).

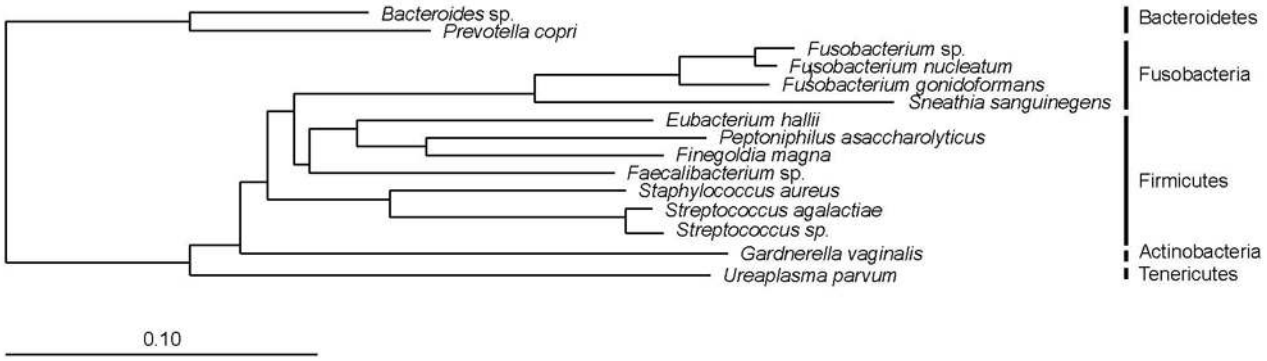

Figure 3. Bacterial taxa detected by PCR in those samples (n=26) for which culture data were incomplete

Phylogeny is based on a neighbor-joining algorithm with Felsenstein correction and a 682-column filter. The scale bar represents evolutionary distance (10 substitutions per 100 nucleotides).

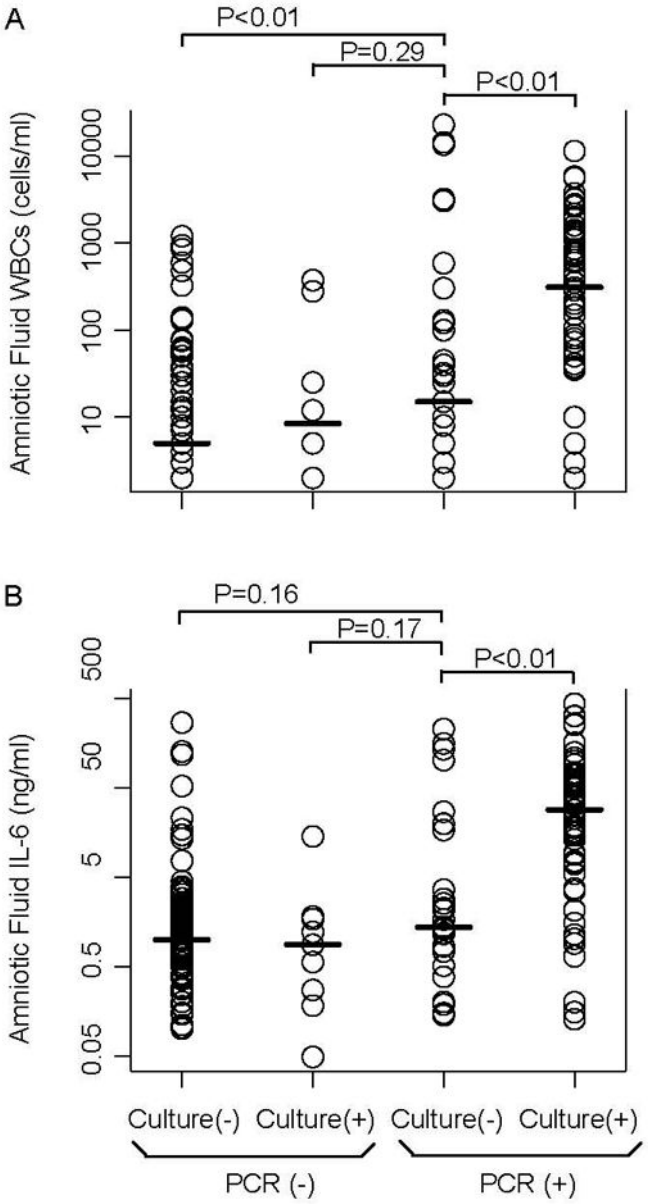

Figure 4. Inflammatory markers in amniotic fluid at presentation, according to PCR and culture results

Panel A presents white blood cell counts. **Panel B** presents interleukin-6 concentrations. For both panels, P values were calculated using the Mann-Whitney *U* test. Data are from amniocentesis at presentation of 204 subjects for whom results from all culture assays were available.

