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Survey on the genital Mycoplasmosis by multiplex PCR

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ABSTRACT

Mycoplasmas hominis, *Mycoplasmas genitalium* and *Ureaplasma urealyticum* are associated with infections of the genitourinary tract, reproductive failure, and neonatal morbidity and mortality. A multiplex PCR was developed for simultaneously detection of these *Mycoplasmas* species in a single amplification reaction. The total number of 104 samples was collected from 104 women's genital specimens with urogenital infections for identification of *M.hominis*, *M.genitalium* and *U.realyticum* by multiplex PCR. The High Pure PCR Template Preparation Kit purified nucleic acids from 100 µl of specimen (American, Roche Company). In addition to the kit, boiling method was used to extraction of DNA from samples. UUA2 and UUS2 primers were used for urease gene amplification of *U.urealyticum*, MH1 and MH2 used for 16S rRNA gene amplification of *M.hominis*, and Adhesion protein gene (MgPa) used for 16S rRNA gene amplification of *M.genitalium* in all samples. The number of 28 samples (27%) was positive for *Mycoplasmas*. *M.hominis, M.genitalium,* and *U.urealyticum* were detected in 8.7, 3.9, and 14.5 percent of samples, respectively. The accumulated frequencies for *M.hominis, M.genitalium,* and *U.urealyticum* were 9(8.7%), 4(3.9%), and 15(14.5 %), respectively. The results of this study revealed that Multiplex PCR is a highly sensitive, specific and cost-effective test for screening of genitourinary tract infections.

1. Introduction

Mycoplasmas are the smallest cell free-life microorganisms (Mohseni et al.,2014). They can be isolated as commensals or pathogens from plants, insects, animals and humans. Some are considered as normal flora of the respiratory or genitourinary tract (Christofolini, et al., 2012). Seven species of mycoplasmas can be isolated from genitourinary tract but only *Mycoplasma hominis, Mycoplasma genitalium,* and *Ureaplasma urealyticum* have been implicated in human disease (Rodríguez-Preval et al., 2007). *Mycoplasmas* are associated with infections of the genitourinary tract, reproductive failure, and neonatal morbidity and mortality (McIver et al., 2009). *Mycoplasmas* lack a cell wall, the target of beta-lactam

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antibiotics and vancomycin. Genital mycoplasmas are commonly found in the genitourinary tract of pregnant and non-pregnant women (Bayraktar et al., 2010). They have been associated with various pathological conditions and intrauterine infections, including pyelonephritis, pelvic inflammatory disease, chorioamnionitis, endometritis, and postpartum fever, leading to important complications such as preterm birth, low birth weight, spontaneous abortion, stillbirth, premature birth, infertility, and perinatal mortality (Tita et al., 2010; Waites et al., 2005; Stellrecht et al., 2004). Normal levels of estrogen and progesterone in healthy, non-pregnant women protect them against the infection, but during pregnancy and contraceptive use, the level of hormones will increase and make changes in vagina which could predispose to infection by mycoplasmas (Hel et al., 2010; Kim et al., 2011). *Ureaplasma urealyticum* appears to cause some of nonchlamydial and nongonococcal urithritis cases (Ballini et al., 2011). The general laboratory methods to diagnose *Mycoplasma* are microscopic examination of colonies, serology techniques and molecular biology methods (Zhang et al., 2011). Genital mycoplasma infections are commonly diagnosed by culture (Young et al., 2010). Microscopic examination is the most commonly used method to diagnose this organism on the surface of solid media. However, expertise is required and the material used for culture is expensive. It can take two to five days to culture *U.urealyticum* and *M.hominis*, and up to eight weeks to culture *M.genitalium* (Uphoff and Drexler, 2011). Infectious agents can be detected in less than 8 hours by nucleic acid amplification techniques. PCR methods have been developed to identify each of these bacteria (Hopert and Uphoff, 1993). The aim of this study was to evaluate the molecular genetic-based multiplex PCR for the high throughput screening of clinical samples for simultaneously detection of *U.urealyticum*, *M.hominis*, and *M.genitalium*.

