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Original Article

Comparison of Culture and Real-time Polymerase Chain Reaction Methods for Detection of *Mycoplasma hominis* in Amniotic Fluids Samples

F Keskin, S Ciftci, SA Keceli[1], MO Koksal[2], E Caliskan[3], Y Cakiroglu[4], A Agacfidan[2]

Department of Microbiology, Faculty of Dentistry, Istanbul University, Istanbul, [1]Department of Medical Microbiology, Faculty of Medicine, Kocaeli University, Kocaeli, [2]Department of Medical Microbiology, Istanbul Faculty of Medicine, Istanbul University, [3]Department of Obstetrics and Gynecology, Faculty of Medicine, Bahcesehir University, Istanbul, [4]Department of Obstetrics and Gynecology, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey

Abstract

Background: *Mycoplasma hominis* is often present in the amniotic cavity with microbial invasion associated with spontaneous preterm labor. Conventional culture method is the gold standard for detection of Mycoplasmas, but real-time polymerase chain reaction (real-time PCR) has revolutionized the diagnosis of *M. hominis*. **Objective:** The purpose of this study is the comparison of the culture methodology with real-time PCR for the detection of *M. hominis* in amniotic fluid samples. **Methods:** Amniotic fluid samples were collected from 65 pregnant women (age range: 25–45 years) previously followed at an infertility clinic. They were collected by transabdominal genetic amniocentesis during 16–21 weeks of gestation. Amniotic fluids were inoculated in SP4 broth for 48–72 h, and after becoming alkaline, culture suspension was spread on A7 agar plate for 1 week till the typical colonies seen in "fried-egg" morphology under stereomicroscope. DNA was extracted using a QIAGEN Mini DNA kit. The real-time-PCR was performed using Rotor-Gene Q Real-time PCR instrument. A melting-curve analysis was also performed. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were measured by real-time PCR by taking culture as gold standard. **Results:** Sixty-five women in 16–21 weeks of gestation, with a mean age of 33 ± 5.06 years, were enrolled into this study. *M. hominis* detected by culture and real-time PCR assay was 72% (47/65) and 69% (45/65), respectively. 66% (43/65) specimens were positive by both methods. Real-time PCR sensitivity was 91.5%, specificity 88.9%, PPV 95.6%, and NPV 80%. **Conclusion:** Rapid detection of Mycoplasmas causing maternal complications such as neonatal infections and preterm labor in pregnancy by real-time PCR may be important and necessary. The high sensitivity and shorter time requirement of real-time PCR support its further development for diagnosis of Mycoplasma infections.

Keywords: *Amniotic fluids, culture, Mycoplasma hominis, real-time polymerase chain reaction*

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Introduction

Mycoplasmas belonging to the class of *Mollicutes* are the smallest living prokaryotes known. Characteristic features of the *Mollicutes* class are self-replication and a lack of the cell wall found in other bacteria.[1-3] Genetic distinction is the small genome size and low G + C content.[4] Most Mycoplasma and Ureaplasma are species of the family *Mycoplasmataceae*.[5] These organisms have intrinsic resistance to cell wall-active beta-lactam antibiotics due to the lack of a typical cell wall containing peptidoglycan renders.[6] These organisms are weakly

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Address for correspondence: Dr. F Keskin, Department of Microbiology, Faculty of Dentistry, Istanbul University, Istanbul, Turkey.
E-mail: fahriye.keskin@gmail.com

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stained by bacterial stains.[7] Mycoplasmas may cause infections in humans, but they can also colonize many animals and plants. Only a few types of Mycoplasma and Ureaplasma species are pathogenic for humans. These host organisms colonize primarily in mucous membranes of the upper respiratory and genitourinary tract causing atypical pneumonia, pyelonephritis, pelvic inflammatory disease, abortion, infertility, postpartum fever, bacterial vaginosis, neonatal bacteremia, meningitis, and abscesses.[8,9] Mostly, three species have been isolated from the genitourinary tract: *Mycoplasma hominis*, *Ureaplasma urealyticum*, and *Mycoplasma genitalium)*. They are commonly referred to as "genital Mycoplasmas" as the infection comes out via sexual contact. The effect of Mycoplasmas in inflammatory sites of the genitourinary organ is still in dispute. Culture is the reference standard for detection of *M. hominis*,[10] but high cost and the need for specialized media and proficiency complicate the use of culture. Confirmed culture results can be obtained within 2–5 days. Mycoplasma species such as *M. hominis* producing fried-egg colonies on A8 or SP4 agar can be identified based on growth rates, hydrolysis of arginine, and body site of origin. Considerable attention has been given in the application of the polymerase chain reaction (PCR) assay in primary detection of perinatal infections of Mycoplasma and Ureaplasma. PCR is essential if fastidious, slow-growing organisms such as *M. genitalium* or *M. fermentans* are sought and is also valuable in differentiating Ureaplasma species and serotypes, as described earlier.[7] Real-time PCR, a form of PCR, is a sensitive and specific option for the detection of microorganisms and generates data as the reaction proceeds.

