Eukaryote-Made Thermostable DNA Polymerase Enables Highly Sensitive and Reliable PCR-Based Detection of Bacteria, Mycoplasma, Ureaplasma and Fungi in the Amniotic Fluid of Preterm Labor Cases

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ABSTRACT

The percentage of premature delivery before 28 weeks of pregnancy is increasing, which is a serious issue in the obstetric field. The primary cause of it is the chorioamnionitis based on the intrauterine infection. Using the current culture-based identification of the pathogenic microorganisms, it usually takes about one week, so it is difficult to treat patients with optimal antibiotics. As a result, not a few patients with chorioamnionitis often results in the premature delivery.

In this research, we made it possible to detect bacteria, *Mycoplasma*, *Ureaplasma*, and fungi in amniotic fluid with highly sensitivity and reliability using the eukaryote-made thermostable DNA polymerase which is free from bacterial DNA contamination, and using the originally designed nested PCR assay. In addition, we devised to do quantitative comparison among bacteria, *Mycoplasma* and *Ureapasma* in a patient sample, which is useful information for the choice of antibiotic agents.

To estimate our original nested PCR assay, we checked the number of detected bacteria, mycoplasma, ureaplasma and fungi in 305 amniotic fluid samples compared with the conventional culture results. As a result, the rates of matched results were as follows, bacteria: 87.9 %, *Mycoplasma*: 93.1%, *Ureaplasma*: 94.4%, and fungi: 99.7%.

In addition, the nested PCR assay detected pathogens a lot more than the conventional culture method.

In conclusion, the use of the eukaryote-made thermostable DNA polymerase and the original designed nested PCR assay enables highly sensitive and reliable detection of bacteria, *Mycoplasma*, *Ureaplasma* and fungi in the Amniotic Fluid, which would contribute to the treatment of Preterm Labor Cases.

The earliest possible detection of pathogenic microorganisms in amniotic fluid is critical for selecting an appropriate antimicrobial therapy and for obtaining a favorable outcome for preterm labor cases (1-3). Intrauterine infection, such as chorioamnionitis, is a primary cause of premature delivery before 28 weeks of pregnancy, a definitive diagnosis of which requires the detection of pathogenic microorganisms (4). However, as the current pathogen-identification methods using microbial culture require several days, empirically selected antimicrobial agents are administered until the pathogenic microorganisms are identified (5, 6). As a result, the use of inappropriate antimicrobial agents often leads to premature delivery in patients with intrauterine infections (7). Thus, there are significant risks associated with the treatment of intrauterine infections. Therefore, there is a critical need to develop a method that can rapidly detect pathogenic microorganisms. If such a rapid method is developed, more informed use of antimicrobial agents would be possible, and this would reduce the premature delivery before 28 weeks of pregnancy (8, 9).

We herein report the development of a rapid, highly sensitive, and reliable method to detect bacteria, *Mycoplasma*, *Ureaplasma*, and fungi in amniotic fluid samples using the eukaryote-made thermostable DNA polymerase (10, 11) and the originally designed nested PCR assay. Using this assay, not only highly sensitive and

reliable detection of bacteria, *Mycoplasma*, *Ureaplasma*, and fungi, but also quantitative comparison of them becomes possible.

RESULTS

Construction of highly sensitive and reliable detection of bacteria, Mycoplasma, Ureaplasma and fungi in the amniotic fluid samples.

The flow chart of our detection assay is divided into two parts (**Fig. 1**). One part is the detection of bacteria, *Mycoplasma* and *Ureaplasma*, and the other part is the detection of fungi. To prevent the occurrence of unclear results in PCR-based assaying of Amniotic fluid samples for both bacterial and fungal pathogens because of contamination by bacterial or fungal DNA, a two-step usage of thermostable DNA polymerase was performed. That is, to detect bacterial pathogens including Mycoplasma and Ureaplasma, eukaryote-made thermostable DNA polymerase can be used in combination with bacterial universal primers. In contrast, to detect fungal pathogens, conventional bacterially made thermostable DNA polymerase, which is usually free from fungal DNA contamination, can be used in combination with fungal universal primers. Although bacterially made thermostable DNA polymerase contains trace

amounts of DNA from bacterial host cells, no fungal universal primers can bind to bacterial genomic DNA, because fungi are eukaryotes. Consequently highly sensitive and reliable detection of bacteria and fungi without any unclear results would make it possible to obtain more rapid (within three hours of amniotic fluid sample collection) and accurate diagnostic results, which would thereby improve the management of preterm labor cases.

