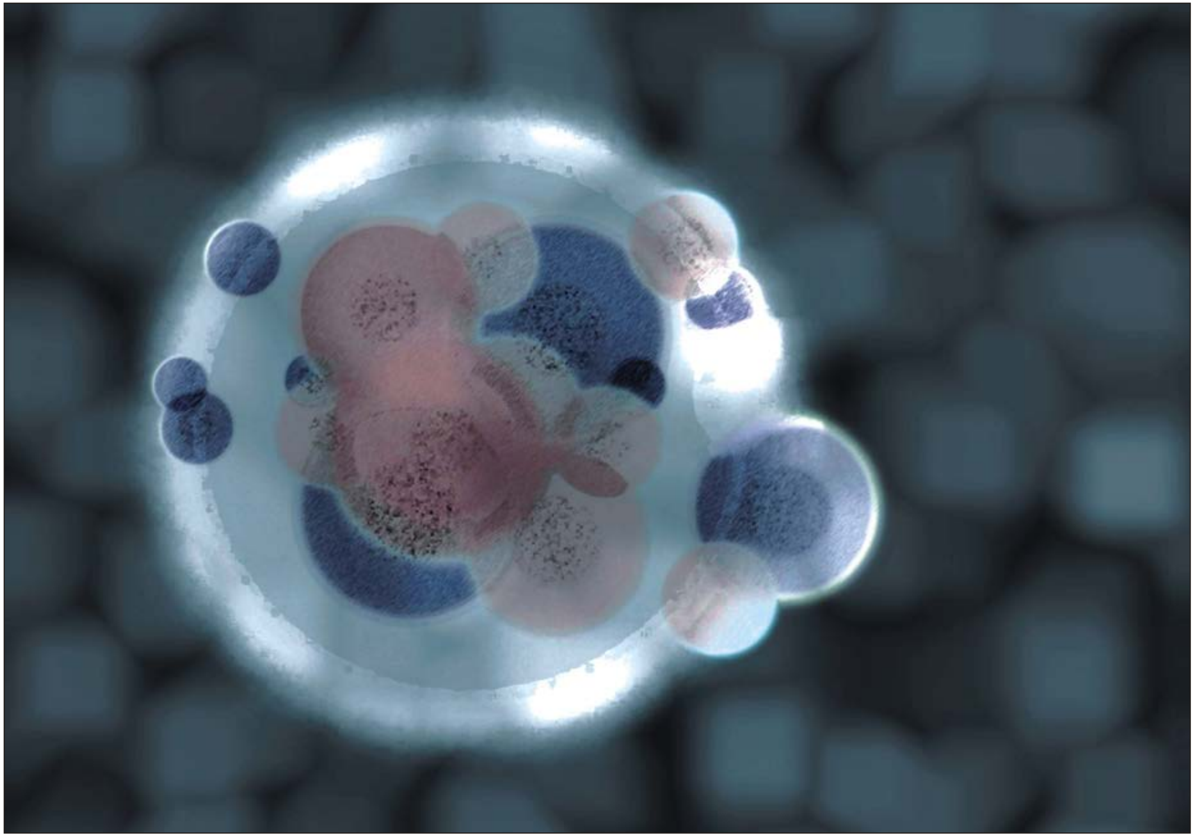

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Simultaneous Detection of Genital Mycoplasma in Women with Genital Infections by PCR

[1]N. Amirmozafari, [1,2]R. Mirnejad, [3]B. Kazemi, [4]E. Sariri, [1]M.R. Bojari and [5]F. Dehdar Darkahi
[1]Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[2]Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[3]Cell and Molecular Biology Research Center, Shahid Beheshti University of Medical Science, Tehran, Iran
[4]Gynecology Ward of Rasool Hospital, Iran University of Medical Sciences, Tehran, Iran
[5]Shahid Beheshti University of Medical Science, Tehran, Iran

