

Diagnostic Efficacy of Multiplex PCR for Detection of *Mycoplasma genitalium*, *Mycoplasma hominis*, and *Ureaplasma urealyticum* in Urogenital Samples

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(Received: 09 September 2015; accepted: 06 December 2015)

Mycoplasma genitalium, *Mycoplasma hominis*, and *Ureaplasma urealyticum* are associated with infections of the genitourinary tract, reproductive failure. A multiplex PCR was used for detection of these *Mycoplasmas* species in a single amplification reaction simultaneously. 156 sample were collected from 104 women's genital and 52 semen specimens (from infertile men) with urogenital infections, referred to gynecological and general outpatient obstetrics clinics. The Specimens were analyzed with species specific primers for identification of *M. genitalium*, *M. hominis*, and *U. urealyticum* by multiplex PCR. The primer sets; amplified a 78 bp fragment (*M. genitalium*), 280 bp fragment (*M. hominis*), and 418 bp fragment (*U. urealyticum*). Data from the patients were analyzed in order to confirm their relationship of isolates with each disorders. Of the 156 samples examined, the total positive rates of *Mycoplasmas* were 25% (*M. genitalium* isolates, 3.8 %; *M. hominis* isolates, 8.4 %; and *U. urealyticum* isolates, 12.8 %), by using multiplex PCR. Genital infections caused by mycoplasmas are the most important causes of reproductive disorders. Multiplex PCR was shown to be a highly sensitive, specific test, when specific primer sets were used for screening and diagnosis of genital *Mycoplasmas*.

Key words: *Mycoplasma genitalium*, *Mycoplasma hominis*,
Ureaplasma urealyticum, Multiplex PCR, Urogenital diseases.

Mycoplasmas are the smallest and simplest self-replicating known free-life microorganisms with a size between 150 and 250 nm being about 300 nm in diameter. They are fungi-like in nature, hence the prefix myco- and lack a cell wall, hence the suffix-plasma. *Mycoplasmas* refer to the plasticity of bacterial forms resembling fungal elements. The absence of a rigid cell wall in them is

responsible for the lack of a gram stain reaction¹⁻³. The term *Mycoplasma* is usually used as a synonym for the class of Mollicutes that represents a large group of highly specialized bacteria and are all characterized by their lack of a rigid cell wall⁴. They have a triple layered membrane and unlike conventional bacteria, and lack a rigid cell wall⁵. Some *Mycoplasmas* considered as normal flora of the respiratory or genitourinary tract⁶. They may be the only prokaryotes which can symbiotically grow in the eukaryotic host cells⁷. Their characteristics and a molecular explanation for their pathogenesis have been reviewed quite recently.

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The primary habitats of human are the mucous surfaces of the respiratory and urogenital tracts^{5,7}. Genital Mycoplasmas will be used to denote that discussion encompasses all the Mycoplasmataceae that are commonly found in the genitourinary tract and may occur in the female genital tract of pregnant and non-pregnant women⁸. Seven species of *Mycoplasma* can be isolated from genitourinary tract, but only *Mycoplasma hominis*, *Mycoplasma genitalium*, and *Ureaplasma urealyticum* have been implicated in human disease and related with pathologies of the urogenital tract, and particularly with non gonococcal urethritis (NGU) in men^{9,10}. *Mycoplasma* have been associated with various infections of the genitourinary tract, pathological conditions, reproductive failure and intrauterine infections, including pyelonephritis, urethritis, pelvic inflammatory disease, chorioamnionitis, endometritis, postpartum fever, and leading to important complications such as infertility, puerperal infection, preterm birth, still birth, premature birth, low birth weight, septic and spontaneous abortion, neonatal morbidity and perinatal mortality¹¹⁻¹⁴. During pregnancy and contraceptive use, the level of estrogen and progesterone hormones will increase higher and make changes in vagina which could predispose to infection by mycoplasmas¹⁵. Among these three species of *Mycoplasma* only *Mycoplasma genitalium* was significantly more frequent in men with *Nongonococcal* urethritis (NGU)¹⁶. NGU is one of the most common sexually transmitted diseases (STDs) diagnosed among men. *Ureaplasma urealyticum* appears to cause some of nonchlamydial nongonococcal cases^{9,17}. The general laboratory methods for diagnosis *Mycoplasma* are microscopic examination of colonies, serology techniques and molecular biology methods^{18,19}. However, the main method of detecting Mycoplasmas is by culture, but it can take two to five days for culture *U. urealyticum* and *M. hominis*, and up to eight weeks to culture *M. genitalium*²⁰. These infectious agents can be detected in less than 8 hours by nucleic acid amplification techniques¹². PCR methods have been developed to identify each of these bacteria. The aim of this study was to evaluate the multiplex PCR for the high throughput screening of clinical samples for simultaneous detection of *U.*

