Polymicrobial Amniotic Fluid Infection with *Mycoplasma/Ureaplasma* and Other Bacteria Induces Severe Intra-Amniotic Inflammation Associated with Poor Perinatal Prognosis in Preterm Labor

Noriko Yoneda[1], Satoshi Yoneda[1], Hideki Niimi[2], Tomohiro Ueno[2], Shirou Hayashi[2], Mika Ito[1], Arihiro Shiozaki[1], Daichi Urushiyama[3,4], Kenichiro Hata[3], Wataru Suda[5], Masahira Hattori[5,6], Mika Kigawa[7], Isao Kitajima[2], Shigeru Saito[1]

[1]Department of Obstetrics and Gynecology, University of Toyama, Toyama, Japan;
[2]Clinical Laboratory Center, Toyama University Hospital, Toyama, Japan;
[3]Department of Maternal-Fetal Biology, National Research Institute for Child Health and Development, Tokyo, Japan;
[4]Department of Obstetrics and Gynecology, Faculty of Medicine, Fukuoka University, Fukuoka, Japan;
[5]Laboratory of Metagenomics, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Japan;
[6]Graduate School of Advanced Science and Engineering, Waseda University, Tokyo, Japan;
[7]Department of Public Health Faculty of Medicine, University of Toyama, Toyama, Japan

Keywords
Eukaryote-made thermostable DNA polymerase, intra-amniotic infection, polymerase chain reaction , polymicrobial infection, preterm labor, *Ureaplasma* spp

Correspondence
Shigeru Saito, Professor and Chair, Department of Obstetrics and Gynecology, University of Toyama, 2630 Sugitani, Toyama, Toyama 930-0194, Japan.
E-mail: s30saito@med.u-toyama.ac.jp

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Problem
To study the relationship between perinatal prognosis in cases of preterm labor (PTL) and polymicrobial infection in amniotic fluid (AF) and intra-amniotic (IA) inflammation using a highly sensitive and reliable PCR-based method.

Method of Study
To detect prokaryotes using a nested PCR-based method, eukaryote-made thermostable DNA polymerase without bacterial DNA contamination was used in combination with bacterial universal primers. We collected AF aseptically from 118 PTL cases and 50 term subjects.

Results
The prevalence of microorganisms was 33% (39/118) by PCR and only 7.6% (9/118) by culture. PTL caused by a combination of positive *Mycoplasma/Ureaplasma* and other bacteria had significantly higher AF IL-8 levels and a significantly shorter amniocentesis-to-delivery interval.

Conclusions
Our newly established PCR method is useful for detecting IA microorganisms. Polymicrobial infection with *Mycoplasma/Ureaplasma* and other bacteria induces severe IA inflammation associated with poor perinatal prognosis in PTL.

Introduction

Preterm birth is the leading cause of perinatal mortality, perinatal morbidity, and childhood neurological morbidity.[1–3] The preterm birth rate is 11.1% of all livebirths worldwide, and it is increasing in most countries.[1,2] The causes of preterm birth before 28 weeks of gestation appear to be multifactorial,

but many studies indicate that microbial invasion into a pregnant uterus is one of the important risk factors for preterm birth.[4–7] *Mycoplasma* and *Ureaplasma* spp are among the organisms most frequently isolated from both placenta and amniotic fluid (AF) in spontaneous preterm delivery.[8–10]

The standard microbial culture method for detecting microorganisms, such as *Mycoplasma, Ureaplasma,* and other aerobic or anaerobic bacteria, requires 1 week to obtain the results. Moreover, it is difficult to detect *Mycoplasma/Ureaplasma* by conventional culture systems.

Recently, molecular microbiology techniques based on the polymerase chain reaction (PCR) have emerged as a rapid and sensitive methods for the detection of microorganisms, such as *Mycoplasma* and *Ureaplasma* (e.g., *U. urealyticum* and *U. parvum*) in clinical samples, including AF.[8–16] Indeed, it has been observed that the detection rate of microorganisms in AF by PCR-based methodology is higher than by standard microbial cultures.[13,14,17–22] However, concerning bacterial universal PCRs, it is difficult to obtain highly sensitive and reliable detection of other bacteria (almost all kinds of bacteria other than *Mycoplasma* and *Ureaplasma*) with a clear negative control. This is because the commercially available thermostable DNA polymerase (e.g., recombinant *Taq polymerase*) contains host (prokaryote)-derived trace amounts of bacterial DNA, and therefore, false positives are detected when PCR cycles increase.[23]

We have established a nested PCR-based method using eukaryote-made thermostable DNA polymerase that is free from bacterial DNA contamination.[23] Using the eukaryote-made thermostable DNA polymerase, a sensitive and reliable detection of other bacteria has become feasible. For the detection of microorganisms in the AF of preterm labor (PTL) cases, we have established a methodology for detecting *Mycoplasma*, *Ureaplasma*, other bacteria, and fungi using devised primer sets.[24]

In this study, using this sensitive and reliable PCR-based method, we evaluated the microbial footprints for *Mycoplasma/Ureaplasma*, other bacteria, and fungi in the AF of patients with PTL. In particular, we compared the infection results with various maternal characteristics, placental histological findings, AF interleukin-8 (IL-8) levels, and neonatal outcomes. The main focus in this study was which microorganisms are associated with severe intrauterine inflammation causing preterm delivery.

Materials and methods

Study Design

The study population consisted of 168 patients who were admitted to the participating institution between November 2001 and January 2014 with the diagnosis of PTL ($n = 118$), term cesarean section ($n = 50$), and singleton gestation and who underwent amniocentesis for the assessment of microbiologic status of AF. The patients met diagnostic criteria for PTL, that is, the presence of regular uterine contractions (6/hour or more) and changes in cervical dilatation or cervical length. Gestational age ranged between 18 and 40 weeks. At Toyama University Hospital, amniocentesis is offered to patients admitted with the diagnosis of PTL and intact membranes. We excluded cases with preterm premature rupture of membranes (PPROM), congenital abnormalities, multiple pregnancy, and maternal severe complications such as diabetes mellitus, preeclampsia, placenta previa, placental abruption. The indications for delivery in PTL patients in our institute are onset of labor, clinical chorioamnionitis (CAM), and non-reassuring fetal status.

Composite severe neonatal morbidity was defined as intraventricular hemorrhage (IVH) ≧grade 2, bronchopulmonary dysplasia (BPD), and necrotizing enterocolitis (NEC) during hospitalization in the neonatal care unit. Written informed consent was obtained from all subjects to donate AF for research purposes. The study was conducted with the approval of the institutional review board of our institution.

AF Sample Collection and DNA Extraction

A total of 5–10 mL AF samples were collected from pregnant women in PTL by transabdominal amniocentesis, or at the time of term cesarean section at Toyama University Hospital. In cases of cesarean section, amniocentesis was performed before cutting the fetal membrane.

One millilitre of AF or, in the case of extraction control, 1 mL of distilled water (water deionized and sterilized for molecular biology; Nacalai Tesque, Inc. Kyoto, Japan) was centrifuged at 20,000× ***g*** for 20 min to spin down the microorganisms, and 950 μL of the supernatant fraction was carefully removed so as not to rupture the pellet, leaving the pellet with 50 μL of supernatant. DNA was

isolated from the resulting pellet using a DNA extraction kit (High Pure PCR Template Preparation Kit, Roche Applied Science, Basel, Switzerland) in accordance with the supplier's instructions. Finally, the bacterial DNA was eluted with 100 μL of elution buffer.

