Original Article

Evaluation of PCR for the Identification of *Mycoplasma spp.* and *Mycoplasma genitalium* congenital infection in cord blood

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Abstract

Background: Congenital infections are the most important threat to human fetal health caused by bacterial, viral, and fungal agents. In this study, PCR diagnostic method has been used on umbilical cord samples to diagnose bacterial agents such as *Mycoplasmas*, especially *Mycoplasma genitalium*. **Materials and Methods:** This study was performed on 100 cord blood samples from cesarean section patients. Limit of detection (LOD) and specificity tests of both PCR was performed. DNA samples were extracted by the DNG plus method and amplified by the PCR technique. The optimized PCR product of 715 bp for *Mycoplasma spp.* and 427 bp for *M. genitalium* was amplified and observed on 1.5% gel electrophoresis. DNA specificity testing of seven other organisms revealed a 100% specificity of these primers. The detection limit was set at 100 copy / reaction for *Mycoplasma.spp* and 1000 copy / reaction for *M. genitalium*. **Results:** Of the 100 samples examined, 5 samples were positive for *Mycoplasma spp*, and no positive cases were observed for *M. genitalium*. Maternal infections can have important consequences on the fetus. **Conclusion:** Molecular methods such as PCR could be used for rapid identification of important factors in congenital infections, such as *mycoplasma*, and provide a good prognosis.

Keywords: PCR, Cord Blood, Maternal Infection, *Mycoplasma*, *Mycoplasma genitalium*

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Introduction

Congenital infections are the most important threat to human fetal health caused by bacterial, viral, and fungal factors. However, the majority of congenital infections are caused by viral factors such as the cytomegalovirus, varicella-zoster virus, herpes simplex virus, rubella virus, etc., which may be present in the cord blood. Genital or respiratory infections caused by bacterial agents such as *Mycoplasmas* are an important part of medical reports.[1-3]. Genital *Mycoplasmas* are one of the major contributing factors of vaginitis, cervicitis, and pelvic inflammatory disease (PID) in women referred to obstetric clinics. These organisms attach to the mucosal surfaces of the genital tract. Due to the high potency of colonization in endocervixes, they are likely to cause adverse effects for the mother and her baby [4, 5]M. genitalium was first isolated from the urinary tract of men with non-gonococcal urethritis (NGU). This *Mycoplasma* is very similar to *M. pneumonia* and causes PID, Urethritis, endometritis, and cervicitis[6, 7]. Among the 16 species isolated from human, only *M. pneumonia* has been identified as a genuine human pathogen [8, 9].This bacteria is an important and common cause of acute respiratory infections[10, 11] and has been isolated from human urogenital tracts [12].In the reproductive system, M. hominis and also *M. genitalium* can sometimes

exacerbate bacterial vaginosis or nonspecific (non-gonococcal) urethritis or even miscarriage [13]. Frequent isolation of *U. urealyticum* from men with non-gonococcal urethritis revealed that it causes other infections in addition to genital infections[14], such as endocarditis and osteomyelitis [15]and even chronic lung infections in infants, bacteremia, and death[16]. However, due to the problems encountered in the cultivation and inoculation of these bacteria in the laboratories, the role of these organisms in causing human infection, and in particular the adverse effects have been underestimated in our country. *Mycoplasmas* cause various diseases in the genitourinary and respiratory tracts and cause abnormal reproductive and neonatal mortality [17]. There are various types of Mycoplasmas in the genitourinary tract that act as opportunistic organisms[18].

Genital Mycoplasmas, especially *M. hominins* and *U. urealyticum*, are natural residents of the genitourinary tract of men and women who have sexual activity. These bacteria are more common in women than men and are transmitted by sexual intercourse or from mother to baby at birth. According to research by Kacerovsky et al. in 2014 on cord blood samples of pregnant women studied, microorganisms such as *Ureaplasma spp*. And M. hominis was about 9%. No significant relationship was observed between the microbial load of the amniotic fluid and the cord blood because, under normal conditions, the amniotic fluid should be sterile[19]. Stellrecht used PCR instead of culture in his study, and the results showed that PCR could be a suitable method for the detection of *M. genitalium* and other genital *Mycoplasmas* in clinical samples. A study by Luki et al., using PCR on samples from pregnant women, showed 3.6% of M. genitalium[20].In a study on the infection caused by genital *Mycoplasma* in 174 women with bacterial vaginosis is performed by Vatani and colleagues, the predominance of molecular methods such as PCR (77.6%) compared to culture (about 40.8%) was proven[21].