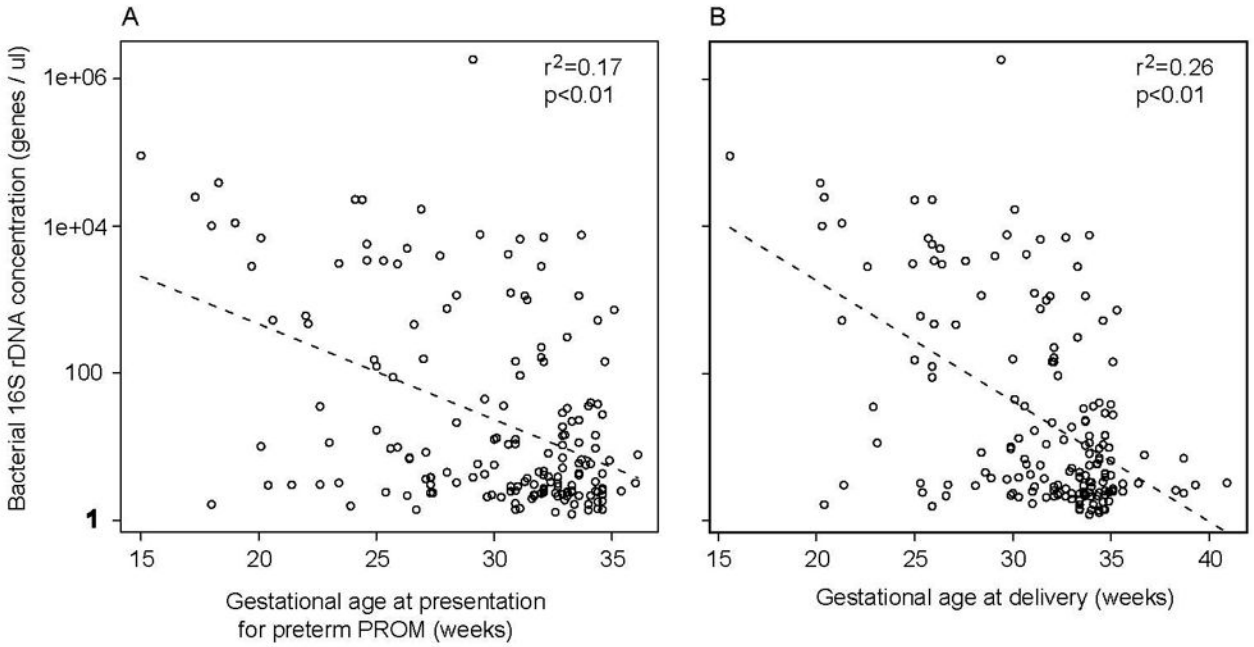

Figure 5. Correlation of bacterial 16S rDNA concentration with pregnancy outcomes

Panel A presents results for gestational age at which subjects presented with preterm PROM. **Panel B** presents results for gestational age at delivery.