Nested PCR Assays for Detecting *Mycoplasma*, *Ureaplasma,* and Other Bacteria

The following is the nested PCR procedure (first PCR: 30 cycles ➡ dilute 500-fold ➡ second, nested PCR: 30 cycles). The LightCycler Nano (Roche Applied Science) was used for the amplification and real-time detection of the target DNA. We used 1.5-mL PCR-clean Eppendorf tubes that were RNase and DNase free (Eppendorf, Hamburg, Germany) and 0.1-mL PCR tubes (Roche Diagnostics). All oligonucleotide primers were designed using a multiple alignment software program (Clustal X) comparing more than 200 kinds of bacterial 16S rRNA sequences and were synthesized by Life Technologies Japan, Ltd. (Tokyo, Japan). The primer information has been described previously.[24]

During the first PCR procedure, all reactions were performed in one tube. The PCR reaction mixture (20 μL) contained 2 μL of DNA template or, as a positive control, 2 μL (8.0 ng/μL) of DNA extracted from *Escherichia coli* (ATCC 25922) or, as a negative control for the PCR step, 2 μL of distilled water (water deionized and sterilized for molecular biology; Nacalai Tesque, Inc.) in 200 μM of each dNTP (CleanAmp Hot Start dNTP Mix, Sigma-Aldrich, St. Louis, MO, USA) filtered using an Amicon Ultra 50K centrifugal filter (Merck Millipore, Darmstadt, Germany), 50 mM KCl, 2.25 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 0.3 μM each of Bacterial Universal Primer for 1st PCR, 1×EvaGreen (Biotium Inc. CA, USA), and 1.0 unit (0.5 μL) of eukaryote-made thermostable DNA polymerase supplemented with stock buffer solution. The generation of eukaryote-made thermostable DNA polymerase using *Saccharomyces cerevisiae* has been described previously.[23]

The sample was incubated for 5 min at 95°C to activate the Hot Start dNTPs, then was denatured for 10 s at 95°C, annealed for 15 s at 57°C, extended for 30 s at 72°C, and subjected to fluorescence acquisition for 2 s at 82°C for 30 cycles. The PCR product was diluted 500-fold with distilled water (water deionized and sterilized for molecular biology; Nacalai Tesque, Inc.) and then used as a template for the second (nested) PCR procedure. Even if no amplification curve was observed by the 30th cycle in the first PCR, the second (nested) PCR was still performed.

For the second (nested) PCR, all reactions were performed in four tubes for detecting bacteria, *Mycoplasma*, *Ureaplasma*, and bacteria other than *Mycoplasma* and *Ureaplasma*, respectively. The PCR reaction mixture (20 μL) contained 8 μL of DNA template of the diluted first PCR product in 200 μM of each dNTP (CleanAmp Hot Start dNTP Mix; Sigma-Aldrich) filtered using an Amicon Ultra 50K centrifugal filter (Merck Millipore), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 0.25 μM each of each primer (Bacterial Universal Primer for 2nd PCR, Mycoplasma-specific primer, Ureaplasma-specific primer, NotMycoUrea Bacterial Universal Primer), 1×EvaGreen (Biotium, Inc., Hayward, USA), and 1.0 unit (0.5 μL) of eukaryote-made thermostable DNA polymerase supplemented with stock buffer solution. Each sample was incubated for 5 min at 95°C to activate the Hot Start dNTPs, then denatured for 10 s at 95°C, annealed for 15 s at 57°C, extended for 10 s at 72°C, and subjected to fluorescence acquisition for 2 s at 82°C for 30 cycles. If no amplification curve was observed by the 30th cycle in the second (nested) PCR, we defined the sample as containing no bacteria other than *Mycoplasma*/*Ureaplasma*. The presences of *Mycoplasma*, *Ureaplasma,* and/or other bacteria were judged according to the real-time detection of target DNA. In addition, amplicons were further confirmed by agarose gel electrophoresis (2% agarose gel, ethidium bromide staining) or microchip electrophoresis (MCE-202 MultiNA; Shimadzu, Tokyo, Japan). The term 'other bacteria' means aerobic, anaerobic bacteria except for *Mycoplasma*/*Ureaplasma*.

PCR Assays for Detecting Fungi

To detect fungi, the LightCycler Nano (Roche Applied Science) and PCR-clean tubes were used as described above. During the PCR, the PCR reaction mixture (20 μL) contained 2 μL of DNA template or 2 μL (8.0 ng/μL) of DNA extracted from *Candida albicans* as a positive control, or distilled water (Nacalai Tesque, Inc.) as a negative control in 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 200 μM of each deoxynucleoside triphosphate (dNTP), 0.25 μM each of fungal universal primer, 1×EvaGreen (Biotium Inc.), and 2.0 units (0.4 μL) of conventional

thermostable DNA polymerase (r-Taq: Toyobo, Osaka, Japan) supplemented with stock buffer solution. Each sample was incubated for 3 min at 95°C, then denatured for 10 s at 95°C, annealed for 15 s at 57°C, extended for 20 s at 72°C, and subjected to fluorescence acquisition for 2 s at 82°C for 40 cycles.

Highly Sensitive and Reliable PCR-Based Method for Detecting *Mycoplasma*, *Ureaplasma*, Other Bacteria, and Fungi in AF Samples

Using this PCR-based method, pathogens can be detected within 3 hr of AF sample collection (Figure S1a,b). The workflow of the detection method is divided into two parts. One part is the detection of *Mycoplasma*, *Ureaplasma,* and other bacteria, and the other part is the detection of fungi. To prevent the occurrence of unreliable results in PCR-based assays of AF samples for both bacterial and fungal pathogens, because of contamination by bacterial or fungal DNA, two kinds of thermostable DNA polymerase are used. That is, to detect prokaryotes such as *Mycoplasma*, *Ureaplasma,* and other bacteria, eukaryote-made thermostable DNA polymerase, which is free from bacterial DNA contamination[23,24], is used in combination with bacterial universal primers. In contrast, to detect eukaryotes such as fungi, conventional bacterially made thermostable DNA polymerase, which is usually free from fungal DNA contamination, is used in combination with fungal universal primers. Consequently, highly sensitive and reliable detection of bacteria and fungi with a minimum contamination risk makes it possible to obtain more accurate diagnostic results, which can be useful for the management of PTL cases.

To construct a sensitive and specific detection assay for *Mycoplasma*, *Ureaplasma,* and other bacteria in AF samples, we applied a nested PCR assay employing devised primer sets (Figure S1b). Using the current protocols, the limit of detection (*Escherichia coli*) of this assay is 0.74 CFU/PCR tube (37 CFU/mL of AF).

Nucleotide Sequence-Based Analysis of Bacterial Genomic DNA

Amplicons from the samples amplified in the first PCR procedure were purified (QIAquick PCR Purification Kit; Qiagen, Venlo, Netherlands) and then sequenced (3500 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) using the forward Bacterial Universal Primer for 1st PCR and the reverse Bacterial Universal Primer for 1st PCR. An online homology search was performed for strain identification using the BLAST nucleotide database tool of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/index-j.html). The presence of several species of bacteria in a sample was confirmed based on overlapping sequence data (reads). This sequencing method can be used to identify the dominant bacteria in a sample; however, when the sample contains similar amounts of two or more species of bacteria, the sequencing method cannot identify the bacterial isolate due to the presence of multiple overlapping reads.