To construct highly sensitive and specific detection of bacteria, Mycoplasma and Ureaplasma in amniotic fluid samples, we devised original nested PCR assay (**Fig. 2A**) using the unique primer sets (**Supplemental Table S1**). The sequence homology between the bacterial universal primers and the target regions of bacteria, Mycoplasma and Ureaplasma species are shown in **Fig. 2B**, which means the strategy of our original approach. For the first PCR, bacterial universal primer 1 can amplify almost all kinds of bacteria, Mycoplasma and Ureaplasma species. For the second nested PCR, bacterial universal primer 2 can also detect almost all kinds of bacteria, Mycoplasma and Ureaplasma species. On the other hand, bacterial universal primer 3 can detect almost all kinds of bacteria, but cannot detect Mycoplasma and Ureaplasma species because of primer's low sequence homology. As a result, bacterial universal primer 3 can detect almost all kinds of bacteria except for *Mycoplasma* and *Ureaplasma* species. Using

these bacterial universal primers and *Mycoplasma* / *Ureaplasma* specific primers, target species can be correctly detected (**Fig. 2C**). Especially, eukaryote-made thermostable DNA polymerase makes it a possible to get clear results by preventing bacterial DNA contamination in highly sensitive nested PCR assays.

Evaluation of the detection sensitivity compared with the conventional culture method.

To evaluate the detection sensitivity of our PCR-based assay, we compared with conventional culture method about the detection number of bacteria, Mycoplasma, Ureaplasma and fungi in the same 305 amniotic fluid samples based on the emergence of the amplicons (**Table 1**). As a result, the rates of matched results were as follows, bacteria: 87.9 %, *Mycoplasma*: 93.1%, *Ureaplasma*: 94.4%, and fungi: 99.7%. In addition, the nested PCR assay detected pathogens a lot more than the conventional culture method.

The quantitative comparison of bacteria, Mycoplasma, and Ureaplasma in a amniotic fluid sample.

We measured cycle number of amplification plot of each amplicons by real-time PCR-based assay (**Table 2**). As a result, we can judge the quantitative comparison of bacteria, *Mycoplasma*, and *Ureaplasma*, which would be useful information for the choice of antibiotic agents.

DISCUSSION

Our nested PCR assay detects bacteria, *Mycoplasma*, *Ureaplasma*, and fungi with highly sensitively and reliably in an amniotic fluid sample. Because no culture is used, the ratio of the number of bacteria, *Mycoplasma*, and *Ureaplasm*a in a clinical sample is accurately reflected, so the quantitative comparison of them can be detected using real time PCR-based quantification. The time to detect those pathogens in amniotic fluid is within three hours of patient sample collection. In this regard, using the rapid detection, we can quickly choose the antibiotics that are suitable for the intrauterine infection (12).

In conclusion, the use of eukaryote-made thermostable DNA polymerase and originally designed nested PCR assay makes it possible to detect bacteria, *Mycoplasma*, *Ureaplasma*, and fungi with highly sensitivity and reliability in amniotic fluid samples. This assay is useful for the intrauterine infection that require prompt treatment, and

would contribute to the rescue of unborn children, as well as a decrease in the number of the premature delivery.

MATERIALS AND METHODS

Study participants and clinical sample collection.

Amniotic fluid samples were collected for analysis from 99 women who underwent a transabdominal or transvaginal amniocentesis with the diagnosis of preterm labor, and from 206 women who had a caesarean section or vaginal delivery. Written informed consent was obtained from the patients for the collection and use of the clinical samples. This study was conducted with the approval of the Ethics Committee on Genomic Research of the University of Toyama.

DNA extraction from amniotic fluid samples.

In each of the following processes, The QIAcube system (Qiagen, Germany) provided automated processing of QIAGEN spin columns. For the amniotic fluid, 1 mL samples were collected transabdominally, transvaginally, or at the time of cesarean

section. Amniotic fluid samples were centrifuged at 20,000×g for 20 min to spin down the microorganisms, and DNA was isolated from the pellets using a DNA extraction kit (QIAamp DNA Mini Kit, Qiagen) in accordance with the supplier's instructions.

