

Comparison of polymerase chain reaction and culture for detection of genital mycoplasma in clinical samples from patients with genital infections

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ABSTRACT

الأهداف: مقارنة (BCR) والمزرعة في اكتشاف الفطريات التناسلية في العينات السريرية من المرضى المصابين بالتهابات تناسلية.

الطريقة: تم أخذ عينات مكررة من 210 مريضة، اللواتي تم تحويلهن من عيادة أمراض النساء لمستشفى الرسول -مدينة طهران- إيران خلال الفترة ما بين ديسمبر 2007م ويونيو 2008م. وتم نقل العينات إلى المختبر بواسطة طريقة نقل مختارة للفطريات. تم تلقيح العينات إلى مرق زرعى وصلب للزراعة. تم تحديد خصائص المستعمرات باستعمال صبغة دينيس وفحصها بالميكروسكوب. كما تم تحليل عينات (BCR) بواسطة أنواع المواد البطانية المحددة. هذا الطقم الذي تم تصميمه في مختبرنا وتكبير (465bp) قطعة (*Mycoplasma genitalium*) ، و (559bp) قطعة (*Ureaplasma urealyticum*) و (630bp) قطعة (*Mycoplasma hominis*). تم تعريض العينات التي تحتوي على رباط من صبغات أحجام الفطريات المتوقعة إلى عقار دايجوستين مع أنزيم إندونوكليس المحدد.

النتائج: من 210 عينة تم عزل صبغات الفطريات من 83 مريضة (39.5%) ، 23 معزولات الفطريات، 11% : و 69 معزولات يوربلازما بنسبة 32.9% باستعمال أسلوب عزل اختياري للفطريات. تبين اكتشاف إجمالي عدد 120 عينة (57.1%) موجبة الفطريات 28 فطريات (SPP) بنسبة 13.3%، و 67 فطريات (*ureaplasma spp*) بنسبة 31.9% وعدوى مشتركة في 25 عينة (11.9%).

خاتمة: تبين أن لدى (BCR) حساسية عالية عند استعمال نوع المواد البطانية التمهيدية المحددة لتشخيص الفطريات التناسلية بالمقارنة مع الزراعة.

Objectives: Comparison of polymerase chain reaction (PCR) and culture for detection of genital mycoplasma (*Mycoplasma hominis*, *Mycoplasma genitalium*, and *Ureaplasma urealyticum*) in clinical samples from patients with genital infections.

Methods: Duplicate genital swabs were taken from 210 patients, referred to the gynecology clinic of Rasool Hospital, Tehran, Iran between December 2007 and June 2008. They were transported to

the laboratory in a selective mycoplasma transport medium and in phosphate buffer solution. The specimens were inoculated into specific broth and solid medium for culture. Characteristic mycoplasma colonies were determined with Diennes' stain and examined microscopically. For PCR, samples were analyzed with genus specific primers. The primer sets, which were originally designed in our laboratory, amplified a 465 bp fragment (*Mycoplasma genitalium*), 559 bp fragment (*Ureaplasma urealyticum*), and 630bp fragment (*Mycoplasma hominis*). Samples containing a band of the expected sizes for mycoplasma strains were subjected to digestion with a restriction endonuclease enzyme.

Results: Of the 210 samples, mycoplasma strains were isolated from 83 patients (39.5%), (23 mycoplasma isolates, 11%; and 69 ureaplasma isolates, 32.9%) by using a selective mycoplasma isolation media. Using PCR, a total of 120 (57.1%) samples were found to be positive for mycoplasmas (28 *mycoplasma spp.*, 13.3%; and 67 *ureaplasma spp.*, 31.9%) and co-infections with both species were detected in 25 samples (11.9%).

Conclusion: The PCR was found to be highly sensitive when genus specific primers were used for diagnosis of genital mycoplasmas in comparison with culture.