The aim of the study was to evaluate the performance of real-time PCR against culture, as a method for the detection of *M. hominis* in amniotic fluids.

Materials and Methods

Clinical samples

Amniotic fluid samples were collected from pregnant women who underwent infertility investigation before gestation at the Infertility Clinic of Medical Faculty of Kocaeli University, Turkey. Pregnant women ranging from 25 to 45 years old underwent transabdominal genetic amniocentesis during 16–21 weeks of gestation, and they were asymptomatic for any intraamniotic or genital infections. Participants who developed an infection and were receiving prophylactic or therapeutic antibiotics were excluded from the study. All participants were negative for bacterial vaginosis before amniocentesis, for at least a period of 4–6 weeks.

In the study, 65 amniotic fluid samples were analyzed. Every individual sample was taken with 20–22 gauge and 9–15 cm length amniocentesis injector under sterile conditions. About 2–3 ml of amniotic fluid was taken for karyotype analysis and culture. Samples were kept at 4°C until they were delivered to the microbiology laboratory within 6 h for bacterial examination. This study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethical Committee Medical Faculty of the Kocaeli University (Number 2005; 172/19).

Mycoplasma hominis culture

Amniotic fluid samples (0.5–1 ml) were instilled into 3 ml SP4 broth as described in Tully and Whitcomb's study.,[11] supplemented with 10% L-arginine and incubated at 37°C in atmospheric conditions of 5% CO_2 for 48–72 h. The change in pH was determined using a pH electrode when the growth was first evident as indicated by a change in the color of pH indicator (phenol red). The color changed from red to yellow (Russell, PHM–10-070N). Optical density was measured at 550 nm in a Gallenkamp Visi–spectrophotometer. 100 µl of culture suspension was spread on A7 agar plate when the broth became alkaline (indicating an alkaline pH shift) (BioMerieux, Marcy l'Etoile, France) for *M. hominis*. Plates were incubated at 37°C in atmospheric conditions of 5% CO_2 for 1 week and examined every 2 days under stereomicroscope for typical colonies in "fried-egg" morphology. Plates displaying this morphology were considered as positive culture for *M. hominis*. *M. hominis* is morphologically variable but takes a characteristic fried-egg form because of penetrating deeply into the agar in central region of the colony. *M. hominis* ATCC 23114 strain was used as positive control for culture.

DNA extraction

DNA was extracted from standard strains and clinical samples using a QIAGEN Mini DNA kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The DNA sample was eluted in 200 µl of elution buffer, and 5 µl aliquot was used for real-time PCR.

Real-time polymerase chain reaction assay

The oligonucleotide primers M1 (5'-CAA TGG CTA ATG CCG GAT ACG C-3') and M2 (5'-GGT ACC GTC AGT CTG CAA T-3') were used for amplification from the 16S rRNA gene of *M. hominis*.[12] The real-time PCR test was performed using a Rotor-Gene Q Real-time PCR instrument (Qiagen, Hilden, Germany). One negative and one positive control were included in each run. Reaction mixture volume was 25 µl and it contained 1

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µM forward and reverse primers, 12.5 µl 2× Rotor-Gene SYBR Green PCR Master Mix, 5 µl RNase-free water, and 5 µl template DNA. PCR conditions were 95°C for 5 min denaturation steps followed by 40 cycles of 95°C for 15 s and 60°C 30 s. A melting-curve analysis was also performed (60°C to 95°C, 1°C/5 s). Data analysis was performed, as defined in the Rotor-Gene Q Real-time PCR detection system user's guide.

Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for real-time PCR, with culture method considered as the gold standard.

Results

The 65 women recruited were aged between 25 and 45 years (with a mean age of 33 ± 5.06 years). Demographic characteristics of the patients are shown in Table 1.

M. hominis was detected in 47 (72%) and 45 (69%) individuals by culture and real-time PCR assay, respectively. The results are shown in Figure 1.