Nested PCR assays for detecting bacteria, Mycoplasma, and Ureaplasma.

In each of the following processes, The QIAgility system (Qiagen) provided automated PCR setup. The Rotor-Gene Q (Qiagen) was used for 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, Germany), and 0.2 mL PCR Tubes (Qiagen). All oligonucleotide primers were synthesized by Life Technologies Japan Ltd. (Tokyo, Japan). The primer information is shown in Supplemental Table S1.

During the first PCR, the PCR reaction mixture (20 μL) contained 2 μL of DNA template or 2 μL (80 ng/mL) of DNA extracted from *Escherichia coli* (ATCC 25922) as a positive control, or distilled water (water deionized and sterilized for molecular biology, NAKALAI TESQUE, INC. Kyoto) as a negative control in 50 mM KCl, 2.25 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 μM of each deoxynucleoside triphosphate (dNTP), 0.3 μM each of bacterial universal primer 1, 1×EvaGreen (Biotium Inc. CA,

USA), and 1.0 unit (0.5 µL) of eukaryote-made thermostable DNA polymerase supplemented with storage buffer. The generation of eukaryote-made *Taq* DNA polymerase using *Saccharomyces cerevisiae* was described previously*.

Each sample was preincubated for 5min at 95℃, then denatured for 10 s at 95℃, annealed for 15 s at 57℃ and subjected to extension for 30 s at 72℃ for 30 cycles. The PCR product was diluted 500-fold with distilled water, and was then used as a template for the second nested PCR.

For the second nested PCR, the PCR reaction mixture (20 µL) contained 8 µL of DNA template of a clinical sample or *Escherichia coli* (ATCC 25922) as a positive control, or 8 µL of distilled water as a negative control in 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 µM of each dNTP, 0.25 µM of each primer (Bacterial universal primer 2, Bacterial universal primer 3, Mycoplasma specific primer, Ureaplasma specific primer), 1×EvaGreen, and 1.0 unit (0.5 µL) of eukaryote-made *Taq* polymerase supplemented with storage buffer. Each sample was preincubated for 5min at 95℃, then denatured for 10 s at 95℃, annealed for 15 s at 57℃ and subjected to extension for 10 s at 72℃ for 30 cycles.

A quantitative comparison was subsequently performed, and a quantification

cycle value was calculated using the Rotor-Gene Q software program. Amplicons were further analyzed by gel electrophoresis (2% agarose gel, ethidium bromide staining).

PCR assays for detecting fungi.

To detect fungi, the Rotor-Gene Q and PCR-clean tubes were used as described above. During the PCR, the PCR reaction mixture (20 μL) contained 2 μL of fungal DNA template or 2 μL (80 ng/mL) of DNA extracted from *Candida albicans* as a positive control, or distilled water (NAKALAI TESQUE, INC.) as a negative control in 50 mM KCl, 1.5 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 *Taq* DNA polymerase (r-Taq: Toyobo, Osaka, Japan) supplemented with storage buffer. Each sample was preincubated for 3 min at 95℃, then denatured for 10 s at 95℃, annealed for 15 s at 57℃ and subjected to extension for 20 s at 72℃ for 40 cycles.

Culture-based detection of bacteria, Mycoplasma and fungi.

Amniotic fluid samples were analyzed according to standard methods used by the

Clinical Laboratory Center at Toyama University Hospital. At first, 1mL amniotic fluid samples were centrifuged at 1,880×g for 15 min to spin down the microorganisms, and resulting pellets were subcultured in the appropriate media and incubated aerobically or anaerobically until sufficient growth was present to proceed with testing. For all samples, the specific identification methods differed according to the organism, but include the MicroScan WalkAway system (Siemens Healthcare Diagnostics, IL, USA), RapID ANA II (Thermo Fisher SCIENTICIC, UK) and various latex agglutination and biochemical spot tests.

Culture-based detection of Ureaplasma.