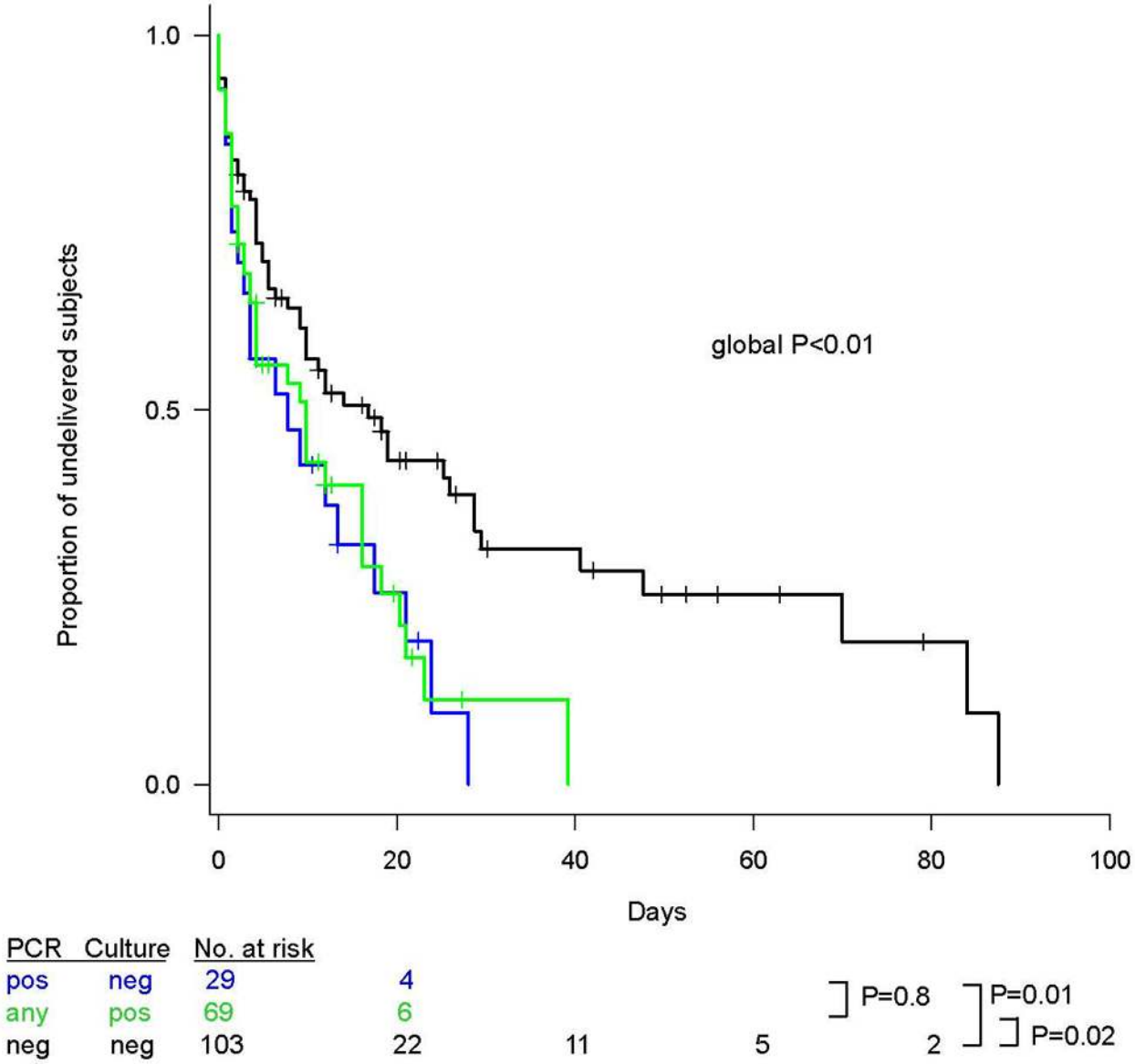

Figure 6. Kaplan-Meier survival plot of amniocentesis-to-delivery interval according to results of PCR and culture of amniotic fluid

Observations were right-censored for subjects who underwent cesarean section prior to labor onset, or who underwent labor induction. Prior to analysis, influential outliers, as defined by a dfbetas residual >3 standard deviations, were excluded (n=3). P values for differences in survival curves were calculated by means of the Mantel-Haenszel log-rank test.