Metagenomic Sequencing of 16S rRNA Gene

Hypervariable regions (V1-2) of 16S rRNA gene were PCR-amplified with universal primers (27Fmod and 338R).[25] The 16S metagenomic sequencing libraries were constructed by DNA stored at −80 Celsius (°C) and negative control (Nuclease-Free Water; Ambion, Foster City, CA, USA), according to the manufacturer's manual (16S Metagenomic Sequencing Library Preparation, Part # 15044223 Rev. A, Illumina, San Diego, CA, USA). Annealing temperature was modified from 55 to 60°C because KAPA HiFi HotStart Ready Mix (Kapa Biosystems, Boston, MA, USA) protocol recommends high annealing temperature. After 25 cycles of PCR, the V1-2 amplicons were purified with Agencourt AMPure XP Kit (Beckman Coulter, Koto-ku, Tokyo, Japan), and then, indices and sequencing adapters were attached using Nextera XT Index Kit (Illumina). After purification, each PCR product was quantified by high-sensitivity D1000 Screen Tape (Agilent Technologies, Santa Clara, CA, USA). Equal amount of purified PCR products from samples was mixed, and the products of the size from 400 to 1000 bp were selected by Pippin Prep (Sage Science, Beverly, MA, USA). The selected products were subjected to MiSeq sequencing platform (paired-end, 300 base pairs) with 20% of PhiX Control kit v3 (Illumina). Average number of passed-filter reads was 345,322 in case samples and 120,320 in blank control samples.

OTU Analysis of Metagenomic Sequencing Data

Two paired-end reads were merged using the fastq-join program. Low-quality reads (quality value <25) and suspected chimeric reads (BLAST match length of <90% with reference sequences in the database;

Ribosomal Database Project v. 10.27 and/or in-house 16S sequenced database in Tokyo University) were filtered out. After trimming off adapter sequences, we randomly selected 3000 reads. Selected reads were clustered into operational taxonomic units (OTUs) using a 96% pairwise-identity cutoff with the UCLUST program (Edgar 2010) version 5.2.32 (http://www.drive5.com/). To assign OTUs to taxonomy, representative sequences of each OTUs were classified by BLAST search against the databases described above. We have reported that 96% pairwise-identity cutoff is subjected to OTU analysis at the species level.[25] After removing the minority OTUs having <0.1% abundance in any samples,[25] positive decision criterion is that OTU was dominant in each sample and not detected in any negative control samples.

Culture-Based Detection of *Mycoplasma*, Bacteria Other Than *Mycoplasma* and *Ureaplasma*, and Fungi

The AF samples were analyzed according to the standard methods used by the Clinical Laboratory Center (certified ISO15189) at Toyama University Hospital. First, 1 mL AF sample was centrifuged at 1880× *g* for 15 min to spin down the microorganisms, and 800 μL of the supernatant fraction was carefully removed so as not to rupture the pellet, leaving the pellet with 200 μL of supernatant. One drop of the resulting pellet with 200 μL of supernatant was placed on the appropriate agar media (PPLO agar for *Mycoplasma*, Brucella HK agar for anaerobic bacteria, blood agar, BTB agar, and chocolate agar, respectively) and incubated aerobically or anaerobically until sufficient growth was present to proceed with testing (PPLO agar was incubated anaerobically at 35°C with 10% CO_2 for up to 7 days, Brucella HK agar was incubated anaerobically at 35°C with 10% CO_2 for up to 72 hr, blood agar and BTB agar were incubated aerobically at 35°C for up to 72 hr, and chocolate agar was incubated aerobically at 35°C with 5% CO_2 for up to 72 hr). For all samples, the specific identification methods differed according to the organism, although they included the MicroScan WalkAway system (Siemens Healthcare Diagnostics, IL, USA), RapID ANA II (Thermo Fisher Scientific, Waltham, UK), and various latex agglutination and biochemical spot tests.

Culture-Based Detection of *Ureaplasma*

One millilitre AF sample was centrifuged at 1880× *g* for 15 min to spin down the microorganisms, and 800 μL of the supernatant fraction was carefully removed so as not to rupture, leaving the pellet with 200 μL of supernatant. The resulting pellet with 200 μL of supernatant was suspended in UMCHs medium: *Mycoplasma* broth base (Becton, Dickinson and Co., Baltimore, MD, USA) 1.47% (wt/vol), 2.5% (wt/vol) yeast extract (Becton, Dickinson and Co.), 20% (vol/vol) horse serum (Biowhittaker, Walkersville, MD, USA), 1.0% (vol/vol) supplement VX (Becton, Dickinson and Co.), 0.04% (wt/vol) urea, 0.001% (wt/vol) phenol red, 0.01% (wt/vol) L-cysteine hydrochloride, and 1000 U/mL penicillin G. After incubation at 35°C for up to 72 hr, the color of the medium changed from yellow to red due to the hydrolysis of urea, and these color changes were regarded as indicating positivity for *Ureaplasma* spp. To confirm the presence of *Ureaplasma* spp., we also detected *Ureaplasma* spp. by colony formation.

Amniotic Fluid Studies

The levels of IL-8 in the AF (AF IL-8) were measured by an enzyme-linked immunosorbent assay (ELISA) as reported before.[26] The detection limit of AF IL-8 by ELISA was 32 pg/mL. On average, intra-assay and interassay coefficients of variation were 4.8% and 7.5%, respectively. The remaining sample of the AF was stored at −80°C.

Placental Histological Examination

CAM was defined in the presence of acute inflammatory changes in chorion–decidua and amnion, respectively, by Blanc's classification.[27] The classification is as follows: Stage I: patchy-diffuse accumulation of neutrophils in the subchorionic plate or decidua. Stage II: more than a few scattered neutrophils in the chorionic plate or membranous chorionic connective tissue. Stage III: neutrophils reach the subamniotic connective tissue and the amniotic epithelium. Funisitis was diagnosed in the presence of neutrophil infiltration in umbilical vessel walls or Wharton's jelly.

Statistical Analysis

Generalized log-rank test for survival test for survival analysis was used to compare the onset-to-delivery interval. Chi-square test or Fisher's exact test was used to compare the incidence of microorganism among groups. The Kruskal–Wallis and

Mann–Whitney *U*-tests were used to test for differences in continuous variables. All analyses were performed using statistical analysis software (JMP 11.0; SAS Institute Inc, Tokyo, Japan). Statistical significance was defined as $P < 0.05$.

Results

Table I and Figure S2 show the results of AF PCR assay and conventional culture assay. The prevalence of any positive AF microorganisms detected by PCR was 33.1% (39 of 118 cases) of PTL cases and 4.0% (2 of 50) of full-term delivery subjects. Microorganism isolated by PCR included other bacteria only (12.7%, 15/118), *Ureaplasma* spp. only (5.1%, 6/118), *Mycoplasma* spp. only (0.8%, 1/118), fungus only (0%, 0/118), polymicrobial infection such as *Mycoplasma/Ureaplasma* spp. and other bacteria (14.4%, 17/118), and fungus (0%, 0/118) in PTL cases.

According to the PCR results, patients with preterm delivery had a significantly higher prevalence of polymicrobial infection with *Mycoplasma* and/or *Ureaplasma* spp. and other bacteria (18.6%, 16/86) than those with normal term delivery (0%, 0/50) ($P = 0.001$).

Most of the subjects with normal term delivery were negative for any microorganism (96%, 48/50), and 2 (4%, 2/50) were positive for *Ureaplasma* spp. only (Table I).