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Mycoplasmales are associated with infections of the genitourinary tract, reproductive failure, and neonatal morbidity and mortality.¹⁻³ *Mycoplasma hominis* (*M. hominis*) has been associated with bacterial vaginosis, cervicitis, pelvic inflammatory disease (PID), endometritis, and post partum septicemia.⁴⁻⁶ *Mycoplasma genitalium* (*M. genitalium*) has also been associated with PID, endometritis, arthritis, cervicitis, and is one the main cause of non-chlamydial urethritis.⁷⁻⁹ *Ureaplasma urealyticum* (*U. urealyticum*) is a causative agent of non-gonococcal, non-chlamydial urethritis in men.^{10,11} In pregnancy and non-pregnant women, ureaplasma can cause chorioamnionitis and preterm delivery, abortion, preterm birth, bacterial vaginosis, and cervicitis.^{12,13} Clinical studies have demonstrated that infants born to infected mothers became infected with these bacteria, and colonization of the respiratory tract of infants has been associated with pneumonia, respiratory distress, and meningitis.^{6,14,15} The diagnosis of genital mycoplasmas is commonly made by culture, but culture is costly and requires special media and expertise. It can take 2-5 days to culture *U. urealyticum* and *M. hominis*, and up to 8 weeks for *M. genitalium* to grow into visible colonies. Genital infectious agents can be detected in 8 hours by nucleic acid amplification techniques. At present, polymerase chain reaction (PCR) is revolutionizing the diagnosis of organisms that are difficult to cultivate.^{16,17} In this study, PCR assay was used for detection of all 3 genital mycoplasmas in a single amplification reaction and its detection rate was compared by culture method.

Methods. *Clinical specimens.* Specimens were taken from a total of 210 patients with clinical signs who were referred to the gynecology clinic of Rasool Hospital, Tehran, Iran between December 2007 and June 2008. Patients were first visited by gynecology specialists, and those who had cervical bleeding and any visible genital lesions, or received antibiotics within the previous 2 weeks were excluded. Duplicate endocervical swab samples were collected. One swab was inoculated into transport medium (pleuropneumonia-like organisms [PPLO] broth supplemented with inactivated horse serum [5%] and penicillin G [5000 u/ml], without urea, arginine, and glucose) and immediately was transported to the laboratory.¹⁸ The other swab was placed in phosphate buffer solution (PBS) and immediately frozen at -70°C for later PCR assays.

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Culture. In order to eliminate normal flora fungi and bacterial specimens, the inoculated transport media was filtered through 0.45 µm pore size disposable filters and the filtrates were inoculated into PPLO broth supplemented with horse serum (20%), 10 ml yeast extract (25%), 2 ml phenol red (0.2%), penicillin G (5000 u/ml), 20 ml urea (10%), or 20 ml arginine (10%).^{18,19} The pH was adjusted to 7.5 for PPLO broth containing L-arginine (specific for growth of *M. hominis*), and to 6.0 for PPLO broth containing urea (specific for *U. urealyticum*). All inoculated culture media was incubated in an atmosphere with 5% CO₂ at 37°C for 48-72 hours. The color changes were taken as the criteria for bacterial growth. Following detection of any pH changes, 0.5 ml of the medium sample was transferred to PPLO agar (the specific solid medium for mycoplasmas) containing all PPLO broth components in addition to 1% agar. The agar plates were incubated at the same atmospheric conditions (5% CO₂ at 37°C) for 48-72 hours. Characteristic *M. hominis* and *U. urealyticum* colonies were determined by staining with the Diennes' method and examined by microscope (x10). Media that did not develop typical colonies within 5-10 days was considered as culture negative.^{19,20} In the case of *M. genitalium*, successful cultivation could be achieved by 8 weeks of incubation in CO₂ environment or co-cultivation with Vero cell cultures. This complex methodology is certainly inadequate for routine cultivation of *M. genitalium*, leaving the door open for the application of molecular techniques, such as PCR.¹⁷ For these reasons, *M. genitalium* was not cultured in this investigation.