Abstract: The aim of this investigation was to simultaneously detect *Mycoplasma hominis*, *Mycoplasma genitalium* and *Ureaplasma urealyticum* in female patients suffering from genital complications (vaginitis, cervicitis and PID) by PCR. Genital swabs were collected from 210 female patients and subsequently suspended in PBS. Following DNA extraction, PCR assay were performed, using a genus specific primer pair. These primer set, which were originally designed in our laboratory, amplified a 465 bp fragment (*M. genitalium*), 559 bp fragments (*U. urealyticum*) and 630 bp fragment (*M. hominis*). Samples containing bands of the expected size for mycoplasma strains were subjected to digestion with restriction endonuclease enzyme. Of the 210 genital swabs tested, 120 sample (57.1%) showed positive reactions in PCR. Sixty eight samples were positive for *Ureaplasma* sp. (32.3%), 28 for *Mycoplasma* sp. (13.3%) and 25 samples had mixed infections (11.9%). In case where specific primers were utilized, PCR has proved to be a simple, fast and relatively inexpensive method for simultaneous detection of all three clinically important genital mycoplasmas.

Key words: Genital mycoplasmas, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma urealyticum*, PCR

INTRODUCTION

Genital mycoplasmas are very prevalent causes of various female genital infections such as vaginitis, cervicitis, Pelvic Inflammatory Disease (PID), pyelonephritis and post partum septicemia (Taylor-Robinson, 1996; Taylor-Robinson and Furr, 1998; Schlicht *et al*., 2004). Through their cell surface appendages, these organisms attach to genital tract epithelium and due to the high degree of colonization in endocervical tissues, they induce various complications in pregnant women and in the newborns as well (Taylor-Robinson, 2007; Waites *et al*., 2005; Razin *et al*., 1998). Colonization varies in relation with several parameters including age, race, hormonal status and the lifetime number of sexual partners and is greater among women, especially during pregnancy. *Ureaplasma urealyticum* has been detected in the genital tract of 30-70% of women and *M. hominis* in less than 20% of women. Several studies have implicated *M. ginitalium* could be found in genital tract of 3.5 to 10% of women visiting STD clinics (Keane *et al*., 2000; Taylor-Robinson and Furr, 1998; Razin *et al*., 1998).

Mycoplasma hominis is associated with cervicitis, PID, pyelonephritis, post partum septicemia and vaginitis (Arya *et al*., 2001; Zdrodowska-Stefanow *et al*., 2006; Abele-Horn *et al*., 1996). *Mycoplasma genitalium* is the causative agent of PID, endometritis, urithritis, arthritis, cervicitis (Lawton *et al*., 2008; Uuskula and Kohl, 2002; Taylor-Robinson, 2002). *Ureaplasma urealyticum* is also a known infectious agent responsible for non-gonococcal, non-chlamydial urethritis, acute prostatitis and acquired arthritis in men (Daxboeck *et al*., 2005; Yoon *et al*., 2000). In women, this organism causes chorioamnionitis, abortion, low birth weight child delivery, vaginitis and cervicitis (Hillier *et al*., 1995). Clinical studies indicated that the respiratory tracts of infants born to mothers who were infected with these bacteria get inflamed, displaying sings of pneumonia, respiratory distress and meningitis (Abele-Horn *et al*., 1996; Keane *et al*., 2000). In order to elucidate the potential roles of genital mycoplasma in various female, male and newborn infections, it is important to utilize a fast and efficient bacterial detection system. In the past, bacterial culture was considered to be the gold standard method for mycoplasma detection. Culture is however

Corresponding Author: Nour Amirmozafari, Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Corner of Chamran and Hemmat Expway, Iran
Tel/Fax: +98 (21) 88058649

very tedious and time consuming taking 2 to 5 days for *U. urealyticum* and *M. hominis* and over 8 weeks for *M. genitalium*. Various DNA amplification techniques have proven to be suitable alternative to culture, for detection of genital mycoplasmas in clinical samples (Sung *et al*., 2006; Jensen *et al*., 1991; Waites *et al*., 2001).