Amniotic fluid samples were suspended in UMCHs medium: *Mycoplasma* broth base (Becton, Dickinson and Co., Baltimore, MD) 1.47% (wt/vol), yeast extract (Becton, Dickinson and Co.) 2.5% (wt/vol), horse serum (Biowhittaker, Walkersville, MD) 20% (vol/vol), supplement VX (Becton, Dickinson and Co.) 1.0% (vol/vol), urea 0.04% (wt/vol), phenol red 0.001% (wt/vol), L-cysteine hydrochloride 0.01% (wt/vol), and penicillin G 1000 U/Ml. After incubation at 35°C for up to 72 h, the color of the medium changed from yellow to red due to hydrolysis of urea, and these color changes

were regarded as indicating positively for *Ureaplasma spp*. We identified *Ureaplasma spp.* by colony formation and subsequent PCR-based assays using modified Kong's method*.

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SUPPLEMENTAL MATERIAL

Table S1, DOC file, 0.2 MB.

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Table 1

Bacterial universal primer 2	Bacterial universal primer 3	Mycoplasma specific primer	Ureaplasma specific primer	Judgment of detection, or non-detection
Amplicon size (bp) 287	Amplicon size (bp) 120	Amplicon size (bp) 170	Amplicon size (bp) 124	
–	–	–	–	None
+	+	–	–	Bacteria
+	–	+	–	Mycoplasma
+	–	–	+	Ureaplasma
+	–	+	+	Mycoplasma and Ureaplasma
+	+	+	–	Bacteria and Mycoplasma
+	+	–	+	Bacteria and Ureaplasma
+	+	+	+	Bacteria and Mycoplasma and Ureaplasma

Table 2

Sample	Detected cycle No. by real-time PCR				Judgment of relative quantification	Diseases
	Bacterial universal primer 2	Bacterial universal primer 3	Mycoplasma specific primer	Ureaplasma specific primer		
Control						
E. coli	9.91	10.25			Bacteria	
L. crispatus	4.72	5.20			Bacteria	
M. hominis	11.37		11.72		Mycoplasma	
U. parvum	2.97			3.28	Ureaplasma	
Patient						
1	11.88	13.62	24.75		Bacteria > Mycoplasma	Previous cesarean section
2	13.04	13.89	26.03		Bacteria > Mycoplasma	Fetal distress
3	14.40	15.10	23.79		Bacteria > Mycoplasma	Previous cesarean section
4	14.73	15.17	24.13		Bacteria > Mycoplasma	Previous cesarean section
5	15.52	16.34	23.18		Bacteria > Mycoplasma	Hypertension, Gestational diabetes mellitus
6	15.78	17.07	27.71		Bacteria > Mycoplasma	Preterm labor
7	22.38	22.99	27.87		Bacteria > Mycoplasma	Preterm labor
8	12.63	13.31	21.04		Bacteria > Mycoplasma	Previous cesarean section
9	2.56	2.86		7.03	Bacteria > Ureaplasma	Premature rupture of membranes
10	2.78	3.34		12.00	Bacteria > Ureaplasma	Dichorionic diamniotic twins
11	2.85	15.87		3.41	Ureaplasma > Bacteria	Preterm labor
12	3.07	8.00		5.49	Ureaplasma > Bacteria	Premature rupture of membranes
13	3.48	17.95		4.47	Ureaplasma > Bacteria	Preterm labor
14	4.12	15.71		4.16	Ureaplasma > Bacteria	Premature rupture of membranes
15	6.00	8.43		21.65	Bacteria > Ureaplasma	Premature rupture of membranes
16	7.09	7.89		12.11	Bacteria > Ureaplasma	Premature rupture of membranes
17	7.20	8.76		17.77	Bacteria > Ureaplasma	Intrauterine fetal death
18	12.91	14.65		13.55	Ureaplasma > Bacteria	Dichorionic diamniotic twins
19	14.04	15.35		14.9	Ureaplasma > Bacteria	Formation of the bag
20	14.07	14.28		22.24	Bacteria > Ureaplasma	Preterm labor
21	2.48	4.53	2.98	10.49	Mycoplasma > Bacteria > Ureaplasma	Premature rupture of membranes
22	2.61	3.63	4.44	7.47	Bacteria > Mycoplasma > Ureaplasma	Premature rupture of membranes
23	3.02	6.58	11.86	3.74	Ureaplasma > Bacteria > Mycoplasma	Premature rupture of membranes
24	6.47	8.26	24.99	13.35	Bacteria > Ureaplasma > Mycoplasma	Formation of the bag
25	12.16	15.10	13.32	17.29	Mycoplasma > Bacteria > Ureaplasma	Previous cesarean section
26	12.71	16.96	13.35	16.53	Mycoplasma > Ureaplasma > Bacteria	Preterm labor
27	14.11	15.30	17.67	18.35	Bacteria > Mycoplasma > Ureaplasma	Previous cesarean section
28	14.27	15.81	20.23	16.52	Bacteria > Ureaplasma > Mycoplasma	Previous cesarean section