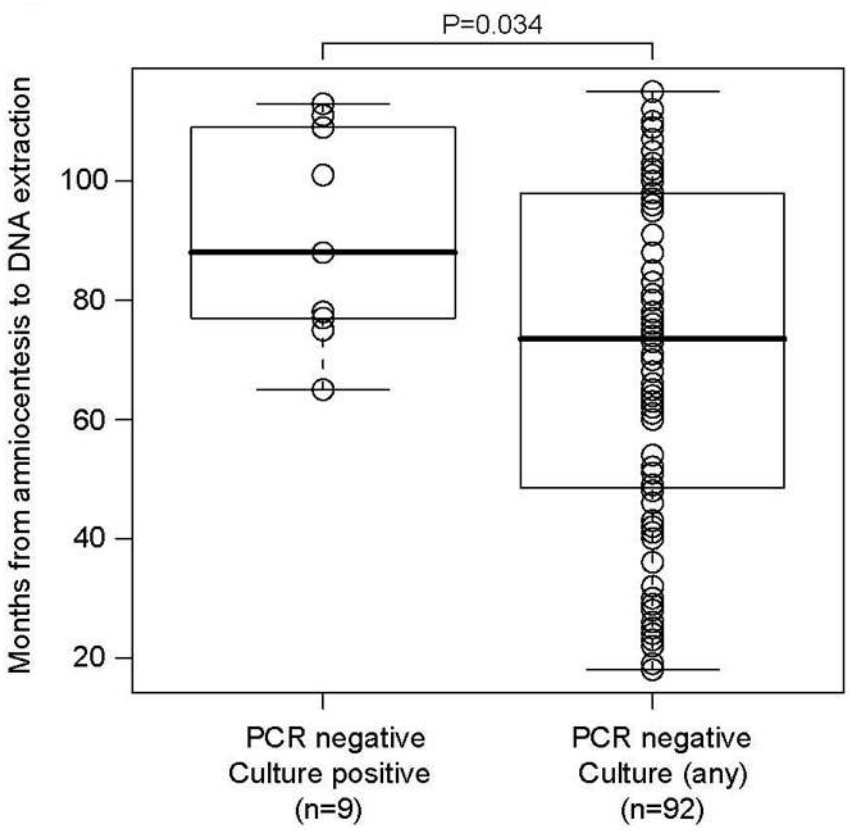

Figure 7.
Time interval from amniotic fluid collection to DNA extraction.

Table 1

PCR assays used in this study.

Approximate taxonomic level	End-point PCR taxonomic specificity	Lower detection limit (gene copies per μL)	Oligonucleotide name	Use	Sequence (5′ – > 3′)	Gene target	Reference
domain	*Bacteria*	100	Bact-8FM	FP	AGAGTTTGATCMTGGCTCAG	16S rDNA	53
			Bact-806R	RP	GGACTACCAGGGTATCTAAT		54
genus	*Ureaplasma*	10	Urease185F	FP	GCTGCTGACGTTGCAAGAAG	urease gene	present study
			Urease756R	RP	CTCCTGGTTCAAAACGAATAGC		present study
genus	*Fusobacterium*	100	Fuso-422F	FP	CGGAATGTAAAGTGCTTTC	16S rDNA	present study
			Fuso-710R	RP	CCCATCGGCATTCCTAC		present study
genus	*Sneathia/Leptotrichia*	10	SsLa-140F	FP	TAGACTGGGATAACAGAGG	16S rDNA	present study
			SsLa-406R	RP	AGTCCTAAAACCTTCTTCACAC		present study
species	*Streptococcus agalactiae*	10	Sag059F	FP	TTTCACCAGCTGTATTAGAAGTA	*cfb*	55
			Sag190R	RP	GTTCCCTGAACATTATCTTTGAT		55
species	*Mycoplasma hominis*	10	Mh-148F	FP	CAATGGCTAATGCCGGATACG	16S rDNA	mod. from 56
			Mh-463R	RP	GGTACCGTCAGTCTGCAATC		mod. from 56
genus	*Candida*	10	Cand-ITS2-42F	FP	GGGTTTGCTTGAAAGACGGTA	ITS2	present study
			Cand-ITS2-125R	RP	TTGAAGATATACGTGGTRGACGTTA		present study
	Real-time PCR taxonomic specificity	**Dynamic range (gene copies per μL)**					
domain	*Bacteria*	15 to 1e8	Bact-8FM	FP	AGAGTTTGATCMTGGCTCAG	16S rDNA	53
			Bact-338K *	Probe	CCAKACTCCTACGGGAGGCAGCAG		53
			Bact-515R	RP	TTACCGCGGCKGCTGGCAC		57
domain	*Archaea*	100 to 1e8	Arch333F	FP	TCCAGGCCCTACGGG	16S rDNA	58
			Univ-515F *	Probe	GTGCCAGCMGCCGCGGTAA		57
			Arch958R	RP	YCCGGCGTTGAMTCCAATT		59

FP = forward primer, RP = reverse primer, Probe = TaqMan probe

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

Table 2

Baseline characteristics of analyzable subjects (n=204), according to results of PCR and culture of amniotic fluid.