Multiplex-PCR is a technique that is able to detect two or more DNA targets simultaneously in a single amplification reaction. It is used for rapid detection of different pathogens simultaneously, as well as for identification of different genetic disorders (Edwards and Gibbs, 1994; Obata-Yasuoka *et al*., 2002). In this study, a PCR assay was developed in order to identify and differentiate three of the most prevalent genital mycoplasmas; *Mycoplasma hominis*, *Mycoplasma genitalium* and *Ureaplasma* urealyticum in women suffering from various genital disorders in a single amplification reaction.

MATERIALS AND METHODS

In this investigation, 210 female patients who referred to the gynecology ward of a general hospital in Tehran, Iran (Hazrat Rasool Hospital), during November 2007 till August 2008 were selected. After registration and receiving oral consents, the patients were initially examined by a gynecology specialist. Following performance of various laboratory tests which excluded the involvement of numerous other bacterial and non-bacterial etiological agents of female genital tract infections, endocervical swab samples were collected from each patient. The swabs were suspended in PBS and immediately were transferred to laboratory for DNA extraction (Lee *et al*., 2007; Stellrecht *et al*., 2004).

Sample preparation and PCR: Extraction of DNA was performed using a commercial kit (high pure PCR template preparation, Roche Co., USA).

Oligonucleotide primers, which were originally designed in our laboratory, were chosen from the published nucleotide sequences of the conserved intergenic spacer region in 16S-23S rRNA of mycoplasmas and modified in order to be able to detect all three genital mycoplasmas simultaneously (Table 1).

Table 1: Sequences of oligonucleotide primers specific for 16S-23S rRNA gene of mycoplasmas

Primers	Oligonucleotide sequences
MyUu F	5`-TGG AGT TAA GTC GTA ACA AG-3`
MyUuR	5`-CTG AGA TGT TTC ACT TCA CC-3`
RNH F	5`CAATGGCTAATGCCGGATACGC-3`
RNH R	5`GGTACCGTCAGTCTGCAAT-3`

For performing PCR, a ready made Master Mix solution (Ampliqon Co., Denmark) was used. The PCR reaction mixture (50 μL total volume) included: 15 μL Master mix solution 1X that contained 1X PCR buffer, 1.5 mM $MgCl_2$, 1 μL template DNA (0.5 μg), 0.15 mM dNTP, 1.25 U Taq DNA polymerase, 20 pmol of each forward and reverse primers and sterile distilled water up to 50 μL. PCR were performed in a GenAmp PCR system (Corbeit, Germany) according to the following program: predenaturation for 5 min at 95°C followed by 30 cycles each containing denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and Extension at 72°C for 60 sec, followed by final extension at 72°C for 5 min.

Analysis of PCR product: The PCR products were electrophoresed on 3% agarose gel and visualized followed by SYBER green staining (Fig. 1). For final confirmation, the amplified bands were subjected to DNA sequencing as well as restriction endonuclease digestion of *M. genitalium* and *U. urealyticum* by the Cac8I and TaqI enzymes, respectively.

Due to the fact that the primer pair (MyUu F 5`-TGG AGT TAA GTC GTA ACA AG-3` and MyUu R 5`-CTG AGA TGT TTC ACT TCA CC-3`) did not anneal with *M. hominis* genomic sequence in the GenBank; whereas, a 630 bp amplified PCR product band was detected which was not digestible with the restriction endonucleases (TaqI and Cac8I) that were used for digestion of the PCR products of *M. genitalium* and *U. urealyticum*, therefore The PCR products were PCR again by the primer pair (RNHF-5`CAATGGCTAATGCCGGATACGC-3` andRNH R-5` GGTACCGTCAGTC TGCAAT-3`) (Stellrecht *et al*., 2004) and sequenced that specifically amplified *M. hominis* genome and the amplified band indeed belonged to this bacterium.