Supplementary Table S1 PCR primers and amplicon sizes in base pairs

Primer name	Primer sequence (5'→3')	Amplicon size (bp)
Bacterial universal primer 1	F- AGAGTTTGATCATGGCTCAG	1377
for 1st PCR	R- CCGGGAACGTATTCACC	
Bacterial universal primer 2	F- AGCAGCCGCGGTAATA	287
for 2nd nested PCR	R- GGACTACCAGGGTATCTAATCCT	
Bacterial universal primer 3	F- TGGTTTAATTCGATGCAACGC	120
for 2nd nested PCR	R- GAGCTGACGACAGCCAT	
Mycoplasma specific primer	F 1- GTGTAGCTATGCTGAG	170
for 2nd nested PCR	F 2- GTTTAGCCGGGTCGAG	
(F1, F2, and R1, R2 are mixed in one PCR)	R 1- TTCTTCCCAAATAAAAGAACTTT	
	R 2- TTCTTCCCTTATAACAGCACTTT	
Ureaplasma specific primer	F- TAACATCAATATCGCATGAGAAG	124
for 2nd nested PCR	R- CAGTACAGCTACGCGTCATT	
Fungal universal primer	F- CTTTCGATGGTAGGATAGTGG	615
	R- GCTTTCGCAGTAGTTAGTCTTC	

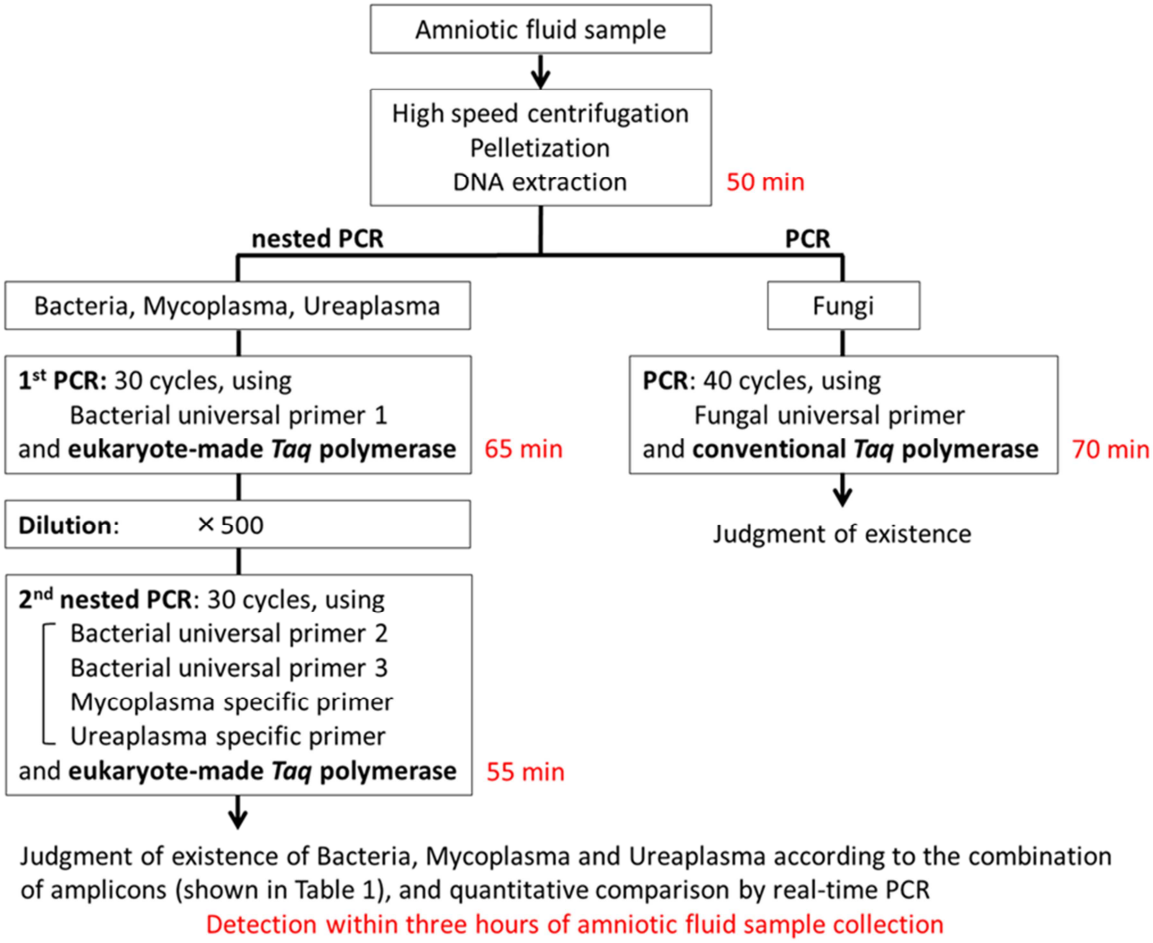
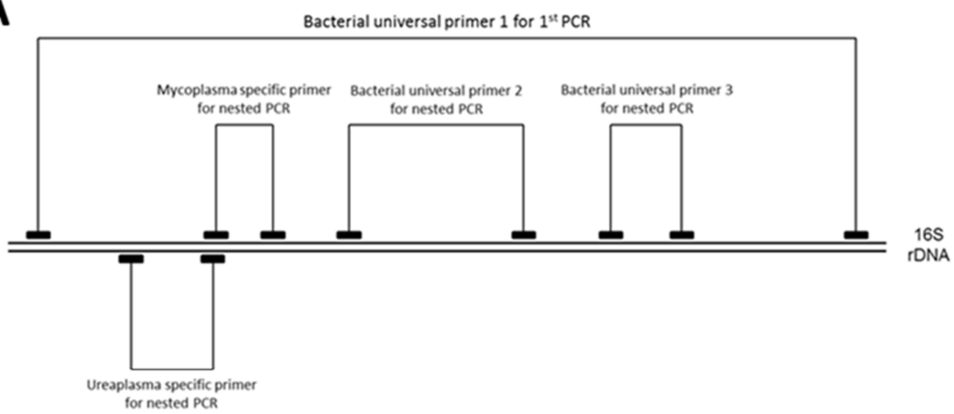


