We amplified bacterial 16S rRNA encoding DNA (rDNA) with the polymerase chain reaction (PCR) to detect amniotic fluid infection in 69 women in premature labor whose membranes were intact. Bacterial rDNA was detected by PCR in samples from 15 (94%) of 16 patients with positive amniotic fluid cultures. Bacteria were detected by PCR in samples from 5 (36%) of 14 patients with negative cultures and elevated interleukin (IL)-6 levels vs. 1 (3%) of 39 patients with negative cultures and IL-6 levels of ≤2,000 pg/mL (P < .01). The median amniotic fluid cytokine levels and the pregnancy outcomes were similar for patients with positive amniotic fluid cultures and those with negative cultures and positive rDNA PCR assays. The association between amniotic fluid infection and premature labor may be underestimated on the basis of amniotic fluid culture results. The broad-spectrum bacterial 16S rDNA PCR assay may prove useful for diagnosing amniotic fluid infection.

Preterm birth is the leading cause of neonatal morbidity and mortality in the United States [1]. The association between premature labor and amniotic fluid infection is well established [2–5]. Women in premature labor whose amniotic fluid cultures are positive have high amniotic-fluid levels of IL-6 and other proinflammatory cytokines [4, 5]. However, 40%–70% of patients with elevated IL-6 levels have negative amniotic fluid cultures [4, 5]. Women in premature labor with either positive amniotic fluid cultures or elevated IL-6 levels invariably give birth prematurely, often within 1–3 days of presentation, while patients with negative cultures and low IL-6 levels usually give birth weeks later [4, 5]. Elevated IL-6 levels in culture-negative amniotic fluid could be the result of amniotic fluid infection that was not detected by culture because of unculurable microorganisms, a low infectious inoculum, or previous antibiotic treatment. Amplification of bacterial 16S rRNA encoding DNA (rDNA) by PCR is a sensitive technique for detecting bacteria that may not be isolated in culture, and PCR has been used to detect unculurable bacteria causing other infectious processes [6–8].

To gain insight into the relationships between amniotic fluid infection, amniotic fluid cytokine levels, and preterm delivery, we examined amniotic fluid samples from women in premature labor by using both optimal culture techniques [2] and a broad-spectrum bacterial 16S rDNA PCR assay. We tested the hypothesis that an elevated IL-6 level in culture-negative amniotic fluid is associated with occult amniotic fluid infection, as detected by PCR.

Methods

Study population. We performed bacterial 16S rDNA PCR assays on amniotic fluid samples from 69 afebrile women who were selected from a cohort of 200 patients admitted to our hospital in premature labor between 1 August 1991 and 30 June 1995. The study population was selected to oversample patients with proven or possible amniotic fluid infection. Amniotic fluid was still available for 132 patients after cultures and cytokine assays were completed; bacterial rDNA PCR was performed on samples from all 16 women with positive cultures, all 14 women with negative cultures and IL-6 levels of >2,000 pg/mL, and 39 (38%) of 102 women with negative cultures and IL-6 levels of ≤2,000 pg/mL. The 39 patients with negative cultures and low IL-6 levels were enrolled at later gestational ages than the women who were not included (median gestational age, 32 weeks vs. 31 weeks, respectively; P < .05), but the two groups did not otherwise differ significantly in terms of demographic or reproductive characteristics.

All participants were at gestational ages of 22–34 weeks, as determined by menstrual dating or by ultrasonography. Premature labor was defined as uterine contractions occurring

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Informed consent was obtained from the patients, and the study was approved by the University of Washington Institutional Review Board.

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at least every 10 minutes, with cervical dilatation of \( \geq 1 \) cm or effacement of \( \geq 50\% \). Women with ruptured membranes were excluded. Nine patients had multiple gestations. For
these women, amniotic fluid samples were analyzed only from the presenting sac. Labor records were abstracted after delivery.

Ultrasonographically guided transabdominal amniocentesis was performed with use of sterile technique. Five (31\%) of 16
patients with positive amniotic fluid cultures, three (21\%) of 14 patients with negative cultures and high IL-6 levels, and four (10\%) of 39 patients with negative cultures and low IL-6 levels received antimicrobial treatment before they underwent amniocentesis (\( P = .2 \)).

**Amniotic fluid studies.** Amniotic fluid was injected into an anaerobic transport vial (Port-A-Cul; BBL, Cockeysville, MD) and inoculated onto culture media for aerobic and anaerobic bacteria and genital mycoplasmas within 12 hours of collection. The methods for culture and identification of organisms were designed to detect low quantities of bacteria through the use of broth enrichment. These methods have been previously described in detail [2].