urealyticum, *M. hominis*, and *M. genitalium* in the west of Mazandaran, Iran.

MATERIALS AND METHODS

Specimen collection

In this study all specimens were taken from 156 patients aged 18 to 50 years (including 104 cervical and vaginal swabs and 52 semen samples) consecutively attending for visits to the gynecological and general outpatient obstetrics clinics and medical laboratories in Western Mazandaran province, Iran between January 2013 and July 2014. Patients for vaginal and cervical samples were first visited by gynecologists. Those who had visible genital lesions, cervical bleeding, or receiving antibiotics within the last two weeks were excluded. Vaginal and cervical specimens were collected with sterile packed cytobrushes and inoculated in 2 ml of Phosphate Buffer Saline (PBS) Solution. Semen samples were collected from infertile men, with urogenital infection, then transported to laboratory and frozen at -20° C for PCR assays. Therefore Sample size calculated from Prashant Kadam study formula²¹.

Primer design

Species specific primer sets were used in this study. The 140-kDa adhesion protein gene (MgPa) primers were used for the 16S rRNA gene amplification of *M. genitalium*, MH1 and MH2 primers were used for the 16S rRNA gene amplification of *M. hominis*, and UUA2 and UUS2 primers were used for the urease gene amplification of *U. urealyticum*. The amplification products are 78-bp (Mg), 280-bp (Mh), and 418-bp (Uu) in length, respectively. Nucleotide sequences of primers used, shown in table 1.

DNA extraction

The High Pure PCR Template Preparation Kit purified nucleic acids from 100 µl of specimen (American, Roche Company) The kit contains 5 different buffers, Proteinase K, High Pure tubes with pre-packed silica filters and collection tubes. Briefly, the starting material is lysed by incubation with Lysis Buffer and Proteinase K to break open cell membranes and expose DNA and RNA. Binding Buffer is added to inactivate nucleases and the solution is transferred into filter tubes and briefly centrifuged. After three washes with different buffers, the nucleic acid bound to the glass fiber

filter is pure and can be eluted with the Elution Buffer into a sterile 1.5 microcentrifuge tube. Eluted DNA can be stored at 4°C, frozen at -20°C or used right away for further experiments. Boiling method for extraction of genomic DNA was used too.

Multiplex PCR amplification assay

Multiplex PCR were performed by a BioRad thermal cycler (USA) in 25 µL reaction mixtures containing 12.5µL of master mix (Takara Bio Inc., Japan, Code RR310-Lot A1301-1, Emerald Amp GT PCR Master Mix (2× premix), 5.0µL of Template DNA (1µg), 2.5µL of Primer Mix (10 pmol of each primer), and 5.0 µL double distilled water.

Initial denaturation step at 95°C for 5 minutes followed by 1 cycle, 35 cycles at 95°C for 40 seconds, annealing at 58°C for 40 seconds, extension at 72°C for 60 seconds, followed by final extension at 72°C for 10 min. Amplified PCR products (5 µL) were visualized and photographed under UV light after electrophoresis in 2% agarose gel containing ethidium bromide (1 µg/mL) for 30 minutes, at 130 V.