Sample preparation for PCR and PCR amplification. The swab samples placed and frozen in PBS were thawed, and DNA extractions were performed by high pure PCR template preparation kit (Roche Co, New York, USA). Oligonucleotide primers, which were originally designed in our laboratory, could detect all 3 genital mycoplasmas simultaneously. Because the primer pair (MyUu F 5'-TGGAGTTAAGTCGTAACAAG-3' and R 5'-CTGAGATGTTTCACTTCACC-3') did not anneal with *M. hominis* genomic sequence in the GenBank, and a 630 bp amplified PCR product band was detected that 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 subjected to an additional PCR reaction using the primer pair (RNH F 5'-CAATGGCTAATGCCGGATACGC-3' and RNH R 5'-GGTACCGTCAGTCTGCAAT-3').²¹ The DNA sequencing of the resulting amplicon indicated that the amplified bands indeed belonged to *M. hominis*. Each PCR reaction mixture contained 15 µl master mix 1x (Ampliqon Co, Skovlunde,

Denmark), which is composed of 1x PCR buffer, 1.5 mM magnesium chloride, 1µl template DNA (0.5 µg), 0.15 mM deoxy-nucleotide-tri phosphate, 1.25 U Taq DNA polymerase, 20 pmol of each forward and reverse primers, and sterile distilled water up to 50 µL. The PCR reactions were performed in a GenAmp PCR system (Corbiet, Sydney, Australia) according to the following program: pre denaturation for 5 minutes at 95°C followed by 30 cycles each containing denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 60 seconds, followed by final extension at 72°C for 5 minutes.

Analysis of the PCR product. The PCR products were electrophoresed on 3% agarose gel for one hour at 85 volts and 25mA, stained by SYBER green and visualized under an UV transilluminator. Amplification products were further evaluated by sequencing and restriction digestion procedure.

Statistical analysis was conducted to determine how many samples were positive for each bacterium, as well as those positive for 2 or 3 bacterial species. Perspective analyses were performed, and data rounded numerical values (percentage) were documented.

Results. In this study, 210 samples were collected from patients with an age ranging from 19-65 years, 94.3% of them were married, 67.7% had not experienced any child delivery, and only 2.9% had previous cesarean deliveries. In these patients, vaginal discharges, urticarial, and vulva or vaginal irritations were common clinical symptoms. Vaginitis and cervicitis were common complications of the sampled women. The specimens DNA was extracted and analyzed by PCR (Figure 1). The amplification products were confirmed further by DNA sequencing and restriction fragment length polymorphism (RFLP). The nucleotide sequence accession number for *U. urealyticum* was GQ375145, and for *M. genitalium* was GQ367563). The restriction enzyme selection was based on our PCR product sequencing results. Briefly, PCR products of *M. genitalium* were digested by Cac8I and yielded 72 bp and 393 bp fragments, and PCR products of *U. urealyticum* were digested by TaqI and yielded 227 bp and 332bp fragments. In the case of *M. hominis*, the PCR products were subjected to an additional PCR reaction using the primer pair (RNH F-5' and RNH R-5').²¹ Sequencing of the amplified reaction product indicated that the amplified band indeed belonged to this bacterium. The nucleotide sequence accession number for *M. hominis* was GQ411532). Of the 210 patients surveyed, 83 were culture positive (39.5%) and 120 were PCR positive (57.1%). *Mycoplasma hominis* were cultured from 23 of the samples (11%) by using specific mycoplasma isolation media. *Ureaplasma*

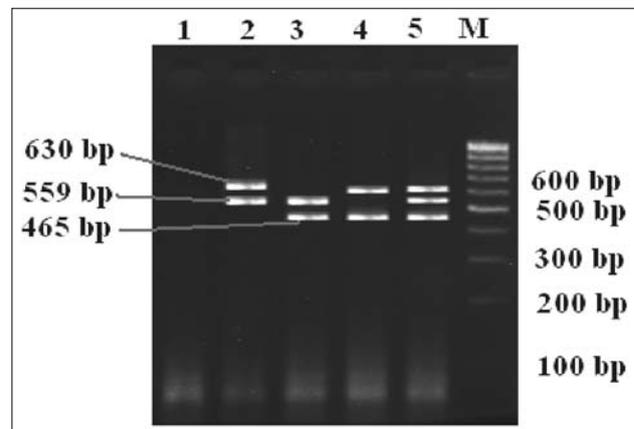


Figure 1 - Agarose gel electrophoresis of PCR amplified products generated from patient DNA samples. Lanes 1 is the negative control showing no mycoplasma infection. Lane 2 shows 559 bp *U. urealyticum* and 630 bp *M. hominis* amplification product. Lane 3 shows 465 bp *M. genitalium* and *U. urealyticum*.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