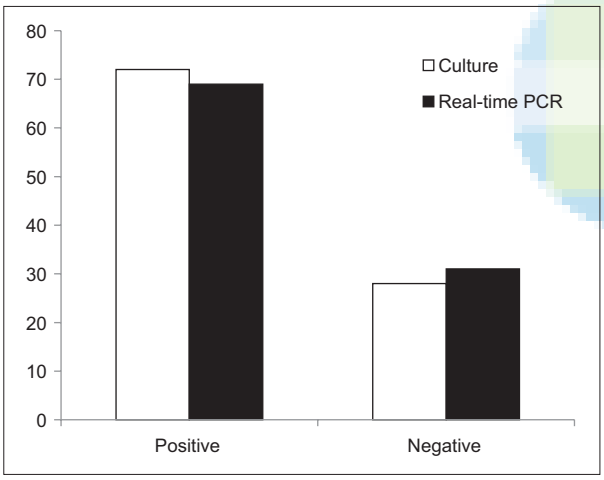

Figure 1: Percentages of *Mycoplasma hominis* positivity and negativity in culture and real-time polymerase chain reaction

Patients in the age group of 30–34 years showed the highest presence for *M. hominis* by both culture and real-time PCR methods (20 [42.6%] vs. 18 [40%] patients). Age distribution of patients being positive and negative for *M. hominis* in culture and real-time PCR are summarized in Figure 2a and b.

A comparison of all results obtained by real-time PCR procedure with those from culture method is shown in Table 2. The results of 65 patients were analyzed and 43 (66%) specimens were positive by both culture and real-time PCR. Forty-five patients (69%) were positive only by real-time PCR and 47 (72%) were positive only by culture method.

The real-time PCR sensitivity and specificity were 91.5% and 88.9%, respectively; PPV and NPV were 95.6% and 80%, respectively. It was found that four samples were culture positive and real-time PCR negative [Table 2]. In these samples, colony counts in cultures were in the range of 1–4 × 10^1 CFU/ml.

Table 1: Demographic characteristics of the patients

Age group	Culture		Real-time PCR	
	Positive, *n* (%)	Negative, *n* (%)	Positive, *n* (%)	Negative, *n* (%)
25-29	9 (19.1)	9 (50.0)	8 (17.8)	10 (50.0)
30-34	20 (42.6)	5 (27.8)	18 (40.0)	7 (35.0)
35-39	11 (23.4)	2 (11.1)	11 (24.4)	2 (10.0)
40+	7 (14.9)	2 (11.1)	8 (17.8)	1 (5.0)

PCR=Polymerase chain reaction

Table 2: Comparison of culture and real-time polymerase chain reaction results of amniotic fluid samples

M. hominis Real-time-PCR	*M. hominis* culture		
	Positive	Negative	Total
Positive	43	2	45
Negative	4	16	20
Total	47	18	65

M. hominis=*Mycoplasma hominis*; PCR=Polymerase chain reaction

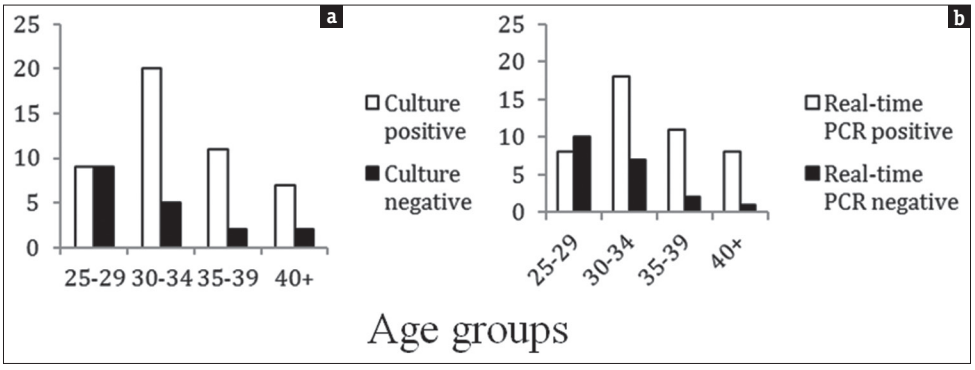

Figure 2: (a and b) Age distribution of patients being positive and negative for *Mycoplasma hominis* in culture and real-time polymerase chain reaction