For each patient with detectable genomic material by genome sequence or 16S rRNA metagenomic sequencing method or positive AF cultures, the microorganisms identified, inflammatory markers in AF, delivery information, and the presence or absence of histological CAM are shown in Table II. The most frequent microorganisms identified by PCR by genome sequence method were *Ureaplasma parvum*. In positive culture cases, all the cases were positive for our established PCR method as well, and we identified the species of *Ureaplasma, Mycoplasma,* and other bacteria. In negative AF culture and positive PCR cases ($n = 30$), using the nucleotide sequence-based analysis and 16S rRNA genome sequencing method (asterisk), we identified the species of microorganisms in 20 cases, but could not identify any species of microorganisms in 10 cases; however, our established highly sensitive PCR method identified the species of microorganisms in all 30 cases (Table II).

Table III shows the clinical characteristics of the each population such as *Mycoplasma*, *Ureaplasma*

Table I Comparison of Microorganisms in Amniotic Fluid Culture and PCR Results

	Preterm labor ($n = 118$)						Normal term delivery ($n = 50$)		
	(A) Preterm delivery ($n = 86$)		(B) Term delivery ($n = 32$)		(A) + (B) ($n = 118$)				
	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	*P**
Mycoplasma/Ureaplasma and bacteria	0/86 (0%)	16/86 (18.6%)	0/32 (0%)	1/32 (3.1%)	0/118 (0%)	17/118 (14.4%)	0/50 (0%)	0/50 (0%)	0.001
Ureaplasma only	1/86 (1.2%)	5/86 (5.8%)	0/32 (0%)	1/32 (3.1%)	1/118 (0.8%)	6/118 (5.1%)	0/50 (0%)	2/50 (4%)	NS
Mycoplasma only	0/86 (0%)	1/86 (1.2%)	0/32 (0%)	0/32 (0%)	0/118 (0%)	1/118 (0.8%)	0/50 (0%)	0/50 (0%)	NS
Other bacteria only	8/86 (9.3%)	10/86 (11.6%)	0/32 (0%)	5/32 (15.6%)	8/118 (6.8%)	15/118 (12.7%)	0/50 (0%)	0/50 (0%)	0.013
Fungus	0/86 (0%)	0/86 (0%)	0/32 (0%)	0/32 (0%)	0/118 (0%)	0/118 (0%)	0/50 (0%)	0/50 (0%)	NS
Any microorganisms	9/86 (10.5%)	32/86 (37.2%)	0/32 (0%)	7/32 (21.9%)	9/118 (7.6%)	39/118 (33.1%)	0/50 (0%)	2/50 (4%)	<0.001
All negative	77/86 (89.5%)	54/86 (62.8%)	32/32 (100%)	24/32 (75%)	110/118 (93.2%)	79/118 (66.9%)	50/50 (100%)	48/50 (96%)	<0.001

*Preterm delivery PCR versus normal term delivery without preterm labor PCR.

Table II Amniotic Fluid (AF) Inflammatory Profile, Delivery Information, Placenta Pathology According to the Result of AF Culture and PCR

		AF culture and PCR results			AF inflammatory profile			Delivery information			Histological placenta		
Patient number	Group	AF culture	PCR microorganism by genome sequence method	PCR highly sensitive method	AF IL-8 (ng/mL)	AF glucose	AF WBC	GA at amniocentesis (weeks)	GA at delivery (weeks)	Amniocentesis-to-delivery interval (days)	Histological chorioamnionitis	Histological funisitis	Composite severe neonatal morbidity or stillbirth or perinatal death
1	Positive AF culture and positive PCR (n = 9)	*Peptostreptococcus prevoti Eubacterium lentum Lactobacillus species*	*Sneathia sanguinegens, Leptotrichia amonionii*	B, M, U	168.9	NI	NI	23	23	4	3	Yes	BPD, IVH grade 2
2		*Streptococcus agalactiae (Group B) Peptostreptocossus anaerobius*	*Streptococcus agalactiae, Ureaplasma parvum*	B, U	120.5	30	1000	23	24	9	3	Yes	BPD
3		*Ureaplasma* sp.	*Ureaplasma* sp.	U	96.3	16	120,000	22	23	12	3	Yes	IVH grade 2
4		*Micrococcus species*	*Ureaplasma parvum*	B, U	135.1	43	5000	25	25	0	No	No	IVH grade 3, NEC
5		*Lactobacillus acidophilus*	*Lactobacillus acidophilus*	B	2.5	<25	40,000	33	33	0	1	No	No
6		*Streptocossis constellatus Lactobacillus species*	*Bacillus* sp	B	1.5	NI	NI	27	27	0	No	No	No
7		*E. raffinosus E. faecalis P. bivia G. morbillorum*	*Lactobaccilus crispatus*	B	2.5	51	4000	21	33	84	3	Yes	No
8		*Staphylococcus shieiferi Gardnerella vaginalis Lactbacillus SP*	*Lactobacillus crispatus, Lactobacillus acidophilus*	B	17.8	NI	NI	27	29	11	No	No	No
9		*Streptococcus agalactiae*	*Streptococcus agalactiae*	B	73.7	42	4000	21	23	18	3	Yes	Perinatal death
10	Negative AF culture and positive PCR (n = 30)		*Ureaplasma parvum*	B,U	181	10	540,000	25	25	3	2	Yes	BPD
11			*Ureaplasma parvum*	B, U	413.7	NI	NI	25	25	0	3	Yes	No
12			*Ureaplasma urealyticum*	B, U	95.2	10	500	30	31	6	3	Yes	BPD
13			*Lactobacillus* sp	B, U	18.7	49	1000	21	22	7	No	No	Stillbirth
14			*Ureaplasma parvum*	B, U	124	8	30,000	22	23	5	3	No	Perinatal death
15			**Ureaplasma parvum, Flavobacterium succinicans*	B, U	14.8	10	11,000	21	33	84	1	No	No

Table II (Continued)

		AF culture and PCR results			AF inflammatory profile			Delivery information			Histological placenta		
Patient number	Group	AF culture	PCR microorganism by genome sequence method	PCR highly sensitive method	AF IL-8 (ng/mL)	AF glucose	AF WBC	GA at amniocentesis (weeks)	GA at delivery (weeks)	Amniocentesis-to-delivery interval (days)	Histological chorioamnionitis	Histological funisitis	Composite severe neonatal morbidity or stillbirth or perinatal death
16			*Ureaplasma parvum*	B, U	179.9	2	NI	22	22	4	3	Yes	Stillbirth
17			Not detected	B, U	5.9	24	27,500	32	34	19	No	No	No
18			*Ureaplasma parvum*	B, U	141	27	28,500	18	24	44	2	No	BPD
19			*Ureaplasma parvum*	B, U	39.6	NI	NI	33	33	0	2	No	No
20			*Ureaplasma urealyticum*	B, U	27.4	17	7000	24	27	21	No	No	No
21			**Ureaplasma parvum, Bacteroides thetaiotaomicron, E coli*	B, U	5.7	34	16,250	25	37	81	1	No	No
22			**Pseudomonas sp., UK4*	B, M	14.8	23	8100	21	25	31	2	No	No
23			**Mycoplasma hominis, E coli*	B, M	7.0	50	15,700	26	31	32	1	No	No
24			*Ureaplasma urealyticum*	U	8.5	NI	NI	24	25	12	1	No	No
25			*Ureaplasma parvum*	U	164.1	10	35,000	27	27	0	2	Yes	No
26			*Ureaplasma urealyticum*	U	17.3	10	500	27	28	7	2	Yes	No
27			*Not detected*	U	1.8	18	20,500	26	35	65	No	No	No
28			**Moraxella catarrhalis, Propionibacterium acnes*	U	0.1	37	18,600	30	37	40	No	No	No
29			Not detected	M	6.8	23	5000	25	31	47	3	Yes	No
30			Not detected	B	25.1	29	1000	29	29	0	1	No	No
31			*Moraxella catarrhalis*	B	4.6	66	NI	31	33	18	2	Yes	No
32			Not detected	B	3.1	69	10,500	27	33	47	1	No	No
33			Not detected	B	1.7	41	22,500	22	38	110	NI	NI	No
34			Not detected	B	1.1	59	21,000	23	28	36	2	Yes	No
35			Not detected	B	1.8	60	18,880	27	37	66	NI	NI	No
36			**Pseudomonas* sp. *UK4, Propionibacterium annes*	B	6.2	42	35,000	26	37	72	No	No	No
37			Not detected	B	6	43	22,500	31	37	42	1	No	No
38			Not detected	B	2.4	40	76,000	29	37	52	NI	NI	No
39			*Lactobacillus* sp	B	15.5	35	5000	30	36	48	No	No	No