The remaining amniotic fluid was stored at \(-70^\circ\)C in pyrogen-free containers. TNF-\( \alpha \) and IL-6 levels were determined by commercial ELISAs specific for each cytokine (Genzyme Diagnostics, Cambridge, MA). The mean of two duplicate 25-\( \mu \)L samples was calculated. The coefficient of variation between duplicate samples was \( \leq 20\% \). For each cytokine assay, standard positive controls of recombinant TNF-\( \alpha \) or IL-6 in buffered solution were run simultaneously with the study specimens. The lower limits of detection were 6 pg/mL for TNF-\( \alpha \) and 69 pg/mL for IL-6.

Before PCR amplification, 0.5-\( \mu \)L samples of amniotic fluid were centrifuged at 1,000 \( g \) for 10 minutes. The pellets were resuspended in 50 \( \mu \)L of 1 mg/mL proteinase K in buffer to inactivate inhibitors [9] and incubated at 37\(^\circ\)C for 2 hours, followed by 20 minutes at 95\(^\circ\)C to inactivate the proteinase K. Bacterial \( rDNA \) sequences were amplified by PCR using the primers 91E:GGAATTCAAACGAATTGACGGGGGC and 13B:CGGGATCCCGAGCCGGGAACGTATTCAC at concentrations of 1 \( \mu \)M each [8]. The corresponding *Escherichia coli* positions for the primers are 911–930 for 91E and 1390–1471 for 13B [10]. These primers produce a 475-bp product containing species-variable \( rDNA \) sequences.

Amplification was accomplished with use of Deep-Vent DNA polymerase (1 unit in 50 \( \mu \)L; New England Biolabs, Beverly, MA). The reaction contained 10 mM KCl, 10 mM \( \left( NH_4\right)_2SO_4 \), 10 mM Tris-HCl (pH 8.8), 2 mM MgSO\(_4\), 0.1% Triton X-100 (Union Carbide, distributed by Sigma, St. Louis), and 200 \( \mu \)M of each dNTP, as previously described [11]. Positive samples were amplified a second time, with use of Taq polymerase (1 unit), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl\(_2\), and 200 \( \mu \)M of each dNTP. All reagents were pretreated for 15 minutes with ultraviolet irradiation (245 nm) to inactivate contaminants. Results were considered definitive only if duplicate negative controls did not exhibit any signals and positive controls exhibited signals.

Thermal cycles consisted of 5 minutes at 94\(^\circ\)C, then 30 cycles of sequential variation at 94\(^\circ\)C, 60\(^\circ\)C, and 72\(^\circ\)C (1 minute each) followed by a 7-minute extension cycle at 72\(^\circ\)C. Amplification products were detected by electrophoresis on 1.4\% agarose gels with 10 \( \mu \)g/mL ethidium bromide staining and ultraviolet illumination. Reactions were set up in a separate area from all other activities involving amplified DNA. Pipettes with aerosol-resistant tips were used [12]. Personnel who performed the PCR assays had no knowledge of culture results or clinical findings.

**Statistical analyses.** We used the \( \chi^2 \) test or Fisher’s exact test (two-tailed) for categorical analyses. Differences between groups for IL-6, TNF-\( \alpha \), and pregnancy outcome variables were examined by using nonparametric techniques. Confidence intervals for medians were derived from the ranks of the variables [13]. We used the Kruskal-Wallis analysis of variance to assess global differences among groups, followed by paired comparisons with use of the Mann-Whitney \( U \) test with the Bonferroni correction.

**Results**

Bacterial 16S \( rDNA \) was detected by PCR in samples from 15 (94\%) of 16 patients with positive cultures, 5 (36\%) of 14 patients with negative cultures and elevated IL-6 levels (>2,000 pg/mL), and 1 (3\%) of 39 patients with negative cultures and low IL-6 levels (\( P < .001, \chi^2 \) test for trend). Isolates from PCR-positive specimens included group B streptococci, *Enterococcus* species, *E. coli*, *Klebsiella pneumoniae*, *Mycoplasma hominis*, *Gardnerella vaginalis*, *Fusobacterium nucleatum*, *Bacteroides ureolyticus*, *Prevotella oulora*, *Clostridium* species, and *Peptostreptococcus asaccharolyticus*. Six (37\%) of 16 patients with positive amniotic fluid cultures had more than one organism isolated from the amniotic fluid. Most of these mixed cultures yielded anaerobic flora.

Table 1 summarizes the findings in gram stains of amniotic fluid for patients with positive cultures, negative cultures but positive PCR assays, and negative cultures and negative PCR assays. Polymorphonuclear leukocytes (PMNs) and bacteria were detected more frequently by gram staining for patients containing species-variable \( rDNA \) sequences.