Characteristic	Negative culture and negative PCR (n=103)	P*	Negative culture but positive PCR (n=31)	P†	Positive culture (n=70)	Missing Culture Data (n=26)
Maternal age (y, mean ± SD)	29.4 ± 7.3	0.2	27.5 ± 8.0	0.6	28.4 ± 7.6	28.9 ± 7.1
Nulliparity (N=55)	19/103 (19%)	<0.05	12/31 (39%)	0.2	18/70 (26%)	6/26 (23%)
Cervical insufficiency (N=6)	1/103 (1%)	0.1	2/31 (7%)	0.6	3/70 (4%)	0/26 (0%)
Smoking (N=44)	18/103 (18%)	0.6	7/31 (23%)	0.2	8/70 (11%)	11/26 (42%)
Alcohol use (N=14)	7/103 (7%)	0.7	1/31 (3%)	1	2/70 (3%)	4/26 (15%)
Drug use (N=2)	2/103 (2%)	1	0/31 (0%)	NA	0/70 (0%)	0/26 (0%)

*Comparison of a negative culture and negative PCR with a negative culture but positive PCR.

†Comparison of a negative culture but positive PCR with a positive culture.

P values for comparison of mean maternal age were calculated using Student's t-test, and assumed unequal variances.

P values for proportions were calculated using Fisher's exact test.

Table 3

Relative risk of a positive PCR or culture for histologic chorioamnionitis or funisitis

Analysis was based on 146 subjects for whom data on histologic inflammation were available.

	Histologic Chorioamnionitis			Funisitis			Histologic Chorioamnionitis and/or Funisitis		
	Present (n=61)	Absent (n=85)	Relative risk (95% CI)	Present (n=39)	Absent (n=107)	Relative risk (95% CI)	Present (n=62)	Absent (n=84)	Relative risk (95% CI)
PCR positive (n=65)	38	27	2.1 (1.4 – 3.0)	25	40	2.2 (1.3 – 3.9)	39	26	2.1 (1.4 – 3.1)
Culture (n=46)	29	17	2.0 (1.4 – 2.7)	22	24	2.8 (1.7 – 4.7)	30	16	2.0 (1.4 – 2.8)

Table 4

Pregnancy outcomes according to results of PCR and culture of amniotic fluid.

	Negative culture and negative PCR (n=103)	P*	Negative culture but positive PCR (n=31)	P†	Positive culture (n=70)
Gestational age at presentation for pPROM	31.7 (18.0 – 36.1)	0.08	30.6 (18.3 – 35.4)	0.3	28.2 (15.0 – 35.1)
Gestational age at delivery	33.4 (20.4 – 40.9)	<0.05	31.9 (20.2 – 35.6)	0.08	29.6 (15.6 – 35.3)
Clinical Chorioamnionitis	4/103 (4%)	0.6	2/31 (7%)	0.3	10/70 (14%)

Gestational age reported as median (range)

*Comparison of a negative culture and negative PCR with a negative culture but positive PCR.

†Comparison of a negative culture but positive PCR with a positive culture.

P values for comparison of median gestational age at delivery were calculated using the Mann Whitney U test.

P values for proportions were calculated using Fisher's exact test.

Table 5

Perinatal outcomes according to results of PCR and culture of amniotic fluid.

Outcome	Negative culture and negative PCR n=103	P*	Negative culture but positive PCR n=31	P†	Positive culture n=70
Birth weight (g, mean ± SD)	2021 ± 596	<0.05	1682 ± 641	0.2	1488 ± 703
Admission to neonatal intensive care unit	81/101 (81%)	0.1	28/30 (93%)	1	54/58 (93%)
Bronchopulmonary dysplasia	5/101 (5%)	<0.05	5/30 (17%)	0.5	6/58 (10%)
Respiratory distress syndrome	16/101 (16%)	<0.05	11/30 (37%)	0.6	18/58 (31%)
Pneumonia	6/101 (6%)	1	1/30 (3%)	1	2/58 (3%)
Sepsis	37/101 (37%)	0.5	13/30 (43%)	0.2	34/58 (59%)
Necrotizing Enterocolitis	1/101 (1%)	<0.05	3/30 (10%)	0.3	2/58 (3%)
Intraventricular hemorrhage (≥grade III)	10/101 (10%)	0.7	2/30 (7%)	0.3	9/58 (16%)
Perinatal death	4/101 (4%)	0.08	4/30 (13%)	0.4	15/68 (22%)

*Comparison of a negative culture and negative PCR with a negative culture but positive PCR.

†Comparison of a negative culture but positive PCR with a positive culture.

P values for comparison of mean birth weight were calculated using Student's t-test, and assumed unequal variances.

P values for proportions were calculated using Fisher's exact test.