Detection and analysis of the PCR products

The multiplex PCR performed on the genomic DNA with MgPa F, MgPa R, MH1, MH2, UUA2 and UUS2 primers produced the expected size bands, which were distinguishable on a 2% agarose gel. Multiplex PCR was developed for the simultaneous detection and identification of the *M. genitalium*, *Mycoplasma hominis*, and *Ureaplasma urealyticum* in women urogenital secretion and in men semen specimens. In this study, 104 cervical and vaginal samples were collected from married women (18-47 years old) and 52 semen samples collected from men (21-50 years old). Vaginal discharges, urethritis, vaginitis,

cervicitis, and vulva irritations in women and urethritis and infertility in men are common clinical symptoms. The specimens DNA were extracted and analyzed by multiplex PCR. The products were confirmed by DNA sequencing. Sequencing was performed on both strands at sequencing service (Macrogen Co. Korea). Finally, sequence alignment was carried out using Basic Local Alignment Search Tool (BLAST) algorithm.

Statistical analysis

Statistical analyses of this study were performed using SPSS statistical software package version 16.0 (SPSS Inc, Chicago, IL) and chi-square test. The *P* value was set at < 0.05 prior to beginning the study.

RESULTS

Primer pairs MgPaF and MgPaR amplified a 78-bp DNA fragment from the Adhesion protein (MsrA-Peptide methionine sulfoxide reductase) gene of *M. genitalium*, Primer pairs MH1 and MH2 successfully amplified a 280-bp DNA fragment from the 16S rRNA gene of *M. hominis*, and Primer pairs UUS2 and UUA2 amplified a 418-bp DNA fragment from the urease gene of *U. urealyticum*. The control reaction which lacks the template DNA, did not exhibit any amplification (Fig. 1). Non-specific bands were not detected. All Positive samples were approved by DNA sequencing. Sequencing was performed on both strands at sequencing service (Macrogen Co. Korea). Finally, sequence alignment was carried out using Basic Local Alignment Search Tool (BLAST) algorithm. The multiplex PCR assay could amplify and differentiate between *M. hominis*, *M. genitalium*,

Table 1. Nucleotide sequences of primers used in this study⁹

| Organism, and primers | Primer sequence (5'–3') | Product Length (bp) | Target genes |
|--------------------------|-----------------------------------------|------------------------|----------------------------------|
| <i>M. hominis</i> | | | |
| MH1 F | 5'-TGAAAGGCGCTGTAAGGCGC-3' | 280 | 16S rRNA |
| MH2 R | 5'-GTCTGCAATCATTTCTATTCCAAA-3' | | |
| <i>M. genitalium</i> | | | |
| MgPaF | 5'-GAGAAATACCTTGATGGTCAGCAA-3' | 78 | 140-kDa Adhesion protein gene |
| MgPaR | 5'-GTTAATATCATATAAAGCTCTACCGTTGTTATC-3' | | |
| <i>U. urealyticum</i> | | | |
| UUS2 | 5'-CAGGATCATCAAATCAATTCAC-3' | 418 | Urease gene |
| UUA2 | 5'-CATAATGTTCCCCTTCGTCTA-3' | | |

and *Ureaplasma urealyticum*. Genital specimens were obtained for detection of mycoplasma from 156 patients seen at fertility clinics. From total, the 39 (25%) PCR-positive specimens, 20 (12.8%) were positive for *Ureaplasma urealyticum*, 13 (8.4%) were positive for *M. hominis*, 6 (3.8%) were positive for *M. genitalium*, 5 were positive for both *U. urealyticum* and *M. hominis*, 4 were positive for both *U. urealyticum* and *M. genitalium*, 3 were

positive for both *M. hominis* and *M. genitalium*, and 2 were positive for all.

In cervical or vaginal specimens, PCR detected Mycoplasma in 28 out of the 39

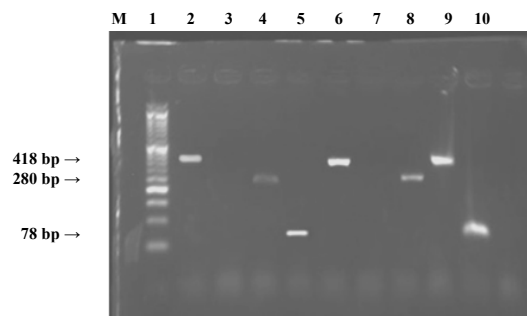


Fig. 1. Ethidium bromide-stained agarose gel electrophoresis of PCR amplified products generated from patients DNA samples. Lane M is the DNA size Marker (50 bp DNA ladder; ClnaClone Co.); lane 1 and 5 shows 418-bp *U. urealyticum* positive sample, ; lane 2 and 6 show the result of negative samples, lane 3 shows 280-bp *M. hominis* positive sample, and Lane 4 shows 78-bp *M. genitalium* positive samples; lanes 7, 8, and 9 show positive controls for these three species; and lane 10 shows negative control