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Table 1. Findings in gram-stains of amniotic fluid from patients in premature labor who had positive amniotic fluid cultures, negative amniotic fluid cultures and positive PCR assays, and negative amniotic fluid cultures and negative PCR assays.

<table>
<thead>
<tr>
<th>Finding</th>
<th>No. with indicated finding/no. with positive culture (%)</th>
<th>No. with indicated finding/no. with negative culture, positive PCR (%)</th>
<th>No. with indicated finding/no. with negative culture, negative PCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymorphonuclear leukocytes</td>
<td>13/16 (81)</td>
<td>4/6 (67)</td>
<td>10/47 (21)*</td>
</tr>
<tr>
<td>Bacteria</td>
<td>11/16 (69)</td>
<td>2/6 (33)</td>
<td>1/47 (2)*</td>
</tr>
</tbody>
</table>

* Global $\chi^2$ test (2 df); $P < .001$. The two-tailed Fisher’s exact test was used to compare the negative-culture, positive-PCR group with the negative-culture, negative-PCR group (1 df); $P < .05$. No significant difference was detected between the positive-culture group and negative-culture, positive-PCR group.

The median IL-6 and TNF-α levels detected for patients with positive amniotic fluid cultures were similar to those for patients with negative cultures and positive PCR assays. The median IL-6 and TNF-α levels for these two groups were significantly higher than the median levels for patients with both negative cultures and negative PCR assays ($P < .01$ for pairwise comparisons). Women with either positive cultures or positive PCR assays presented in premature labor at earlier gestational ages than did patients with negative cultures and negative PCR assays ($P < .01$). The intervals between enrollment and delivery were shorter for patients with infected amniotic fluid, detected by either culture or PCR, and these patients gave birth at earlier gestational ages and had infants with lower birthweights than did patients with both negative cultures and negative PCR assays.

The patient with a positive culture and negative PCR assay had *Stomatococcus* species (a gram-positive coccus occasionally found in the mouth flora) isolated from the amniotic fluid. The IL-6 level in this patient was 5,966 pg/mL, and the TNF-α level was 259 pg/mL; PMNs and gram-positive cocci were identified in the gram stain of her amniotic fluid. We did detect bacterial 16S rDNA on amplification of 5 ng of DNA stock obtained directly from the positive culture. The patient with a positive PCR assay, negative culture, and low IL-6 level (470 pg/mL) did not have measurable TNF-α present and did not have PMNs or bacteria detected in the gram stain of her amniotic fluid.

We calculated the sensitivity and specificity of amniotic fluid culture, gram staining, and PCR for the detection of amniotic fluid bacteria by using an expanded “gold standard” (table 3).

Table 2. Amniotic fluid cytokine levels and pregnancy outcomes for patients in premature labor who had positive amniotic fluid cultures, negative amniotic fluid cultures and positive PCR assays, and negative amniotic fluid cultures and negative PCR assays.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median (95% CI) value for indicated group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive culture ($n = 16$)</td>
</tr>
<tr>
<td>Cytokine level (pg/mL)</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>26,005 (2,897–46,400)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>892 (104–1,101)</td>
</tr>
<tr>
<td>Pregnancy outcome</td>
<td></td>
</tr>
<tr>
<td>No. of weeks’ gestation</td>
<td></td>
</tr>
<tr>
<td>Enrollment</td>
<td>26 (25–31)</td>
</tr>
<tr>
<td>Delivery</td>
<td>26 (25–31)</td>
</tr>
<tr>
<td>No. of days to delivery</td>
<td>1 (1–2)</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>890 (733–1,542)</td>
</tr>
</tbody>
</table>

* Kruskal-Wallis analysis of variance (ANOVA) ($P < .001$) was used for global differences. The paired Mann-Whitney $U$ test with the Bonferroni correction ($P < .01$) was used for the difference between the negative-culture, positive-PCR group and the negative-culture, negative-PCR group.

1 Kruskal-Wallis ANOVA ($P < .01$) was used for global differences. The paired Mann-Whitney $U$ test with the Bonferroni correction ($P < .05$) was used for the difference between the negative-culture, positive-PCR group and the negative-culture, negative-PCR group.
Table 3. Diagnostic indices for the detection of amniotic fluid infection.

<table>
<thead>
<tr>
<th>Amniotic fluid test or finding</th>
<th>No. of specimens positive by test/ total no. positive</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>16/21</td>
<td>76</td>
<td>100</td>
</tr>
<tr>
<td>Bacteria in gram stain</td>
<td>13/21</td>
<td>62</td>
<td>98</td>
</tr>
<tr>
<td>PMNs in gram stain</td>
<td>17/21</td>
<td>81</td>
<td>79</td>
</tr>
<tr>
<td>16S rDNA PCR assay</td>
<td>20/21</td>
<td>95</td>
<td>98</td>
</tr>
</tbody>
</table>

NOTE. PMN = polymorphonuclear leukocyte.