Statistical analysis: Data were analyzed by means of statistical software (SPSS 12.1). The results were analysed as positive or negative PCR amplification reaction for each bacterium separately, as well as for two or three bacteria simultaneously. Descriptive analyses were performed and results are presented as number (%).

RESULTS

The 210 patients tested had a mean age of 31.9 years (SD = 9.7). They were 94.3% married, 76.7% had never experienced pregnancy and only 2.9% of them have had child delivery by caesarean section. In these subjects, vaginal discharges, Urticarial and Vulva or vaginal irritation were common clinical symptoms and vaginitis

Table 2: Clinical symptoms and complication in women with genital mycoplasma infections

Symptoms	No.	%
Vulva or vaginal irritation	22	10.5
Urticarial	24	11.4
Vaginal discharge	171	81.4
Dysuria	7	3.3
Lower abdominal pain	11	5.2
Vaginitis	132	62.9
Cervicitis	55	26.9
Endometritis	2	1.0
PID	2	1.0

Table 3: Result of PCR in vaginal specimens of women with genital mycoplasma infections

Organisms	No.	%
M. genitalium	11	5.2
U. urealyticum	93	44.3
M. hominis	42	20.0

Table 4: Co-infection of *U. urealyticum* with *M. genitalium* and *M. hominis*

Organisms	No.	%
M. genitalium+U. urealyticum	3	1.4
M. genitalium+M. hominis	0	0.0
U. urealyticum+M. hominis	21	10.0
U. urealyticum+M. hominis+M. genitalium	1	0.5

and cervicitis were common complications (Table 2). None of these patients had TORCH syndrome, or were infected with Trichomonas, Candida or Gram negative cocci.

The PCR results indicated that 57.1% (120 cases) of the patients were infected with mycoplasma. The isolation rate for *M. hominis* was 20% (42 cases), *M. genitalium* 5.2% (11 cases) and *U. urealyticum* 44.3% (93 cases) (Table 3). Additionally, 25 specimens were PCR positive for all three organisms, in which only 21 samples were found to by PCR-positive indicating co-infection with both of bacteria *M. hominis* and *U. urealyticum*. None of the patients were co-infected with *M. genitalium* and *M. hominis*; however, one patient was simultaneously infected with all three bacteria (Table 4).

For confirmation of the PCR results, the amplified DNA products (Fig. 1) were subjected to DNA sequencing as well as restriction endonuclease digestion (accession number for *U. urealyticum*: GQ375145 and accession number for *M. genitalium* GQ367563). The amplified bands pertaining to *M. genitalium* and *U. urealyticum* were cut with CaC8I (73 and 393 bp fragments) and TaqI (227 and 332 bp fragments), respectively (Fig. 2 , 3). In the case of *M. hominis*, the PCR products were PCR again by the primer pair (RNH F-5` and RNH R-5`) (Stellrecht *et al*., 2004) and sequenced that specifically amplified *M. hominis* genome and the amplified band indeed belonged to this bacterium (accession number for *M. hominis* GQ411532).

The DNA sequencing results were compared with the known DNA sequences of the 16S-23S rRNA gene of each mycoplasma complied in the Genbank. When the

