OBSTETRICS

Microbial invasion of the amniotic cavity in midtrimester pregnancies using molecular microbiology

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OBJECTIVE: The objective of the study was to determine the frequency of microbial invasion of the amniotic cavity in the midtrimester of pregnancy in patients undergoing amniocentesis for clinical indications.
STUDY DESIGN: This was a prospective investigation of the amniotic fluid of 344 asymptomatic women recruited in midpregnancy for the presence of microbial DNA. Amniotic samples obtained at the time of amniocentesis for genetic testing on women between 15 and 22 weeks of gestation were tested specifically for the presence of *Ureaplasma urealyticum*, *Ureaplasma parvum*, *Mycoplasma hominis*, and *Mycoplasma genitalium* as well as for other bacteria and fungi using broad-range polymerase chain reaction only. Pregnancy outcomes were reviewed independent of all molecular test results.
RESULTS: Using broad-range polymerase chain reaction, the prevalence of microbial invasion of the amniotic cavity in women between 15 and 22 weeks of gestation was 0% (0 vs 344). Early preterm delivery occurred in only 4 women (1%); 1 delivered electively and 3 spontaneously. None were associated with *Ureaplasma urealyticum*, *Ureaplasma parvum*, *Mycoplasma hominis*, or *Mycoplasma genitalium.* In addition, broad range polymerase chain reaction did not reveal the presence of other bacterial or fungal microbes.
CONCLUSION: Microbial invasion of the amniotic cavity in midtrimester gestations of low-risk pregnant women was not detected using molecular methods in 344 patients.

Key words: amniotic fluid, chorioamnionitis, fungi, intraamniotic infection, midtrimester, molecular microbiology, mycoplasma, preterm birth, sterile biological fluid, ureaplasma

Studies have investigated the presence of microorganisms in amniotic fluid among asymptomatic women from amniocentesis performed in the midtrimester for genetic screening purposes. Some[1-3] but not all[4] studies have suggested that *Ureaplasma urealyticum* or *Mycoplasma hominis* in the amniotic fluid may be associated with an increased risk for preterm prelabor rupture of membranes, preterm labor, and/or delivery.

The role of *Mycoplasma* species and their clinical significance in the amniotic fluid of asymptomatic women remain unresolved. Further studies are needed to understand the etiology and pathogenesis of preterm labor in relation to the presence or absence of asymptomatic intraamniotic microbial invasion.

We conducted a longitudinal study on a low-risk cohort of healthy asymptomatic women to determine the prevalence of intraamniotic microbial invasion and to examine their subsequent pregnancy outcomes. Amniotic fluid sampled between 15 and 22 weeks of gestation was assessed for the presence of microorganisms in the amniotic cavity by species-specific polymerase chain reaction (PCR) for *U urealyticum*, *U parvum*, *M hominis*, and *M genitalium*, and the presence of other bacterial and fungal pathogens using broad-range 16S and 18S PCR, respectively.

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Materials and Methods

Patient population

Women were recruited from 2 major tertiary referral centers in Melbourne, Australia (The Royal Women's Hospital, and The Mercy Hospital for Women) between September 2004 and December 2008. Study inclusion criteria were women undergoing amniocentesis for genetic prenatal diagnosis between 15 and 22 weeks of gestation in a singleton pregnancy. Women were excluded if a fetal abnormality was identified on ultrasound or if they had current clinical evidence of infection (of any type) for which they had received antibiotic treatment within the preceding 2 weeks.

Consecutive women were approached and the majority agreed to participate in the study. Gestation was established by ultrasound scan between 8 and 14 weeks. All women provided written informed consent. Ethics approval was received from the Research and Ethics Committees at both hospitals.

Sample processing

Amniotic fluid collected at the time of amniocentesis was stored at −80°C until all samples were collected and then processed over the following 1-2 years. Processing involved 2 different preparation methods prior to DNA isolation: (1) concentration of amniotic fluid whereby 1 mL was centrifuged at 14,000 × *g* for 15 minutes to ensure all biological particles were captured, with the resultant pellet resuspended in 200 μL of phosphate-buffered saline; and (2) 200 μL of neat (undiluted, noncentrifuged) amniotic fluid.

The automated MagNA Pure LC extraction system (Roche Diagnostics, Mannheim, Germany) was used to isolate DNA with the MagNA Pure LC DNA Isolation Kit I on amniotic fluid preparations 1 and 2. To improve the extraction efficiency, 33.3 μg/mL of poly(A) RNA carrier was added to the lysis buffer because of the potential for low concentrations of bacterial and fungal DNA in these samples.

Nucleic acid was eluted into a final volume of 100 μL. To determine cell

adequacy and as an internal control for extraction and amplification procedures, quantitative PCR (qPCR) targeting a 260 bp region of the human β-globin gene was performed.[5,6]

Clinical definitions and outcomes

Microbial invasion of the amniotic cavity was defined as the detection of any bacterial or fungal microorganism in the amniotic fluid using the species-specific and broad-range 16S and 18S assays outlined in the following text.