B, other bacteria; M, *Mycoplasma*; U, *Ureaplasma*; NI, not information; *16S rRNA metagenome sequence; BPD, bronchopulmonary dysplasia; IVH, intraventricular hemorrhage; NEC, necrotizing enterocolitis.

spp., and other bacteria, *Mycoplasma/Ureaplasma* only, other bacteria only, and all negative. There were no significant differences in the clinical characteristics including maternal age or parity. Gestational age at amniocentesis of polymicrobial infection with *Mycoplasma/Ureaplasma* and other bacteria positive cases was significantly earlier than that in microorganism-negative cases ($P = 0.008$).

Clinical data of pregnant women and neonates are shown in Table IV. Patients of polymicrobial infection with *Mycoplasma* and/or *Ureaplasma* spp. and other bacteria had a significantly higher rate of adverse outcomes such as lower gestational age at birth (25 weeks versus 36 weeks, $P < 0.0001$), lower birth weight (1219 g versus 2166 g, $P < 0.0001$), and higher neonatal BPD (33.3% versus 3.8%, $P < 0.01$) than those with negative AF PCR. In *Mycoplasma/Ureaplasma*-only-positive cases ($n = 7$), gestational age at delivery and amniocentesis-to-delivery interval were slightly shorter than those in all negative cases.

Fig. 1 shows the amniocentesis-to-delivery interval according to the results of AF PCR for *Mycoplasma/Ureaplasma* and other bacteria in patients with PTL. Patients with positive PCR for *Ureaplasma* spp. and/or *Mycoplasma* and other bacteria had a significantly shorter period than those with negative PCR ($P < 0.001$); however, the prognosis of other bacteria-only group was similar to that of negative PCR group. *Mycoplasma*- or *Ureaplasma*-positive cases also had a significantly shorter period than those with negative PCR ($P = 0.04$).

Next, we have studied the inflammation such as CAM, funisitis, AF IL-8, AF glucose, and AF WBC (Table V). Patients of polymicrobial infection with positive *Mycoplasma/Ureaplasma* and other bacteria had significantly higher AF IL-8 level (median 95.2 (5.7–413.7) ng/mL) and CAM grade 2 or 3 rate (58.8%, 10/17) than those with a negative AF PCR (median IL-8: 4.5 (0.1–381.5) ng/mL, $P < 0.0001$) and CAM grade 2 or 3 rate (21.3%, 17/79) ($P < 0.01$). However, no significant differences were found between patients with a positive other bacteria only and a negative AF PCR (Table V).

Importantly, AF IL-8 levels in other bacteria only were very low (3 ng/mL) compared with those in *Ureaplasma* and other bacteria superinfection cases, although AF WBC counts in other bacteria only were rather high (19,940/mm^3) compared to those in *Ureaplasma* and bacterium superinfection.

Fig. 2 shows the amniocentesis-to-delivery interval according to the results of AF culture and PCR. Amniocentesis-to-delivery interval of culture (−), PCR (+) group was significantly shorter than that in culture (−), PCR (−) group ($P = 0.03$).

Table VI shows gestational age at delivery, CAM, funisitis, AF IL-8, AF glucose, and AF WBC according to the results of AF culture and PCR. Patients with negative culture but positive PCR had significantly higher AF IL-8 level (median 12 (0–414) ng/mL), CAM grade 2 or 3 (43.3%, 13/30), and significantly lower AF glucose (median 29 (2–69) mg/dL) than those with negative culture and negative PCR (median IL-8: 4.5 (0.1–381.5) ng/mL, $P < 0.05$); CAM grade 2 or 3 (21.5%, 17/79, $P < 0.05$; median 37 (0–91), $P < 0.05$). However, there were no significant differences in the IA inflammation and infection marker between patients with negative culture but positive PCR and positive culture and positive PCR (Table VI).

Discussion

To study the relationship between perinatal prognosis in cases of PTL and the microorganisms in AF, first, we developed an original PCR assay method. This is the first method that can be used to detect mixed AF infections of *Mycoplasma*, *Ureaplasma*, and/or other bacteria. Because we use the eukaryote-made thermostable DNA polymerase, when using bacterial universal primer for PCR detection, our assay can be applied to accurately diagnose the presence or absence of bacteria in AF samples. In addition, employing the devised bacterial universal primer, mixed AF infections of *Mycoplasma*, *Ureaplasma*, and/or other bacteria can be clearly distinguished. This is because the devised primer can detect almost all kinds of bacteria, but does not detect *Mycoplasma* and *Ureaplasma* species, which is a key point for our method.

The most important finding of our study is that polymicrobial infection with *Mycoplasma/Ureaplasma* and other bacteria was associated with severe intrauterine inflammation and neonatal BPD. A shortened amniocentesis-to-delivery interval in polymicrobial infection with *Mycoplasma* and/or *Ureaplasma* spp. and other bacteria was observed compared with that pathogen-negative PTL cases. Gestational age at delivery in polymicrobial infection with *Mycoplasma* and/or *Ureaplasma* spp. and other bacteria was significantly shorter than that in microorganism-

Table III Characteristics of the Preterm Labor Population According to the Results of Amniotic Fluid (AF) PCR for Microorganism

Characteristics	AF PCR (n = 118)			
	Mycoplasma/Ureaplasma, and other bacteria (n = 17)	*Mycoplasma/Ureaplasma* only (n = 7)	Other bacteria only (n = 15)	All negative (n = 79)
Maternal age (years)	31 (20–37)	29 (21–36)	32 (27–41)	31 (21–42)
Nulliparity (n)	7 (41.2%)	4 (57.1%)	4 (26.6%)	34 (43%)
Gestational age at amniocentesis (wks)	24 (18–33)	26 (22–27)	27 (21–33)	27 (20–33)
		P = 0.008		
Antenatal corticosteroid (n/%)	7 (41.2%)	3 (42.9%)	4 (26.6%)	28 (35.4%)

Table IV Perinatal Outcome of the Study Population According to the Results of Amniotic Fluid PCR for Microorganism