Of the *Mycoplasma* species, *M.penetrans*-*M.pirum*- *M.primatum*-*M.spermatophilu* and *M.amphoriforme* have the least threat in pregnancy[22]. In recent years, the evaluation of different species of *Mycoplasma* in the reproductive system such as *M.fermentans*, *M.Penetrans*, and *M.genitalium* has been developed by molecular methods such as PCR[23]. The purpose of this study was to find a suitable diagnostic method for the diagnosis of *Mycoplasma* spp. as well as M. genitalium in congenital cord blood infections, because the failure in timely diagnoses may increase the risk of delayed treatment or overuse of broad-spectrum antibiotics.

Methods

Species used in this study were M. pneumoniae (NCTC 10119), *M. arginini*, *M. hyorhinis*, *M. hominis*, *M. orale*, *Acholeplasma laidlawii*., *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhimurium* provided from Razi Institute and Pasteur Institute of Iran.

Collection of the studied samples. In this study, 100 cord blood samples of cesarean patients were collected from Mustafa Khomeini Hospital. DNA extraction from cord blood samples was performed by the DNG-PLUS method[24].

PCR test. The required compounds for PCR were prepared in 25 µL volume by the following order: Five nanogram (ng) template DNA (in 5 µL) , 0.2 µM of each primers , 1.5 mM Mgcl2 (sinaclon) , 0.2 mM dNTP Mixture, and 1.5 unit of Taq DNA Polymerase (sinaclon),2.5 µL 10x PCR buffer (sinaclone) and 14µl of deionized sterile double distilled water used for reaching to the target volume. The thermal program used and optimized was: heating at 93 ° C for 20 seconds, 60 ° C for 20 seconds, and finally 72 ° C for 30 seconds, which was performed for 40 cycles of amplification. The PCR product with the desired size (715 bp), along with the size marker and positive and negative control, was analyzed on 1.5% agarose gel using SYBR safe (Sinaclon) in the gel documentation system (Major-science).

The primers encode a 715 bp product and their target gene is fixed, stable, or shared sequences (consensus sequence) 16SrRNA fragment of

Mycoplasma[25].

Table1. All primers are used in this study.

Bacteria	Primers	Sequence	Tm (ºC)	Product size (bp)	Reference
M. genitalium	**FMGe427**	5'TACATGCAAGTCGATCGGAAGTAGC-3'	74	427	[26]
	RMGe427	5'-AAACTCCAGCCATTGCCTGCTAG-3'	70		
Mycoplasma spp.	**GPO-1**	5'-ACTCCTACGGGAGGCAGCAGTA-3'	74	715	[27]
	MGSO	5'TGCACCATCTGTCACTCTGTTAACCTC 3'	76		

Limit of Detection (LOD) and specificity. To determine the LOD of the primers used in this study, different dilutions were prepared from *M. genitalium* suspension with specific CFU, their DNA was extracted and finally, the PCR test was performed on the samples with the specified number. Specificity testing using DNA from several organisms such as *M. tuberculosis, P. aeruginosa, S. typhimurium, S. aureus, Hepatitis B virus*, as well as human and mouse DNA for *Mycoplasma spp*. and organisms such as *M. pneumoniae, A. laidlawii, M. orale, M. arginini, M. hyorhinis, M. hominis, and Trichomonas vaginalis* were performed for *M. genitalium*. Optimized PCR test was performed with positive and negative controls.

Results

Using GPO-3, MGSO, and, *Mycoplasmas* DNA such as *M. pneumoniae, M. genitalium, M. hyorhinis, M. orale*, PCR was optimized for *Mycoplasma spp* detection. This PCR test produced a 715bp product with the DNA of all tested *Mycoplasmas* (Figure 1a).By using FMGe427 and RMGe427 primers and *M. genitalium* DNA, the PCR detection test for this bacteria was optimized and 427 bp product was amplified (Figure 1b).In both cases, the 16SrRNA was considered as target gene[28].