In assessing subsequent pregnancy outcomes; early preterm birth was defined as birth occurring prior to 34 completed weeks' gestation, preterm labor was defined as regular uterine contractions and cervical change at <34 completed weeks gestation, in which intervention such as tocolysis, cervical cerclage, or administration of prophylactic steroids for fetal lung maturation were considered to be indicated, and preterm prelabor rupture of membranes was defined as spontaneous rupture of membranes prior to the onset of labor and before 34 completed weeks' gestation.

Medical record reviews were used to determine delivery outcomes for women who delivered at the recruiting hospitals, while outcomes for women who delivered at a nonrecruiting hospital were obtained by telephone interview.

PCR assays used for detection of microbial infection of the amniotic cavity

PCR was performed for each sample using DNA extracted from both pelleted and neat amniotic fluid. Presence of *M genitalium* was assessed using a previously described real-time PCR assay,[7] whereas for *Ureaplasma* species adaptation of real-time PCR, using a Taqman probe 5'-TGGAAGGTGTAGATACAAT GGTTGGT-3', allowed detection of *U urealyticum* and/or *U parvum.*[8]

Amplification was performed in capillaries in a total volume of 10 μL consisting of 5 μL of DNA, 1 μM each primer U4 and U5, 0.5 μM probe, 4 mM $MgCl_2$, 1 × LightCycler Faststart Master HybProbe (Roche Diagnostics). Using the Roche LightCycler samples were heated at 95°C for 10 minutes and cycled 55 times using parameters of 95°C for 20 seconds, 55°C for 20 seconds, and 72°C for 30 seconds. Fluorescence was acquired once each cycle at the end of the extension segment. *M hominis* was assessed as previously described,[9] using the Roche LightCycler 480 (Roche Diagnostics).

A broad-range 16S ribosomal RNA gene qPCR assay was developed in our laboratory on the LightCycler (Roche Diagnostics) using previously described primers fD1mod and 16S1RR-B and a 515F Taqman probe.[10] The amplification reaction consisted of 10 μM of the primers fD1 mod and 16S1RR-B, 0.3 μM of Taqman probe 516F, 1 × LightCycler Faststart Reaction mix (Roche), 4.5 mM $MgCl_2$, and 1 U AmpliTaq DNA Polymerase LD in a total reaction volume of 10 μL. An activation step of 95°C for 2 minutes was followed by 45 cycles at 95°C for 5 seconds and 54–56°C for 10 seconds, increasing at increments of 0.1°C per cycle and 60°C for 30 seconds, at which fluorescence was acquired once each cycle. An 18S real-time qPCR assay targeting highly conserved regions of the fungal 18S rRNA gene enabling a wide range of fungi to be detected was performed as previously described.[11] Stringent precautions, including the use of gamma-irradiated PCR grade water, were used during the setup of broad-range 16S and 18S PCR assays to avoid potential contaminants, resulting in false-positive results.

Results

Overall, 408 women were enrolled: 375 from the Royal Women's Hospital and 33 from the Mercy Hospital for Women. The mean age of the women was 36.3 (22–47) years, with a mean gestation at amniocentesis of 17.1 (14.6–22) weeks.

Complete data on pregnancy outcomes were available for 355 women, while 53 were lost to follow-up. Eleven women underwent termination of pregnancy for an abnormal karyotype and were excluded from the final analysis. Of the 344 women who continued their pregnancy, 4 of 344 (1.2%) had an early preterm birth, prior to 34 weeks (Figure). One woman was delivered electively at 33 weeks' gestation for severe preeclampsia.

Three women delivered spontaneously at 30, 31, and 33 weeks gestation (Table). These 3 had vaginal and placental swabs (from the amniochorionic membrane interface) sent for culture. The woman who delivered at 30 weeks had a cervical cerclage inserted at 14 weeks. Although at delivery her vaginal swab was culture positive for *U urealyticum*, it was not isolated from the placental, amniotic or chorionic membrane swabs. Vaginal and placental swabs were negative in the 2 remaining women. There were no procedure-related pregnancy losses. One woman experienced transient amniotic fluid leak 2 days after her amniocentesis but continued her pregnancy uneventfully to term.

DNA was isolated from all 688 (344 pelleted, 344 fluid) samples, which were positive for the human β-globin gene. There was a significant difference in median β-globin level between pelleted (1290 haploid copies/reaction) and fluid (139 haploid copies/reaction) samples ($U = 21.041$, $P \leq .001$). Although all 688 samples were tested for each of the species specific assays described, no *U urealyticum, U parvum, M hominis*, or *M. genitalium* was detected. Moreover, none of the samples had detectable 16S rRNA or 18S rRNA genes by qPCR.

Comment

Principal findings of the study

This study sought to determine the frequency of asymptomatic carriage of microorganisms in the amniotic fluid in the midtrimester. We intensively investigated amniotic fluid for the presence of these microorganisms, with a particular focus on the genital mycoplasmas, previously reported to be associated with preterm birth.[2,4,12-15] Our assessment included both sensitive species-specific, and broad-range bacterial and fungal PCR assays, separately examining both pelleted and neat amniotic fluid. Our principal finding was that amniotic fluid in the midtrimester of pregnancy was found to be sterile in our population.

Moreover, our findings of no detectable microorganisms in the amniotic

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