	Mycoplasma/ Ureaplasma and other bacteria (n = 17)	*Mycoplasma/ Ureaplasma* only (n = 7)	Other bacteria only (n = 15)	All negative (n = 79)	P^a	P^b	P^c
Gestational age at delivery (wks, median and range)	25 (22–37)	28 (23–37)	33 (23–38)	36 (24–40)	<0.0001	<0.05	<0.01
Amniocentesis-to-delivery interval (days)	7 (0–84)	12 (0–65)	42 (0–110)	46 (0–138)	<0.001	<0.1	<0.1
Birth weight (g, mean ± S.D.)	1219 ± 643	1540 ± 631	1936 ± 712.1	2166 ± 565	<0.0001	<0.05	<0.05
Apgar score 1 min <7	10/15 (66.6%)	2/7 (28.6%)	6/15 (40%)	18/79 (22.8%)	<0.001	NS	NS
Apgar score 5 min <7	6/15 (40%)	1/7 (14.3%)	1/15 (6.7%)	6/79 (7.6%)	<0.001	NS	<0.1
Cord arterial pH at birth (mean ± S.D.)	7.312 ± 0.06	7.286 ± 0.08	7.325 ± 0.06	7.317 ± 0.06	NS	NS	NS
Stillbirth (n)	2/17 (11.8%)	0/7 (0%)	0/15 (0%)	0/79 (0%)	<0.05	NS	NS
Deaths at perinatal period,<28 days (n)	1/15 (6.7%)	0/7 (0%)	1/15 (6.7%)	1/79 (1.3%)	NS	NS	NS
Admission to neonatal intensive care unit (n/N)	14/15 (93.3%)	6/7 (85.7%)	8/15 (53.3%)	54/79 (68.4%)	<0.05	NS	<0.05
Composite severe neonatal morbidity (n/N)	6/15 (40%)	1/7 (14.3%)	1/15 (6.7%)	3/79 (3.8%)	<0.001	NS	<0.1
Congenital neonatal sepsis (n/N)	0/15 (0%)	0/7 (0%)	0/15 (0%)	0/79 (0%)	NS	NS	NS
Respiratory distress syndrome (n/N)	7/15 (46.7%)	4/7 (57.1%)	4/15 (26.7%)	16/79 (20.3%)	<0.05	<0.1	NS
Necrotizing enterocolitis (n/N)	1/15 (6.7%)	0/7 (0%)	4/15 (26.7%)	0/79 (0%)	NS	NS	NS
Intraventricular hemorrhage (>grade 2) (n/N)	2/15 (13.3%)	1/7 (14.3%)	0/15 (0%)	0/79 (0%)	<0.1	NS	NS
Bronchopulmonary dysplasia (n/N)	5/15 (33.3%)	0/7 (0%)	1/15 (6.7%)	3/79 (3.8%)	<0.01	NS	NS

[a]Comparison between *Mycoplasma/Ureaplasma* and bacterium versus all negative.
[b]Comparison between *Mycoplasma/Ureaplasma* only versus all negative
[c]Comparison between *Mycoplasma/Ureaplasma* and bacterium versus bacteria only.

negative cases. In addition, AF IL-8 levels in polymicrobial infection with *Mycoplasma/Ureaplasma* and other bacteria were extremely high (95.2 (5.7–413.7) ng/mL) compared with those in microorganism-negative cases (4.5 (0.1–381.5) ng/mL).

Microorganisms were detected in 33.1% (39/118) in AF of PTL cases, and this frequency was significantly higher than that of term delivery subjects. This finding shows that IA infection is closely associated with preterm delivery as other papers reported.[18–22]

It has been reported that the PCR method detects more species of microorganisms than conventional culture detection methods.[18–22] We have detected microorganisms in the AF of 9 in 118 PTL cases using culture tests, but in the AF of 39 in cases of PTL, microorganisms were detected using PCR method.

The frequencies of histological CAM and the amount of AF IL-8 were significantly higher and AF glucose was significantly lower in positive PCR and negative culture cases than those in negative PCR and negative culture cases, suggesting that microbial footprints detected by PCR were associated with maternal and fetal inflammation and infection (Table VI). Some reports showed that AF cytokines

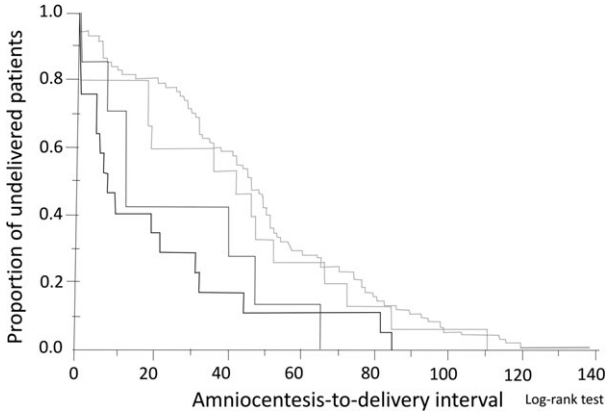

Fig. 1 Kaplan–Meier analysis of amniocentesis-to-delivery interval according to the results of amniotic fluid PCR. Patients with positive PCR for *Mycoplasma/Ureaplasma* and other bacteria had a significantly shorter period than those with negative PCR ($P < 0.001$). *Mycoplasma*- or *Ureaplasma*-only-positive cases had a significantly shorter period than those with negative PCR ($P = 0.04$). Black line shows *Mycoplasma/Ureaplasma* and other bacteria ($n = 17$), black dotted line shows *Mycoplasma/Ureaplasma* spp. only ($n = 7$), gray dotted line shows other bacteria only ($n = 15$), gray line shows all negative ($n = 79$).

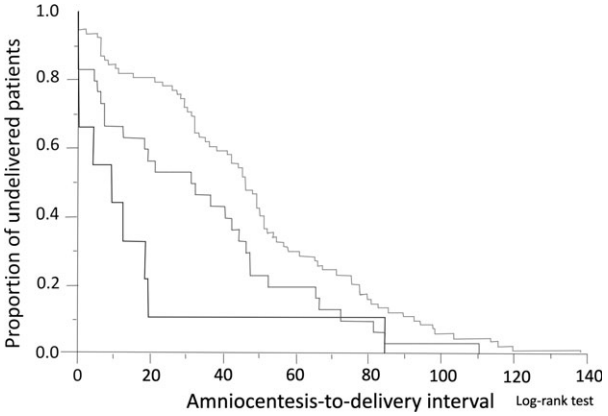

Fig. 2 Kaplan–Meier analysis of amniocentesis-to-delivery interval (days) according to the results of amniotic fluid culture and PCR. There were no significant difference in amniocentesis-to-delivery interval between culture (+), PCR (+) group and culture (−), PCR (−) group. Amniocentesis-to-delivery interval of culture (−), PCR (+) group was significantly shorter period than that in culture (−), PCR (−) group ($P = 0.03$). Black line shows culture (+), PCR (+) ($n = 9$), black dotted line shows culture (−), PCR (+) ($n = 30$), gray line shows culture (−), PCR (−) ($n = 79$).