True positives were considered to be all patients with either a positive culture or a positive PCR and an IL-6 level of >2,000 pg/mL, on the basis of the previously reported sensitivity of elevated amniotic fluid IL-6 levels for detecting amniotic fluid infection [4, 5]. On the basis of this definition, PCR had a sensitivity of 95% and a specificity of 98% for detecting amniotic fluid infection, while amniotic fluid culture had a sensitivity of 76% and a specificity of 100%. The detection of bacteria in a gram stain of amniotic fluid was specific but less sensitive than amniotic fluid culture. The presence of PMNs in a gram stain was more sensitive than amniotic fluid culture but less sensitive and much less specific than PCR.

Discussion

The results of our study suggest that up to one-third of women in premature labor who have negative amniotic fluid cultures and elevated IL-6 levels have amniotic fluid infection, as demonstrated by the bacterial rDNA PCR assay. Possible reasons for negative amniotic fluid cultures and positive PCR assays might include a low infectious inoculum, difficulty in isolating fastidious microorganisms [2], or the presence of un-culturatable bacteria, as has been found for other infectious processes [8]. The frequency of antibiotic treatment before amniocentesis was higher for the culture-positive group than for the culture-negative, PCR-positive group, and this finding suggests that antibiotic pretreatment did not account for the positive PCR assays for women with negative cultures.

It seems likely that a positive 16S rDNA PCR assay indicates amniotic fluid infection. Patients with negative cultures but positive PCR assays had elevated IL-6 and TNF-α levels and frequently had PMNs present in amniotic fluid. Patients with negative cultures but positive PCR assays presented at similar gestational ages and gave birth as rapidly after admission as did patients with positive cultures.

The patient from whom Stomatococcus was isolated and who had a negative PCR assay probably had amniotic fluid infection since high IL-6 and TNF-α levels were detected and bacteria were seen in the gram stain. Even though this organism is an unusual pathogen, amplification of 16S rDNA in the sample taken directly from the culture implies that detection of the organism in the amniotic fluid sample should have been possible. One patient with a negative culture and low cytokine levels probably had a false-positive PCR assay. When we used an expanded gold standard developed for the purpose of comparing diagnostic techniques, we found that the sensitivity of PCR (95%) was higher than that of culture (76%) for the detection of amniotic fluid infection and that the specificities of the two methods were equivalent.

Optimal culture methods were used to recover anaerobic bacteria and genital mycoplasmas. In an earlier study, split samples of amniotic fluid were cultured in our research laboratory as well as in the general clinical microbiology laboratory. Less than 50% of the anaerobes isolated with use of our techniques were identified by the clinical laboratory [2]. The sensitivity of amniotic fluid culture performed in a general clinical microbiology laboratory could therefore be expected to be lower.

The major limitation of the 16S rDNA PCR assay is the difficulty associated with identifying the specific microorganisms detected in amniotic fluid. Our study and others [2, 3] indicate that amniotic fluid infection is often polymicrobial. The polymicrobial nature of amniotic fluid infection makes specific identification of the PCR products challenging. While a direct sequencing approach is useful for the identification of a PCR product from one organism, the presence of bacterial rDNA from two or more species makes sequencing ladders difficult to interpret.

Specific identification of bacteria from a polymicrobial infection such as amniotic fluid infection involves cloning the individual PCR products before sequencing. At present, this process is time-consuming and requires serial cloning and sequencing procedures. An alternative approach might be to develop a customized battery of probes that would allow identification of the more common bacteria that cause amniotic fluid infection. The combination of broad-spectrum bacterial rDNA PCR amplification followed by the use of targeted probes or sequencing techniques may offer the potential for rapid, sensitive detection of amniotic fluid infection in the future.

The association between amniotic fluid infection and preterm delivery is likely to be underestimated on the basis of amniotic fluid culture results, even when optimal methods for the recovery of anaerobic bacteria and genital mycoplasmas are used. The 16S rDNA PCR assay provides evidence of bacterial infection in up to one-third of women in premature labor who have elevated IL-6 levels and negative amniotic fluid cultures. A broad-spectrum bacterial PCR assay may prove to be a useful alternative to culture for the diagnosis of amniotic fluid infection in women in premature labor. The pathophysiology linking elevated amniotic fluid cytokine levels and preterm delivery for patients with negative amniotic fluid cultures and negative bacterial PCR assays requires the study of other infectious agents and inflammatory conditions.
References