Figure 1

Flow chart of the highly sensitive detection of bacteria, Mycoplasma, Ureaplasma and fungi in the amniotic fluid samples

A



B

Bacterial universal primer 1	Forward (5'→3')	Reverse (5'→3')	Difference (bp)	
	AGAGTTTGATCATGGCTCAG	CCGGGAACGTATTCACC	Forward	Reverse
Escherichia coli	AGAGTTTGATCATGGCTCAG	CCGGGAACGTATTCACC	0	0
Lactobacillus aviarius	AGAGTTTGATCCTGGCTCAG	CCGGGAACGTATTCACC	1	0
Lactobacillus crispatus	AGAGTTTGATCATGGCTCAG	CCGGGAACGTATTCACC	0	0
Mycoplasma genitalium	AGAGTTTGATCCTGGCTCAG	CCGGGAACGTATTCACC	1	0
Mycoplasma hominis	AGAGTTTGATCCTGGCTCAG	CCGGGAACGTATTCACC	1	0
Ureaplasma parvum	AGAGTTTGATCCTGGCTCAG	CCGGGAACGTATTCACC	1	0
Ureaplasma urealyticum	AGAGTTTGATCCTGGCTCAG	CCGGGAACGTATTCACC	1	0

Bacterial universal primer 2	Forward (5'→3')	Reverse (5'→3')	Difference (bp)	
	AGCAGCCGCGGTAATA	GGACTACCAGGGTATCTAATCCT	Forward	Reverse
Escherichia coli	AGCAGCCGCGGTAATA	GGACTACCAGGGTATCTAATCCT	0	0
Lactobacillus aviarius	AGCAGCCGCGGTAATA	GGACTACCAGGGTATCTAATCCT	0	0
Lactobacillus crispatus	AGCAGCCGCGGTAATA	GGACTACCAGGGTATCTAATCCT	0	0
Mycoplasma genitalium	AGCAGTCGCGGTAATA	GGACTACTAGGGTATCTAATCCT	1	1
Mycoplasma hominis	AGCAGCCGCGGTAATA	GGACTACCAGGGTATCTAATCCT	0	0
Ureaplasma parvum	AGCAGCCGCGGTAATA	GGACTACTAGGGTATCTAATCCT	0	1
Ureaplasma urealyticum	AGCAGCCGCGGTAATA	GGACTACTAGGGTATCTAATCCT	0	1