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Discussion

In this study, we compared culture and real-time PCR methods in mid-trimester amniotic fluids samples for the detection of *M. hominis*. Genital Mycoplasma species are found to be the most widespread microorganisms related to preterm birth.[13-15] Mycoplasmas are well-known unusual bacteria among all prokaryotes. Attempts have been made worldwide to obtain valuable method for isolation and identification of this organism. However, at the present, there are two main methods used for diagnosis Mycoplasma, culturing and PCR, even though there are some advantages and disadvantages for each method.[16] The diagnosis of bacterial pathogens by the use of traditional methods is still an essential constituent of the diagnostic process. The traditional methods such as culturing fastidious organisms, determination of antimicrobial susceptibility for the organism, culturing clinical samples after antibiotic treatment, and biochemical assays are time-consuming and troublesome. In addition, these methods might increase the duration of diagnosis and treatment.[17] Real-time PCR has revolutionized the way clinical microbiology laboratories diagnose human pathogens. The combination of excellent sensitivity and specificity, low contamination risk, ease of performance, and speed has made real-time PCR technology an appealing alternative to conventional culture-based testing methods used in the clinical microbiology for diagnosing many infectious diseases.[18] PCR has an another advantage in that it can still detect DNA of dead organisms.[19]

In our study, during the amniocentesis period, patients were found to be asymptomatic and had no symptoms of bacterial vaginosis or vaginal infection. According to our results, 43 (66%) pathogen-containing specimens were detected by both culture and real-time PCR methods, 47 (72%) only by culture, and 45 (69%) only by PCR. The results indicate that the use of a PCR assay for Mycoplasma in amniotic fluid samples results in a rate of detection of this microorganism that is similar to observation with the literature for Mycoplasma.[20-23] Interestingly, four patients had positive culture, but they had negative PCR assay results. Other studies in literature also show similar observations.[20,21] This result may be attributed to possible degradation of bacterial DNA or to the presence of inhibitor(s) of PCR reaction in clinical samples. Considering these potential problems, sensitivity of real-time PCR method is 91.5% for the detection of *M. hominis*.

In our study, two samples were found to be positive by real-time PCR and negative with culture. This may indicate that the cultures may be inadequate in samples with low bacterial loads or dead organisms. This result suggests that real-time PCR may be useful in samples with DNA of low-level bacterial loads and dead organisms. The lower level of bacteria detection in cultures may be partly attributable to the generally recognized difficulties in culturing and isolating Mycoplasmas. Even though culture was considered the reference method, PCR is theoretically able to detect fewer organisms; therefore, PCR-positive, culture-negative specimens likely represent true positives.

Nucleic acid-based techniques such as real-time PCR have superiority over culture-based methods and additionally they provide fast and specific organism detection as well. Real-time PCR reduced the assay time from 3–5 days for *M. hominis* to 24 h.[20] Duration for DNA extraction from samples is usually <4 h, and in this research, the results were received in 90 min. In addition, amplification by real-time PCR took around 2 h, which is comparably less than the time required for detection of *M. hominis* by the culture method. Such a finding would be clinically relevant because genetic identification of bacteria capable of invading the amniotic cavity and eliciting preterm labor could result in earlier detection and improvements in therapeutic intervention. Furthermore, identification of the *M. hominis* genes involved in invasion of the amniotic cavity could contribute to a better understanding of the role of this bacterial species in preterm birth. In that manner, real-time PCR is chosen for rapid detection of *M. hominis* in amniotic fluids.

M. *hominis* grows well in SP4 medium and colonies appear on agar within 2–3 days, visible with a stereomicroscope. To confirm species identity for Mycoplasmas growing on agar, additional procedures (e.g., a PCR assay) must be performed because there are no phenotypic tests that can distinguish them.[24] Comparison of PCR method with culture has proved that real-time PCR assay is much more sensitive than culture for the detection of *M. hominis* in clinical samples.[21] Detection of *M. hominis* by PCR method is specific, sensitive, and time saving. This organism is available to be detected in routine plate cultures, but this could not be counted as a rapid and sensitive approach for detection. For same cases such as specialized cultures, it takes a longer time than the defined PCR assay.[25] Other advantages of real-time PCR usage are rapidity and being a closed system, eliminating the risk of carry over.

Conclusion

Conventional culture methods are considered as a gold standard to identify and isolate bacterial pathogens.

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However, culture methods need special laboratories and are time-consuming. Results show that real-time PCR has an important role for the rapid detection of Mycoplasma in clinical samples. The culture is considered as gold standard method in this study and in general, but colonial identification is challenging because of subjectivity of the evaluation by human eye. The morphology could be misidentified as colonies or colonies may be overlooked which could lead to false results. Although real-time PCR is considered to be technically complex, it is an efficient high-throughput technique widely used compared to culture method for a laboratory with PCR experience.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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