2. Materials and Methods

In this experimental and cross sectional study all specimens were taken from 104 patients aged 18 to 48 years (including 104 cervical and vaginal swabs) consecutively attending for outpatient visits to the gynecological and general outpatient obstetrics clinics and medical laboratories in Western Mazandaran province between January 2013 and January 2014. Patients were first visited by gynecology specialists. Those who had visible genital lesions and cervical bleeding were excluded. Vaginal and cervical specimens were collected with sterile packed cytobrush swabs. Swabs were inoculated in 2 ml of Phosphate Buffer Saline (PBS) solution. Samples were transported to laboratory and immediately frozen at -20°C for PCR assays. Therefore sample size calculated from Prashant Kadam study formula (Kadam and Bhalerao, 2010). MH1 and MH2 primers were used for the 16S rRNA gene amplification of *M.hominis*, the 140-kDa adhesion protein gene (MgPa) primers were used for the 16S rRNA gene amplification of *M.genitalium*, and UUA2 and UUS2 primers were used for the urease gene amplification of *U.urealyticum*. Based on these sequences, the amplification products are 280-bp (Mh), 78-bp (Mg), and 418-bp (Uu) in length (Table 1) (Mondeja, 2013). Nucleic acids were purified by High Pure PCR Template Preparation Kit from 100 µl of specimen (American, Roche Company). In addition to the kit, boiling method was used for extraction of DNA from samples. Multiplex PCR were performed by a BioRad thermal cycler (USA) in 25 µL reaction mixtures containing 12.5 µL master mix (Ampliqon Co, Denmark), double distilled water, and 1µl of each forward and reverse primers (10 pM). Initial denaturation for 5 minutes at 95˚C was followed by 35 cycles, each containing denaturation at 95˚C for 40 seconds, annealing at 58˚C for 40 seconds and extension at 72˚C for 60 seconds, followed by final extension at 72˚C for 10 min. Amplified PCR products (7 µL) were analyzed in 2.5% agarose gel and visualized after staining with ethidium bromide. The multiplex PCR performed on the genomic DNA with Mh, MgPa, and UUA2 and UUS2 primers produced the expected size bands, which were distinguishable on a 2.5% agarose gel.

Multiplex PCR was developed for the simultaneous detection and identification of the *M.hominis*, *M.genitalium*, and *U.urealyticum* in women urogenital secretion. Vaginal discharges, vaginitis, cervicitis, urethritis, 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.

Statistical analysis: Statistical analysis was performed using Statistical Package for Science (SPSS 10.0 for Windows).

3. Results

Primer pairs MH1 and MH2 successfully amplified a 280-bp DNA fragment from the 16S rRNA gene of *M. hominis*. 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 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 (Figure 1). Non-specific bands were not detected. All Positive samples were approved by twice sequencing (multiple alignment of nucleotide sequences-Next-generation sequencing, Qiagen).

The multiplex PCR assay could amplify and differentiate between *M.hominis*, *M.genitalium*, and *U.urealyticum*. Genital specimens were obtained for detection of mycoplasma from 104 patients seen at fertility clinics. From total, the 28 (27%) PCR-positive specimens, 9 (8.7%) were positive for *M.hominis*, 4 (3.9%) were positive for *M.genitalium*, 15 (14.5%) were positive for *U.urealyticum*, 5 were positive for both *U.urealyticum* and *M.hominis*, 3 were positive for both *U.urealyticum* and *M.genitalium*, 1 was positive for both *M.hominis* and *M.genitalium***,** and 1 was positive for all. The patient's characteristics have been summarized in table 2. Therefore, the number of positive samples according to the age of woman was shown in table 3.

The studied group age range was 18-48. No significant difference was observed between infected and uninfected individuals ($P > 0.05$

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4. Discussion

M.hominis, *M.genitalium*, and *U.urealyticum* are part of the microbial flora of the genitourinary tract in asymptomatic sexually active women and agents of sexual transmitted diseases (Mohseni et al., 2014; Waites et al., 2011). The isolation rates of these microorganisms in the world are diverse and controversial. As, these bacteria are generally isolated together with other pathogens, so it is too difficult to determine that if they are responsible for any pathogenicity (Sweet, 2011).

All *Mycoplasmas* are phenotypically distinguished from other bacteria by their small size (0.3-0.8 micron in diameter) and lack of cell wall. Rapid laboratory detection of genital mycoplasmosis is very important (Stellrecht et al., 2004). Epidemiologic data indicated that the presence of *Mycoplasma* in the genital tract is associated with incidence of urethritis, vaginitis, cervicitis, pelvic inflammatory disease *(*PID*)*, neonatal morbidity and mortality, and reproductive failure (Kim et al., 2011).

Also is one of the major trait that puts them in the separate taxonomic group of microorganisms, *Mollicutes* class (Latin *mollis*, soft; *cutis*, skin) (Ghosh et al., 2011). The cell membrane is rich in protein (up to two thirds of the membrane mass) that to a great extent consists of highly structured adaptive lipoproteins employed in evading the host immune system, attach to the host cells and invasion process. Most mycoplasmas are non-motile, with exception of a few flask-shaped human and animal pathogens (Mohseni et al., 2014). Genitourinary infections, including sexually transmitted diseases (STDs), are caused by a large number of diverse microbial agents that cause considerable morbidity and mortality worldwide (Schlicht et al., 2004).