Bacterial universal primer 3	Forward (5'→3')	Reverse (5'→3')	Difference (bp)	
	TGGTTTAATTCGATGCAACGC	GAGCTGACGACAGCCAT	Forward	Reverse
Escherichia coli	TGGTTTAATTCGATGCAACGC	GAGCTGACGACAGCCAT	0	0
Lactobacillus aviarius	TGGTTTAATTCGAAGCAACGC	GAGCTGACGACAGCCAT	1	0
Lactobacillus crispatus	TGGTTTAATTCGATGCAACGC	GAGCTGACGACAGCCAT	0	0
Mycoplasma genitalium	TTGCTTAATTCGACGGTACAC	GAGCTGACGACAACCAT	6	1
Mycoplasma hominis	TGGTTTAATTTGAAGATACAC	GAGCTGACGACAACCAT	5	1
Ureaplasma parvum	TTGCTTAATTTGACAATACAC	GAGCTGACGACAACCAT	8	1
Ureaplasma urealyticum	TTGCTTAATTTGACAATACAC	GAGCTGACGACAACCAT	8	1

C

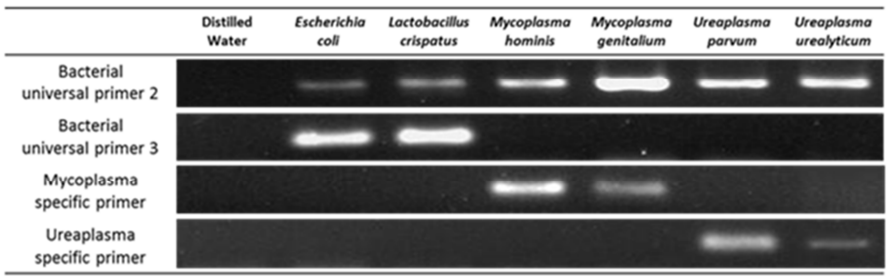


Figure 2

The strategy of primer designs

(**A**) Nested PCR is performed using the bacterial universal primer 1 for the 1st PCR, and for the 2nd nested PCR, bacterial universal primer 2, 3, *Mycoplasma* specific primer, and *Ureaplasma* specific primer are used.

(**B**) This shows the sequence homology between the bacterial universal primers (1, 2, and 3) and the target regions of bacteria, Mycoplasma, and Ureaplasma in 16S ribosomal DNA. Bacterial universal primers 1 and 2 can detect almost all kinds of bacteria, *Mycoplasma*, and *Ureaplasma*. In contrast, bacterial universal primer 3 can detect almost all kinds of bacteria, but cannot detect *Mycoplasma* or *Ureaplasma*.

(C) Gels show the prevention of bacterial contamination using eukaryote-made Taq DNA polymerase and the specificity of each primer set. PCR amplification products were detected according to the presence or absence of the target DNA templates.

A

Bacteria	PCR method		
detection	–	+	Total
Conventional culture method –	224	28	252
Conventional culture method +	9	44	53
Conventional culture method Total	233	72	305

C

Mycoplasma	PCR method		
detection	–	+	Total
Conventional culture method –	278	20	298
Conventional culture method +	1	6	7
Conventional culture method Total	279	26	305

B

Fungi	PCR method		
detection	–	+	Total
Conventional culture method –	302	1	303
Conventional culture method +	0	2	2
Conventional culture method Total	302	3	305

D

Ureaplasma	PCR method		
detection	–	+	Total
Conventional culture method –	269	15	284
Conventional culture method +	2	19	21
Conventional culture method Total	271	34	305

Figure 3

A comparison of detection results between the conventional culture method and the PCR method

(**A**) Number of detected Bacteria in 305 amniotic fluid samples, (**B**) Number of detected Fungi in 305 amniotic fluid samples, (**C**) Number of detected *Mycoplasma* in 305 amniotic fluid samples, (**D**) Number of detected *Ureaplasma* in 305 amniotic fluid samples.