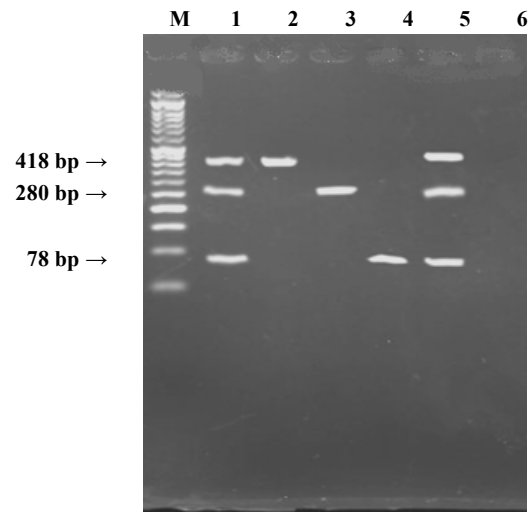


Fig. 2. Ethidium bromide-stained agarose gel electrophoresis of PCR amplified products generated from patients DNA samples. Lane M is the DNA size Marker (50 bp DNA ladder; ClnaClone Co.); lane 1 show the result of multiplex PCR 78-bp *M. genitalium*, 280-bp *M. hominis*, and 418-bp *U. urealyticum* positive samples simultaneously; lane 2 shows 418-bp *U. urealyticum* positive sample; Lane 3 shows 280-bp *M. hominis* positive sample; lane 4 shows 78-bp *M. genitalium* positive sample; lane 5 shows positive control; and lane 6 shows negative control

Table 2. Number of specimens positive for genital *Mycoplasmas*

| Specimen type | Number of specimen Tested | Number of specimen PCR positive (%) | <i>M. hominis</i> | <i>M. genitalium</i> | <i>U. urealyticum</i> |
|---------------|---------------------------|-------------------------------------|-------------------|----------------------|-----------------------|
| Vaginal swab | 71 | 21(30) | 7(10) | 3(4) | 11(16) |
| Cervical swab | 33 | 7(21) | 2(6) | 1(3) | 4(12) |
| Semen | 52 | 11(21) | 4(8) | 2(4) | 5(9) |
| Total | 156 | 39(25) | 13(8.4) | 6(3.8) | 20(12.8) |

Table 3. Number of positive samples according to the age of the man and woman

| Woman Age/Samples | 18-22 | 23-27 | 28-32 | 33-37 | 38-42 | 43-47 | Total |
|----------------------|-------|--------|-------|-------|-------|-------|--------|
| No. Samples | 17 | 26 | 19 | 16 | 15 | 11 | 104 |
| Positive Samples (%) | 4(23) | 11(42) | 7(37) | 3(19) | 2(13) | 1(9) | 28(27) |
| Man Age/Samples | 21-25 | 26-30 | 31-35 | 36-40 | 41-45 | 46-50 | Total |
| No. Samples | 8 | 14 | 10 | 9 | 7 | 4 | 52 |
| Positive Samples (%) | 2(25) | 3(21) | 3(30) | 2(22) | 1(14) | 0(0) | 11(21) |

specimens. In semen specimens, PCR was positive in only 11 specimens. The patients characteristics here have been summarized in table 2 (Table 2). Therefore age and sexual indicators shown in table 3 (Table 3). The studied group age was 18-50 (The mean age was ± 34 years old). No significant was observed between infected and uninfected individuals (P value < 0.05).