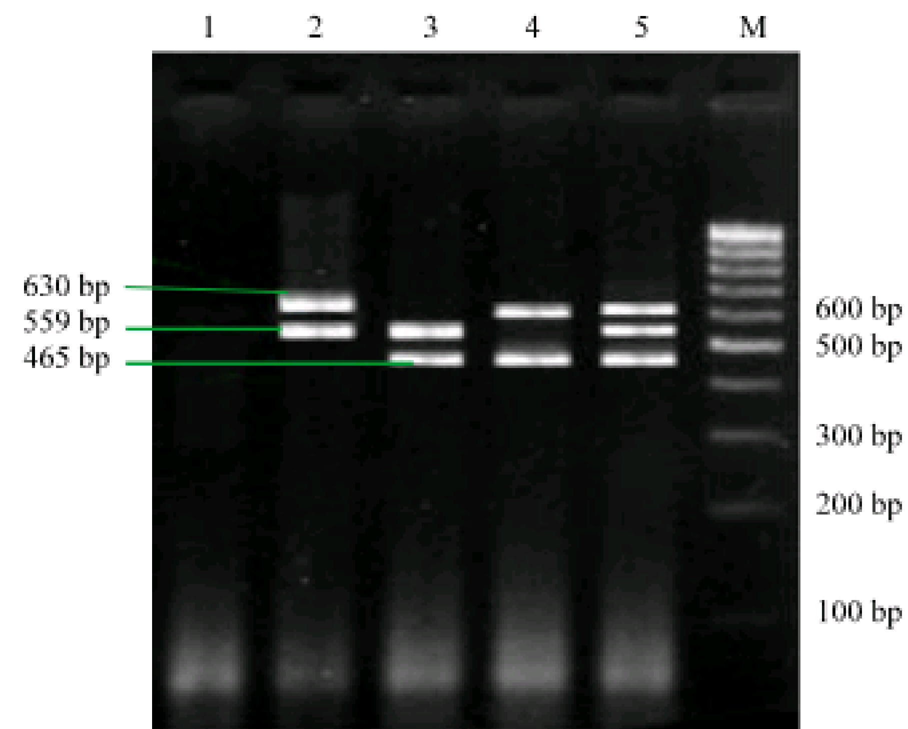

Fig. 1: Agarose gel electrophoresis of PCR amplified products generated from patient DNA samples. Lanes 1 is negative control showing no mycoplasma infection. Lane 2 shows 559 bp *U. urealyticum* and 630 bp *M. hominis* amplification product. Lane 3 shows 465 bp *M. genitalium* and *U. urealyticum*. Lane 4 shows *M. genitalium* and *M. hominis*. Lane 5 shows *M. genitalium*, *U. urealyticum* and *M. hominis*. Lane 6 is DNA size marker (100 bp DNA ladder, SM#333)