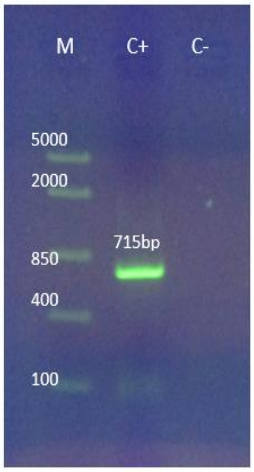

Figure 1a

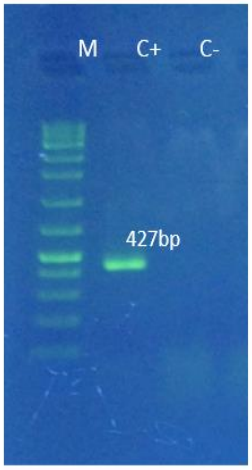

Figure 1b

Figure 1: Optimized PCR tests for *Mycoplasma spp* and *M. genitalium* Figure 1a: Optimized PCR Test for *Mycoplasma spp* detection Column M: Size of DNA marker (Middle Range DNA Ladder Thermo scientific), C +: Positive control (*M. genitalium*), C-: Negative control. Figure 1b: Optimized PCR Test for *M. genitalium* detection Column M: Size of DNA marker (1Kb Ladder bioflux), C +: Positive control (*M. genitalium*), C-: Negative control

Evaluation of test detection limit was performed by dilution of *Mycoplasma* culture with specific colonies. results showed that the sensitivity of the PCR test for Mycoplasma spp was 100 copies (Figure 2a) and the PCR detection sensitivity of M. genitalium was 1000 copies in one test (Figure 2b)

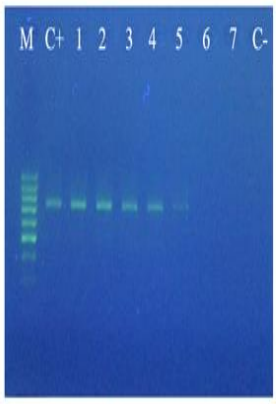

Figure 2a

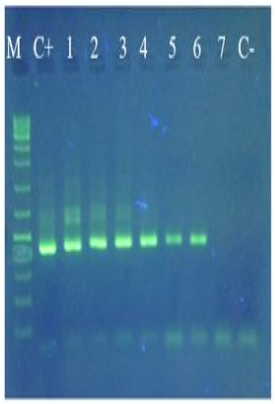

Figure 2b

Figure 2: Limit of detection test of PCR for both *Mycoplasma spp* and *M. genitalium* Figure 2a: Optimized PCR detection test for *M. genitalium*: Column M: Size marker, (100 bp DNA Ladder Thermoscientific) C+: Positive control, Column1: 10^6 CFU, Column 2: 10^5 CFU, Column 3: 10^4 CFU, Column 4: 10^3 CFU, Column 5: 100 CFU, Column 6: 10 CFU, Column 7: 1 CFU, C-: Negative control Figure 2b: Optimized PCR detection test for *Mycoplasma spp*: Column M: Size marker, (100 bp DNA Ladder Thermoscientific) C+: Positive control, Column1: 10^8 CFU, Column 2: 10^7 CFU, Column 3: 10^6 CFU, Column 4: 10^5 CFU, Column 5: 10^4 CFU, Column 6: 10^3 CFU, Column 7: 100 CFU, C-: Negative control

By performing specificity tests for PCR detection of *Mycoplasma spp* revealed that the primers used have made no unwanted product with the DNA of non-*Mycoplasma* bacteria such as *M. tuberculosis*, *P. aeruginosa*, *S. typhimurium*, *S. aureus*, *viruses* such as *B hepatitis*, and also human and rat DNA (Figure 3a).