DISCUSSION

The class *Mollicutes* was established in the 1960s to include the Mycoplasmas and related organisms and it now contains four orders, five families, eight genera, and more than 200 known species that have been detected in humans, vertebrate animals, arthropods, and plants¹¹. Most Mycoplasmas are non-motile, with exception of a few flask-shaped human and animal pathogens. Also is one of the major trait that puts them in the separate taxonomic group of microorganisms, *Mollicutes* class (Latin *mollis*, soft; *cutis*, skin)²⁰. The isolation rates of these microorganisms in the world are diverse and controversial, because these bacteria are generally isolated together with other pathogens, so it is too difficult to determine that if they are responsible for any pathogenicity²². Multiplication of most species on solid media results in the formation of small colonies that have characteristic “fried egg” appearance⁴. *Mycoplasma genitalium*, *M. hominis*, and *U. urealyticum* are part of the microbial flora of the genitourinary tract in asymptomatic sexually active women and agents of sexual transmitted diseases²³. Mycoplasmas and Ureaplasmas species have been associated with genitourinary tract infections including infertility, a complex medical problem whose causes may be diverse. These species are susceptible to protein synthesis-inhibiting tetracyclines, fluoroquinolones and macrolides²⁴. Rapid laboratory detection of genital *Mycoplasmosis* is very important²⁵. Epidemiologic data indicated that the presence of *Mycoplasma* in the genital tract has been associated with incidence of Genitourinary infections, including urithritis, vaginitis, cervicitis, PID, neonatal morbidity and mortality, and reproductive failure¹⁰. Genital *Mycoplasma* infections are commonly diagnosed by culture, as an gold standard, which is time-consuming, costly, and requires expertise²⁶.

In recent years, detection of several Mycoplasmal species in the urogenital tract such as *M. fermentans*, *M. penetrans*, and *M. genitalium* and improved molecular-based detection methods has mandated a reassessment of the possibilities that Mycoplasmas and Ureaplasmas may be of clinical significance in a variety of urogenital infections affecting pregnant women and neonates, which are the focus of this review²⁷. Recently polymerase chain reaction(PCR) has been reported to offer a better diagnostic performance than culture²⁸. PCR was shown to be more sensitive compared to microbiological culture in other study, improving the diagnosis in an average of 5%³. The prevalence of *M. hominis* and *U. urealyticum* were shown to be in an equal range as reported by Günyeli I. et al. in Turkey, Michou IV et al. in Netherlands, and Miron et al. in Romania. There were no prominent differences in rates of infection beside inconsistency in study population being socially different and geographically in reports²⁸⁻³⁰. The high sensitivity of PCR allows the use of various genital specimen types, including endocervical swabs, vaginal swabs, and semen samples for simultaneous detection of genital mycoplasmas³¹. More than 25 years after its initial isolation from men with non-gonococcal urethritis, *M. genitalium* is now recognized as an independent etiologic agent of acute and persistent male urogenital disease was a significant advancement in our knowledge of Sexual transmitted infections³². The high sensitivity of PCR allows the use of various genital specimen types, including endocervical swabs, self-collected vaginal swabs and urine samples, for simultaneous detection of genital pathogens³¹. Multiplex PCR may be a useful approach to laboratory diagnosis of urethritis in men for its high sensitivity and specificity³³. We developed a multiplex PCR assay for the simultaneous detection of *U. urealyticum*, *M. genitalium*, and *M. hominis* in clinical specimens by use of species specific primer sets. All women and men participating in this study did not take any antimicrobial agent prior to sampling which could affect the mycoplasmas. Prevalences of *M. genitalium*, *M. hominis*, and *U. urealyticum* as determined by PCR were 8.4 %, 3.8 %, and 12.8 %, respectively. This optimized method was enough sensitive to detect the specific gene of each of these three species. In this study infertile women

had no symptoms of acute infection of the genital tract, therefore the low prevalence of infection with *M. genitalium* and *M. hominis* were usual. High number of positive cases of *U. urealyticum* infection may be associated with the sexual activity and age in the women selected group. In overall, this results were in the same range of the other reviewed study. Several women may have genital Mycoplasmas in the cervix of the reproductive tract, despite having no symptoms of an ongoing acute inflammation. We determined optimized multiplex PCR is a low cost, suitable, fast and reliable method for diagnosis of genital infections in women and men caused by Mycoplasmas.

ACKNOWLEDGEMENTS

This study is part of Esmaeil Ghorbanalinezhad thesis with grant number 179226 from Islamic Azad University, Science and Research branch, School of Basic Sciences, Tehran, Iran. Authors are appreciative and thankful to the department of microbiology, Islamic azad university of Tonekabon branch, Iran, for laboratory equipments support and technical assistance.

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