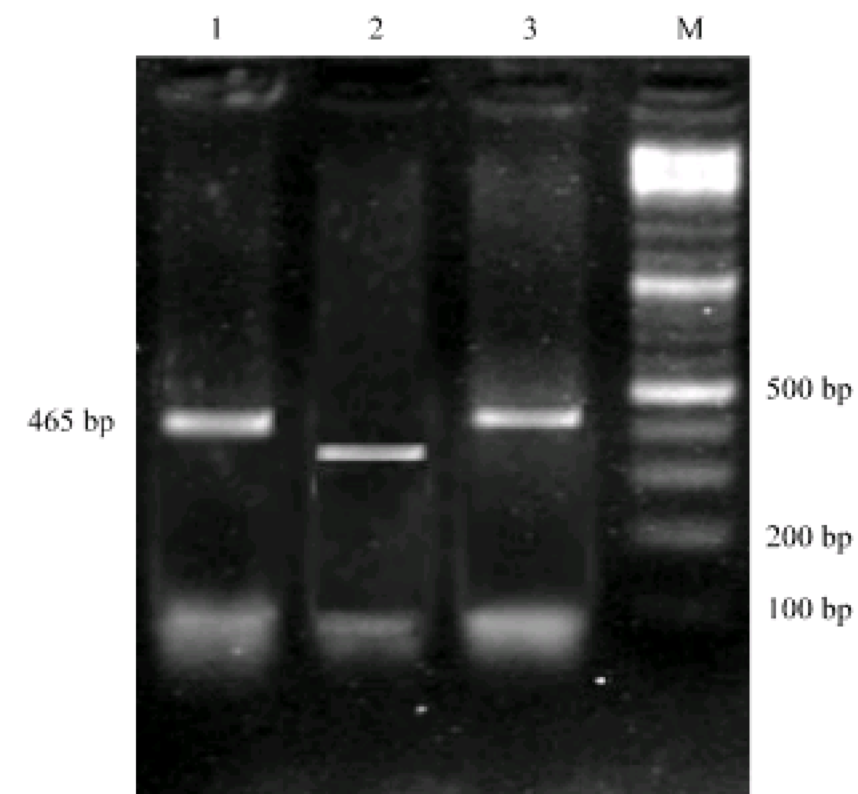

Fig. 2: Restriction Fragment Length Polymorphism (RFLP) patterns of PCR products from *Mycoplasma genitalium* after digestion with CacI restriction enzymes; Lane 1: 465 bp-long PCR products *Mycoplasma genitalium*, respectively; Lane 2: PCR products of *Mycoplasma genitalium* after digestion with CaCI, respectively; Lane 3: PCR products of *Mycoplasma genitalium* after was digested with TaqI (it is not digested); Lane 4 is DNA size marker (100 bp DNA ladder, SM#333)

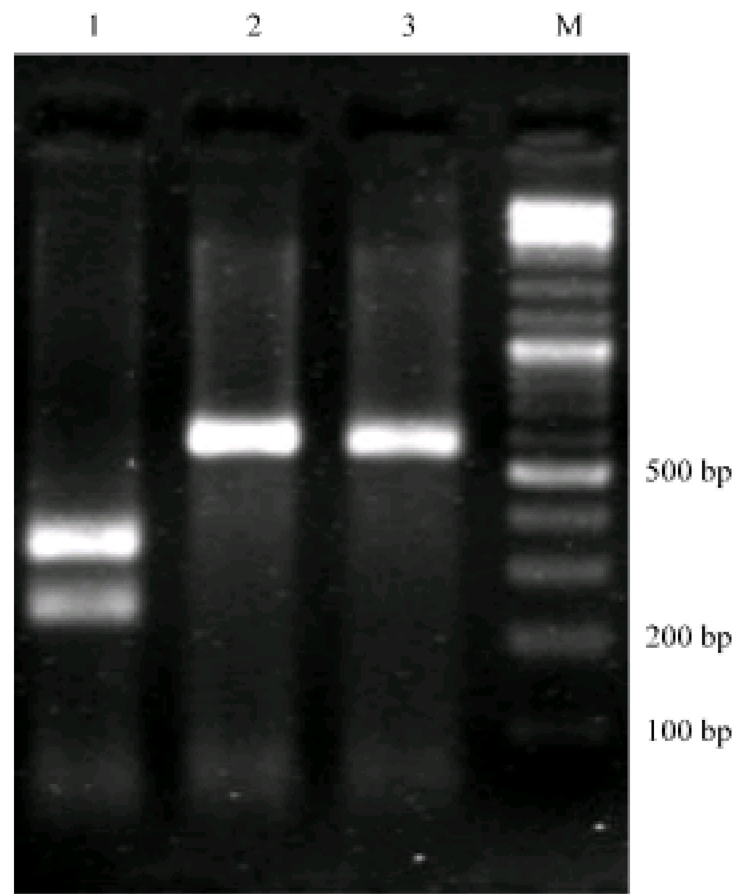

Fig. 3: Restriction Fragment Length Polymorphism (RFLP) patterns of PCR products from *Ureaplasma urealyticum* after digestion with TaqI restriction enzymes; Lane 1: PCR products of *Ureaplasma urealyticum* after digestion with TaqI, respectively; Lane 2: 559 bp-long PCR products *Ureaplasma urealyticum*, respectively; Lane 3: PCR products of *Ureaplasma urealyticum* after was digested with CaCI (it is not digested); Lane 4 is DNA size marker (100 bp DNA ladder, SM#333)

sequences were analysed by the Blast software, it clearly indicated that each of the amplified products did indeed belong to the corresponding mycoplasma.