M. genitalium specificity test with primers used, no unwanted products with DNA of *M. pneumoniae*,*A. laidlawii*,*M. orale*, *M. arginini*, *M. hyorhinis*, *M. hominis* and *T. vaginalis* (Figure 3b).

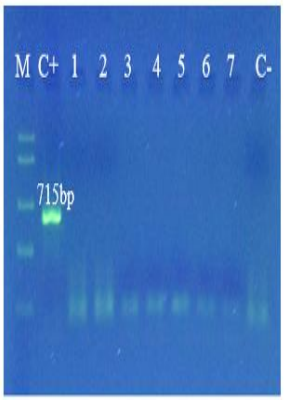

Figure 3a

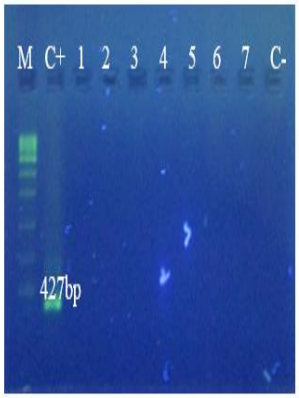

Figure 3b

Figure 3: Specificity of PCR tests for the detection of *Mycoplasma spp* and *M. genitalium* Figure 3a. Specificity of PCR test for *Mycoplasma spp* detection. Column M: Size marker size (Middle Range DNA Ladder Thermoscientific), C+: Positive control, Column 1: Human DNA, Column 2: Rat DNA, Column 3: *M. tuberculosis*, Column 4: *P. aeruginosa*, Column 5: *S. aureus*, Column 6: *S. typhimurium*, Column 7: B and C Hepatitis virus, C-: Negative control Figure 3b. Specificity of PCR test for *M. genitalium* detection. Column M: Size marker (1Kb DNA Ladder (bioflux)), C+: Positive Control, Column 1: *M. pneumoniae*, Column 2: *A.laidlawii*, Column 3: *M. tuberculosis*, Column 4: *M. orale*, Column 5: *M. hyorhinis*, Column 6: *M. hominis*, Column 7: *T. vaginalis*, C-: Negative control

Mycoplasma infection in 100 cord blood samples was searched by PCR. 5 samples (5%) were positive for *Mycoplasma spp* but none of them were positive for *M. genitalium*.

Discussion

This study attempts to investigate the diagnostic potential of PCR testing for *Mycoplasma* infections in neonatal cord blood. Evaluation of cord blood seems to be helpful in the diagnosis of neonatal harms, including sepsis [29, 30]. According to Leena and colleagues research, sensitive and accurate diagnosis of sepsis in newborns is very difficult, and sepsis is the result of an intrauterine infection [31]. Failure in timely diagnose may increase the risk of delayed treatment or overuse of broad-spectrum antibiotics [32]Methods of diagnosis of sepsis include blood culture but there are some disadvantages such as timing, sensitivity, maternal antibiotic use, low sample size, and low pathogen concentration in the sample[33, 34]. Other laboratory tests, such as white blood cell count and CRP, have low specificity [35, 36]. Due to

the lack of reliable diagnostic methods in the NICU, long-term use of antibiotics can lead to dangerous consequences such as necrosis, enterocolitis, antibiotic resistance, fungal infections and hearing loss [37, 38]. Evaluation of cord blood reflects intrauterine inflammation, where premature infants develop sepsis.

Rapid detection of genital *Mycoplasmas*, especially in pregnant women and preterm infants, is essential because these organisms can lead to spontaneous miscarriage, postpartum fever, chorioamnionitis, preterm delivery, and premature birth. Premature infants with low birth weight also may develop respiratory distress syndrome, pneumonia, meningitis, and death. According to research by Han et al., molecular techniques not only identify a wide range of amniotic fluid infections but are also an appropriate method for the diagnosis of mixed infections [39].

A combination of PCR molecular method and culture methods has been proposed by Stakenborg in 2005 for the identification of a wide range of *Mycoplasma* infections [40]. Also, PCR has previously been used to detect other infections such as falciparum and some viruses in cord blood [41]. The culture method which has been used to diagnose Mycoplasmasis very time consuming (1 to 4 weeks), has high false-negative results, and low sensitivity and specificity [42, 43].