Table V Intra-Amniotic Inflammation of the Study Population According to the Results of Amniotic Fluid (AF) PCR for Microorganism

	Mycoplasma/ Ureaplasma and other bacteria ($n = 17$)	*Mycoplasma/ Ureaplasma* only ($n = 7$)	Other bacteria only ($n = 15$)	All negative ($n = 79$)	P^a	P^b	P^c
CAM grade 2 or 3 (n/N)	10/17 (58.8%)	4/7 (57.1%)	4/15 (26.7%)	17/79 (21.3%)	<0.01	<0.05	<0.1
Funisitis (n/N)	6/17 (35.3%)	4/7 (57.1%)	4/15 (26.7%)	12/79 (15%)	<0.1	<0.01	NS
AF IL-8 (ng/mL)	95.2 (5.7–413.7)	13 (2–164)	3(1–74)	4.5 (0.1–381.5)	<0.0001	NS	<0.001
AF glucose (mg/DL)	24 (2–50)	16 (10–23)	43 (29–69)	37(0–91)	<0.01	<0.01	<0.01
AF WBC (cells/mm^3)	11,000 (500–540,000)	20,500 (500–120,000)	19,940 (1000–76,000)	8375 (500–230,000)	NS	NS	NS

[a]Comparison between *Mycoplasma/Ureaplasma* and other bacteria versus all negative.
[b]Comparison between *Mycoplasma/Ureaplasma* only versus all negative.
[c]Comparison between *Mycoplasma/Ureaplasma* and other bacteria versus other bacteria only.

such as IL-6 and IL-8 are increased in positive PCR and negative culture in preterm delivery cases.[13,18,21,22] Our data also supported these findings that showed that IA inflammation is present in positive PCR and negative culture cases.

Toll-like receptor (TLR) 2/6 recognizes *Mycoplasma*-derived lipoprotein, and TLR1/2 recognizes Gram-positive bacteria-derived lipoprotein. Therefore, those interaction between TLR 2/6 and TLR4 or TLR 2/6 and TLR 1/2 may induce severe inflammation resulting in PTL.[10,28,29] Studies have reported the additive effects of inflammation by LPS and Gram-positive bacteria-derived lipoprotein *in vitro*[30] and *in vivo*.[31] Recent data showed that superinfection with viral infection and bacterial infection induced PTL in mice.[32,33] Treatment of vaginal Trichomonas infection by antibiotic agents is one of the risk factors for preterm birth. Finchorova et al. reported that Trichomonas virus in Trichomonas induces severe inflammation via TLR systems.[34] These inflammation may induce severe inflammation in the uterus causing preterm delivery. Bacterial

Table VI Pregnancy Outcome of the Study Population According to the Results of Amniotic Fluid (AF) Culture and PCR for Microorganism

	AF culture and PCR				
	Negative culture and negative PCR (n = 79)	P^a	Negative culture but positive PCR (n = 30)	P^b	Positive culture and positive PCR (n = 9)
Gestational age at delivery (wks, median, and range)	36 (24–40)	<0.01	31 (22–38)	<0.05	25 (23–33)
Amniocentesis-to-delivery interval (days)	46 (0–138)	<0.05	32 (0–110)	NS	9 (0–84)
CAM grades 2, 3 (n/N)	17/79 (21.5%)	<0.05	13/30 (43.3%)	NS	5/9 (55.6%)
Funisitis (n/N)	12/79 (15.2%)	<0.1	9/30 (30%)	NS	5/9 (55.6%)
AF IL-8 (ng/mL)	4.5 (0.1–381.5)	<0.05	12 (0–414)	NS	74 (2–169)
AF glucose (mg/DL)	37 (0–91)	<0.05	29 (2–69)	NS	42 (16–51)
AF WBC (cells/mm^3)	8375 (500–230,000)	<0.1	18,600(500–540,000)	NS	4500 (1000–120,000)

[a]Comparison between a negative culture and negative PCR and a negative culture but positive PCR.
[b]Comparison between a negative culture but positive PCR and a positive culture and positive PCR.

vaginosis is one of the risk factors for preterm delivery. In bacterial vaginosis, *Lactobacillus* spp was decreased in vagina. One of the causes for the absence of *Lactobacillus* spp is phage infection.[35] In this bacterial vaginosis case, superinfection with virus and bacteria may induce preterm delivery. Recent data showed that the viral infection rate was only 1.4% in PTL cases;[22] therefore, superinfection with bacteria and virus may be rare. However, our data showed that the superinfection of *Ureaplasma/Mycoplasma* and other bacteria was observed in 16 cases among 86 preterm deliveries (18.6%). In our study, we did not analyze the viral infection; so further study is necessary to reanalyze the viral infection in the future.

In our study, some cases showed high AF IL-8 in PCR-negative PTL cases. In sterile IA inflammation cases, amniotic HMGB1 is very high.[22] HMGB1 promotes a p38 MAPK-associated non-infectious inflammatory response pathway in human fetal membrane.[36] Thus, we should measure the HMGB1 in such cases.

As one of the limitations of our study, we did not examine the amount of microorganisms using semi-quantitative PCR assay. It is important to evaluate the amount of microorganisms in the AF of PTL cases. Several studies showed that the IA inflammatory responses to *Ureaplasma* species are dose dependent.[37–39] We are now attempting to establish a semi-quantitative PCR assay. It is necessary to clarify whether the amount of microorganisms is associated with poor prognosis of PTL cases in the future.

Another problem is that 16S rRNA sequencing might detect dead bacteria as well as live bacteria. Therefore, we cannot predicate that the sequenced 16S rRNA always shows the pathogens for preterm birth. In the future, it is necessary to distinguish live bacteria from dead bacteria.

In our study, we firstly showed that superinfection with *Ureaplasma, Mycoplasma,* and bacteria induced severe IA inflammation. Superinfection with *Ureaplasma, Mycoplasma,* and bacteria or with both bacteria and virus may be important to induce preterm delivery. For preventing preterm delivery, new therapies against superinfection with *Ureaplasma, Mycoplasma,* and bacteria are needed.

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References

1 World Health Organization: Born to Soon: The Global Action Report Preterm Birth. WHO Library Cataloguing-in-Publication. Geneva, Switzerland, World Health Organization, 2012.