DISCUSSION

Detection of *Mycoplasma* sp. is clinically relevant in light of the increased capability of the bacteria to adhere and colonize the endocervical linings and thereby induce clinical symptoms in women, in fetus, as well as in newborns (Taylor-Robinson, 2007; Waites *et al*., 2005; Razin *et al*., 1998). Despite the fact that their exact role in pathogenesis in poorly understood and in somewhat controversial, epidemiologic data clearly show a definite correlation between their presence in women genital tract and various diseases such cervicitis, vaginitis, pyelonephritis, urethritis and PID (Razin *et al*., 1998; Taylor-Robinson, 1996; Taylor-Robinson and Furr, 1998). Therefore, prompt isolation of these bacteria is clinically important (Luki *et al*., 1998). In this investigation, a PCR assay was developed which can promptly detect all three pathogenic species of genital mycoplasma in a single amplification reaction by only primer set.

Multiplex-PCR in often performed by using primer pairs in order to detect many different organisms, usually one primer pair for each agent (Lee *et al*., 2007). It is often necessary to optimize the reaction conditions for each primer set. The concentrations of Mg^{2+}, dNTPs, DNA polymerase and other reagents should be empirically set at the ideal workable levels. Since, each primer set has its own melting temperature and annealing time, it is often very difficult to arrive at a consensus annealing temperature and time so that all primer pairs could attach to their target sequences. That in why the set up of a PCR assay is often very tedious and requires great deal of scientific expertise (Edwards and Gibbs, 1994; Obata-Yasuoka *et al*., 2002). PCR assays that were developed for detection of genital mycoplasma by Stellrecht *et al*. (2004) and other investigators employ different primer sets and are therefore not very practical to be used in routine clinical laboratory setting (Stellrecht *et al*., 2004; Choppa *et al*., 1998). Whereas the PCR assay developed in this study uses only primer set, which works just like a regular PCR reaction, in order to detect all three important genital mycoplasma species in clinical samples.

In this study, the rate of mycoplasma isolation with the PCR method was 57.1%. Additionally, cases of mixed infections which were detectable with the PCR were considerable (11.9%). The study indicates that a high percentage point of the patients were indeed infected with *U. urealyticum* (44.3%) and to a lesser degree with *M. genitalium* (5.2%). The *M. hominis* infection rate was 20%. The results of this study is very similar to those of Luki *et al*. (1998), who used conventional PCR to detect mycoplasmosis in pregnant women and found 56.3% *U. urealyticum*, 12.7% *M. hominis* and 3.6% *M. genitalium*. The minute difference in the results of these two studies may be due to the number of subjects, type of subjects (pregnant vs. non-pregnant genital symptomatic patients) and the target specificities of the primers used for the amplification reactions.

The results of the present study, just like those of Stellrecht *et al*. (2004) proved that PCR is a suitable method for genital mycoplasma detection. The rate of mycoplasm and Ureaplasma detection in this study was 20 and 44.3%, respectively. Even though, in the Stellrecht *et al*. (2004) investigation, it was reported to be 3.53 and 27%, respectively. Stellrecht *et al*. (2004) did not detect any *M. genitalium* at all and co-infection with both mycoplasma and ureaplasma was reported to be 2.35%. The difference for the results of these two studies may be due to the nature of specimens collection as well as the methodologies of the PCRs. The PCR used for present study employed a single primer pair specific for all three

genital mycoplasmas; whereas, they utilized multiple primer sets in their multiplex assay. Additionally, unlike the study of Stellrecht *et al*. (2004) the specimens used for this study were only collected from the vaginal and endocervical epithelia. Despite the fact that more studies are needed in order to elucidate the efficacy and effectiveness of using a single primer set in PCR assay for detection of multiple targets, the results of this survey clearly indicates that this assay system is a simple, fast and highly specific for detection of genital mycoplasmas. Since rapid detection of mycoplasmosis is low-birth-weight infants born to infected mothers is crucial to prevent serious complication such as meningitis, respiratory distress (Keane *et al*., 2000), the PCR developed in this survey can be a suitable alternative to other mycoplasma identification methods such as bacterial culture or serology.

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