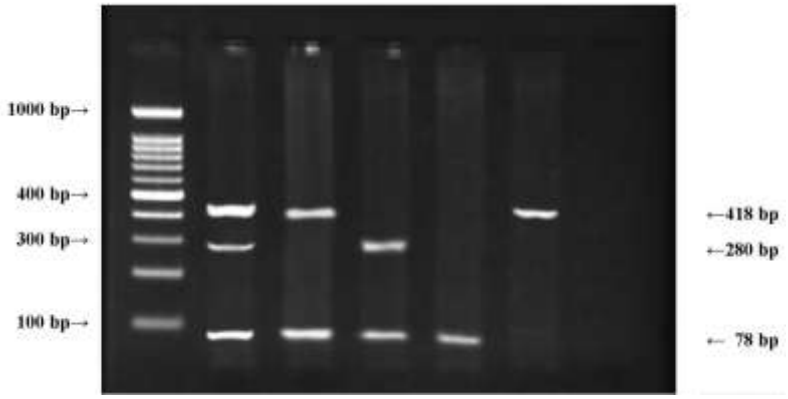

Figure 1. Ethidium bromide-stained agarose gel electrophoresis of PCR amplified products generated from patients DNA samples. Lane M is the DNA size Marker (100 bp DNA ladder; CinaClone Co.); lane 2 show the result of multiplex PCR 78-bp *Mycoplasma genitalium* and 418-bp *Ureaplasma- urealyticum* positive samples simultaneously; Lane 3 shows78-bp *M. genitalium* and 280-bp *M. hominis* positive samples; lane 4 shows 78-bp *M. genitalium* positive sample; lane 5 shows 418-bp *U. urealyticum* positive samples; lane 1 shows positive control; and lane 6 shows negative control.

Table 1. Nucleotide sequences of primers used in this study (3)

Analysis, organism, and primer	**Target or DNA sequence (5´–3´)**	**Length (bp)**
Multiplex PCR		
M. hominis	16S rRNA	280
MH1 F	5'-TGA AAG GCG CTG TAA GGC GC-3'	
MH2 R	5'-GTC TGC AAT CAT TTC CTA TTC CAA A-3'	
M. genitalium	140-kDa Adhesion protein gene	78
MgPaF	5'-GAG AAA TAC CTT GAT GGT CAG CAA-3'	
MgPaR	5'-GTT AAT ATC ATA TAA AGC TCT ACC GTT GTT ATC-3'	
U. urealyticum	Urease gene	418
UUS2	5'-CAG GAT CAT CAA ATC AAT TCA C-3'	
UUA2	5'-CAT AAT GTT CCC CTT CGT CTA-3'	

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

Specimen type	Number of specimen	*M. hominis*	*M. genitalium*	*U. urealyticum*	
	Tested	PCR positive (%)			
Vaginal swab	71	21(30)	7(10)	3(4)	11(16)
Cervical swab	33	7(21)	2(6)	1(3)	4(12)
Total	104	28(27)	9(8.7)	4(3.9)	15(14.5)

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

Woman Age / sampls	**18-23**	**23-28**	**28-33**	**33-38**	**38-43**	**43-48**	**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)

Genital *mycoplasma* infections are commonly diagnosed by culture, which is time-consuming, costly, and requires expertise. Recently polymerase chain reaction (PCR) has been reported to offer a better diagnostic performance than culture (Gdoura et al., 2007).

We developed a multiplex PCR assay for the simultaneous detection of *U.urealyticum*, *M.genitalium*, and *M.hominis* in clinical specimens. All women participating in this study did not take any antimicrobial agent prior to sampling which could affect the mycoplasmas. Prevalences of *M.hominis, M.genitalium, and U.urealyticum* as determined by PCR were 8.7%, 3.9%, and 14.5%, respectively. This optimized method was enough sensitive to detect the specific gene of each of the three species.

The prevalence of *M.hominis* and *U.urealyticum* were shown to be in an equal range as reported. There were no prominent differences in the rates of infection beside inconsistency in study population being socially different and geographically in reports. 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 (Mousavi et al., 2014; Günyeliin et al., 2011, Miron et al., 2013, and Michou et al., 2006).

High number of positive cases of *U.urealyticum* infection may be associated to the sexual activity and age in the selected women group. Low percentage of *M.hominis* infections in results may be due to the lack of any patients suffering from bacterial vaginitis.

The frequency of *M.genitalium* in the infertile women was similar to those reported in poland, and in Iran, but lower than study by Grześko (Tomusiak et al., 2013; Mousavi et al., 2014; Grześko et al., 2009). Though, more studies with large study group of infected human are required to evaluate the rate of *M.genitalium* infection.

In conclusion a high prevalence of *U.urealyticum* and low percentage rate of *M.genitalium* and *M.hominis* infections were reported. These data could be a reflection of the social conditions such as Muslim women with limited partners and regional, which decrease bacterial infection in Iranian population. In overall, these 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 using three pairs of primers, is a low cost and suitable method for diagnosis of genital infections in women and *Mycoplasma* infection in patients.

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Refereces

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