2 Blencowe H: National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet* 2012; 9:2162–2172.
3 Muglia LJ, Katz M: The enigma of spontaneous preterm birth. *N Engl J Med* 2010; 362:529–535.
4 Lahra MM: A fetal response to chorioamnionitis is associated with early survival after preterm birth. *Am J Obstet Gynecol* 2004; 190:147–151.
5 Jones HE: Validity and reliability of using a self-lavaging device for cytology and HPV testing for cervical cancer screening: findings from a pilot study. *PLoS One* 2013; 20:e82115.
6 Goldenberg RL: Intrauterine infection and preterm delivery. *N Engl J Med* 2000; 18:1500–1507.
7 Romero R, Mazor M, Wu YK, Sirtori M, Oyarzun E, Mitchell MD, Hobbins JC: Infection in the pathogenesis of preterm labor. *Semin Perinatol* 1988; 12:262–279.
8 Yoon BH: The clinical significance of detecting *Ureaplasma urealyticum* by the polymerase chain reaction in the amniotic fluid of patients with preterm labor. *Am J Obstet Gynecol* 2003; 189:919–924.
9 Yoon BH: Clinical implications of detection of *Ureaplasma urealyticum* in the amniotic cavity with the polymerase chain reaction. *Am J Obstet Gynecol* 2000; 183:1130–1137.
10 Gerber S: Detection of *Ureaplasma urealyticum* in second-trimester amniotic fluid by polymerase chain reaction correlates with subsequent preterm labor and delivery. *J Infect Dis* 2003; 1:518–521.
11 Jalava J, Mantymaa ML, Ekblad U, Toivanen P, Skurnik M, Lassila O, Alanen A: Bacterial 16S rDNA polymerase chain reaction in the detection of intra-amniotic infection. *Br J Obstet Gynaecol* 1996; 103:664–669.
12 Hitti J, Riley DE, Krohn MA, Hillier SL, Agnew KJ, Krieger JN, Eschenbach DA: Broad-spectrum bacterial rDNA polymerase chain reaction assay for detecting amniotic fluid infection among women in premature labor. *Clin Infect Dis* 1997; 24:1228–1232.
13 DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, Kim CJ, Erez O, Edwin S, Relman DA: Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS One* 2008; 3:e3056.
14 Markenson GR: The use of the polymerase chain reaction to detect bacteria in amniotic fluid in pregnancies complicated by preterm labor. *Am J Obstet Gynecol* 1997; 177:1471–1477.
15 Gardella C, Riley DE, Hitti J, Agnew K, Krieger JN, Eschenbach D: Identification and sequencing of bacterial rDNAs in culture-negative amniotic fluid from women in premature labor. *Am J Perinatol* 2004; 21:319–323.
16 Miralles R, Hodge R, McParland PC, Field DJ, Bell SC, Taylor DJ, Grant WD, Kotecha S: Relationship between antenatal inflammation and antenatal infection identified by detection of microbial genes by polymerase chain reaction. *Pediatr Res* 2005; 57:570–577.
17 Oyarzun E: Specific detection of 16 micro-organisms in amniotic fluid by polymerase chain reaction and its correlation with preterm delivery occurrence. *Am J Obstet Gynecol* 1998; 179:1115–1119.
18 DiGiulio DB, Romero R, Kusanovic JP, Gomez R, Kim CJ, Seok KS, Gotsch F, Mazaki-Tovi S, Vaisbuch E, Sanders K, Bik EM, Chaiworapongsa T, Oyarzun E, Relman DA: Prevalence and diversity of microbes in the amniotic fluid, the fetal inflammatory response, and pregnancy outcome in women with preterm prelabor rupture of membranes. *Am J Reprod Immunol* 2010; 64:38–57.
19 Jones HE, Harris KA, Azizia M, Bank L, Carpenter B, Hartley JC, Klein N, Peebles D. Differing prevalence and diversity of bacterial species in fetal membranes from very preterm and term labor. *PLoS One* 2009; 4: e8205.
20 Wang X, Buhimschi CS, Temoin S, Bhandari V, Han YW, Buhimschi IA: Comparative microbial analysis of paired amniotic fluid and cord blood from pregnancies complicated by preterm birth and early-onset neonatal sepsis. *PLoS One* 2013; 8:e56131.
21 Romero R, Miranda J, Chaiworapongsa T, Chaemsaithong P, Gotsch F, Dong Z, Ahmed AI, Yoon BH, Hassan SS, Kim CJ, Korzeniewski SJ, Yeo L: 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. *Am J Reprod Immunol* 2014; 71:330–358.
22 Romero R, Miranda J, Chaiworapongsa T, Korzeniewski SJ, Chaemsaithong P, Gotsch F, Dong Z, Ahmed AI, Yoon BH, Hassan SS, Kim CJ, Yeo L: Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. *Am J Reprod Immunol* 2014; 72:458–474.
23 Niimi H, Mori M, Tabata H, Minami H, Ueno T, Hayashi S, Kitajima I: A novel eukaryote-made thermostable DNA polymerase which is free from bacterial DNA contamination. *J Clin Microbiol* 2011; 49:3316–3320.
24 Ueno T, Niimi H, Yoneda N, Yoneda S, Mori M, Tabata H, Minami H, Saito S, Kitajima I: Eukaryote-made thermostable DNA polymerase enables rapid PCR-based detection of *Mycoplasma*, *Ureaplasma* and other bacteria in the amniotic fluid of preterm labor cases. *PLoS One* 2015; 4:e0129032.
25 Kim SW, Suda W, Kim S, Oshima K, Fukuda S, Ohno H, Morita H, Hattori M: Robustness of gut microbiota of healthy adults in response to probiotic intervention revealed by high-throughput pyrosequencing. *DNA Res* 2013; 20:241–253.
26 Luo L, Ibaragi T, Maeda M, Nozawa M, Kasahara T, Sakai M, Sasaki Y, Tanebe K, Saito S: Interleukin-8 levels and granulocyte counts in cervical mucus during pregnancy. *Am J Reprod Immunol* 2000; 43:78–84.
27 Blanc WA: Pathology of placenta, membranes and umbilical and in bacterial, fungal, and viral infections in man. *Monogr Pathol* 1981; 22: 67–132.
28 Kim YM, Romero R, Chaiworapongsa T, Kim GJ, Kim MR, Kuivaniemi H, Tromp G, Espinoza J, Bujold E, Abrahams VM, Mor G: Toll-like receptor-2 and -4 in the chorioamniotic membranes in spontaneous labor at term and in preterm parturition that are associated with chorioamnionitis. *Am J Obstet Gynecol* 2004; 191:1346–1355.
29 Abrahams VM, Potter JA, Bhat G, Peltier MR, Saade G, Menon R: Bacterial modulation of human fetal membrane Toll-like receptor expression. *Am J Reprod Immunol* 2013; 69:33–40.
30 Glauser MP: The inflammatory cytokines. New developments in the pathophysiology and treatment of septic shock. *Drugs* 1996; 52 (Suppl 2):9–17.
31 Elovitz M, Wang Z: Medroxyprogesterone acetate, but not progesterone, protects against inflammation-induced parturition and intrauterine fetal demise. *Am J Obstet Gynecol* 2004; 190:693–701.
32 Cardenas I, Means RE, Aldo P, Koga K, Lang SM, Booth CJ, Manzur A, Oyarzun E, Romero R, Mor G: Viral infection of the placenta leads to fetal inflammation and sensitization to bacterial

products predisposing to preterm labor. *J Immunol* 2010; 185:1248–1257.
33 Ilievski V, Hirsch E: Synergy between viral and bacterial toll-like receptors leads to amplification of inflammatory responses and preterm labor in the mouse. *Biol Reprod* 2010; 83: 767–773.
34 Fichorova RN, Lee Y, Yamamoto HS, Takagi Y, Hayes GR, Goodman RP, Chepa-Lotrea X, Buck OR, Murray R, Kula T, Beach DH, Singh BN, Nibert ML: Endobiont viruses sensed by the human host - beyond conventional antiparasitic therapy. *PLoS One* 2012; 7: e48418.
35 Pavlova SI, Kiliç AO, Mou SM, Tao L: Phage infection in vaginal lactobacilli: an in vitro study. *Infect Dis Obstet Gynecol* 1997; 5:36–44.
36 Bredeson S, Papaconstantinou J, Deford JH, Kechichian T, Syed TA, Saade GR, Menon R: HMGB1 promotes a p38MAPK associated non-infectious inflammatory response pathway in human fetal membranes. *PLoS One* 2014; 9:e113799.
37 Kacerovsky M, Pliskova L, Bolehovska R, Musilova I, Homychova H, Tambor V, Jacobsson B: The microbial load with genital mycoplasmas correlates with the degree of histologic chorioamnionitis in preterm PROM. *Am J Obstet Gynecol* 2011; 205: e211–e217.
38 Kacerovsky M, Musilova I, Hornychova H, Kutova R, Pliskova L, Kostal M, Jacobsson B: Bedside assessment of amniotic fluid interleukin-6 in preterm prelabor rupture of membranes. *Am J Obstet Gynecol* 2014; 211:e381–e389.
39 Musilova I, Kutova R, Pliskova L, Stepan M, Menon R, Jacobsson B, Kacerovsky M: Intraamniotic inflammation in women with preterm prelabor rupture of membranes. *PLoS One* 2015; 10: e0133929.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) The workflow of the highly sensitive and reliable detection method for *Mycoplasma*, *Ureaplasma*, other bacteria, and fungi in the amniotic fluid samples. (b) The strategy used for the primer design.

Figure S2. Trial profile.