Based on the results, 715 bp DNA fragments were generated for *Mycoplasma spp* as a result of using GPO-3 and GMSO primers. The result is consistent with the results of Tabatabai Qomi and colleagues research [44].

The results of LOD using Mycoplasma culture dilution showed that PCR is a sensitive method for the detection of *Mycoplasma spp* and *M. genitalium* (100 and 1000 copies in one test, respectively) (Figure 2). These results are consistent with the results of the study in which PCR was used to detect genital *Mycoplasmas*. A study by Kathleen et al., Proves that PCR is a rapid and sensitive method for the detection of this type of infection, but is also superior in comparison to the culture method [45]. However, as it has been demonstrated in some studies, the modified culture method is also highly sensitive for the detection of some species, such as *M. hominis* in patients' endocervix[46].

In the study by Najar Pirayeh et al. in 2007, the sensitivity of the PCR method and culture method was 91.8% and 53%, respectively, indicating high sensitivity and rapidity of PCR in comparison to the culture method for the identification of these bacteria [47] .N-Luki et al. In Canada, performed a study by using PCR and culture techniques, to isolate U. urealyticum, *M. hominis*, and *M. genitalium* in clinical specimens obtained from 47 high-risk pregnant women and 8 newborns. PCR was used to isolate *Ureaplasma* in 31 out of 55 patients and facilitated *M. hominis* in 7 samples and *M. genitalium* in 2 samples. Four PCR-positive patients showed negative culture results [48].

Also, our results showed that the primers used did not produce any unwanted products with the DNA of other non-Mycoplasma bacteria such as *M. tuberculosis, P. aeruginosa, S.typhimurium, S. aureus*, and viruses such as *B hepatitis* and human DNA (Figure 3a). This suggests that the use of these primers in PCR provides high specificity for the detection of Mycoplasma species. It should be noted that PCR has been used for the detection of species such as *M. tuberculosis* [49], *P. aeruginosa* [50], *S. typhimurium* [51], *S. aureus* [52] and viruses such as *B hepatitis* [53]. Although the primers used were different and none of them studied samples were cord blood.

In another part of the study, PCR specificity for differential detection of *M. genitalium* was investigated using RMGe427 and FMGe427 primers. The results showed that no unwanted DNA products of bacteria such as *M. pneumoniae, A. laidlawii, M. orale, M. arginini, M. hyorhinis, M. hominis* and *T. vaginalis* were produced (Fig. 3b). This indicates that these primers are specific for *M. genitalium* and that PCR can also be used as a reliable method for differential diagnosis of this species. Because *M.genitalium* culture is time consuming, studies have shown that PCR is faster and more susceptible in comparison to cultivation [54, 55].For example, using a primer belonging to the gene encoding a binding protein, this species was identified in specimens collected from the throats of patients. On the other hand, other studies have identified the genome of *M. genitalium* in different genotypes by relying on the 16S rRNA coding gene and PCR method. However, it has been proven that the use of primers with genes such as MgPa has the risk of inappropriate primer placement and misdiagnosis of *M. genitalium*. However, the LOD of the PCR test has been reported

to be approximately 1000 copies, which is consistent with the results of the present study [56]. However, all of this research has been done on tissues other than cord blood. It is noteworthy that M. genitalium contamination was not confirmed in any of the collected cord specimens. *M.genitalium* isolation rates, range from zero percent in the UK [57]to 34.4 percent in New Zealand[58]. Various studies have reported different prevalence of *M. genitalium*. This may be due to the type of study, the population studied (antibiotic use, genital infection, multiple sex partners, etc.) sample number, sampling method, age of patients, race, culture, geographic location, laboratory method (s) (Culture and PCR).

Conclusion

Due to the potential impact of *Mycoplasmas* on the infection of pregnant mothers and neonatal transmission through the umbilical cord and important fetal outcomes, the need for timely diagnosis and treatment of these infectious agents is increasingly felt. Molecular methods such as PCR can provide a good prognosis for the rapid identification of important factors in congenital infections such as *Mycoplasmas*.

Conflicts of Interest

The authors declared no conflict of interest.

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