urealyticum were isolated from 69 (32.9%) of the sample by cultivation. *Mycoplasma hominis*, *M. genitalium*, and *U. urealyticum* were detected by PCR in following order; 20%, 5.2%, and 44.3%.

Discussion. Rapid laboratory detection of genital mycoplasmosis in pregnant women is very important mainly because of the ability of the bacteria to colonize the endocervical linings and cause injury to the fetus.^{13,14,22} Epidemiologic data indicated that their presence in the genital tract has been associated with incidences of urethritis, vaginitis, cervicitis, PID, and pathology of pregnancy and newborns; so their rapid and specific diagnosis are clinically very important.^{1,2,22} In this study, PCR and bacterial culture were used for detection of genital mycoplasma in clinical samples from women with genital infections. Bacterial culture is generally considered as the gold standard detection method of genital mycoplasmosis. However, the results of this study as well as those of other investigations, particularly Teng,¹⁹ and Stellrecht,²¹ have indicated that PCR has much higher sensitivity rate than culture. In the present study, the detection rate for *Mycoplasma spp.* by culture was approximately 11%, and for *Ureaplasma spp.* was roughly 32.9%. Whereas, the detection rate by PCR for the 2 species were 20% and 44.3%. The studies of Serin,²³ and Stellrecht,²¹ also indicated that PCR is a more sensitive method for the diagnosis of genital mycoplasmas. Although somewhat rapid culture of *M. hominis* and *U. urealyticum* is possible; however,

in the case of *M. genitalium*, bacteriological culture usually takes up to 8 weeks and numerous studies indicated that an amplified DNA detection system is simple, rapid, and more sensitive than culture. Our study, in total agreement with the results of Stellrecht et al²¹ indicated that detection of *M. genitalium* by PCR is a highly sensitive method for detection of this agent in clinical specimens.

The lower extent of culture detection in this investigation may in part be attributed to the high sensitivity of mycoplasma to environmental factors (pH, temperature, and materials present in culture media, and clinical specimens) as well as loss of viability during specimen collection and/or transport. Organisms that die before incubation, of course cannot be cultured, whereas the DNA of dead organisms can still be detected by PCR.^{21,22} On the other hand, bacterial culture can take 2-5 days for *M. hominis* and *U. urealyticum*, and up to 8 weeks for *M. genitalium*. Genital mycoplasmas not only require special culture media to grow, but also technical expertise is crucial for successful cultivation; whereas, PCR is rapid (<8 hours) and cost effective for simultaneous detection of all 3 genital mycoplasmas in a single amplification reaction.

In this study, 2.9% of the PCR-negative cases were culture-positive, and thus these could be considered as false-negative cases. The PCR false-negative results may be due to the presence of Taq polymerase reaction inhibitors, traces of blood, and antibiotics in clinical specimens. Sequence variability and genome degradation are an alternative source for potential false-negative PCR results. Analysis of PCR amplification products by RFLP indicated that all bacterial species belonged to one type. Furthermore, no sequence divergence has been detected for all the PCR amplified products. Therefore, targeting the 16S rRNA gene for mycoplasma PCR detection has proven to be a specific as well as a conserved target for laboratory probing of all genital mycoplasmas.

This study is unique in that only one primer pair is used for simultaneous detection of all 3 genital mycoplasmas. Whereas, the PCR assay developed by Stellrecht et al²¹ uses multiple primer pairs, one pair for detection of each organism. Utilizing a PCR assay with only one primer pair reduces the costs as well as the set-up time and the complexity of the reaction mixture. Designing multiple primer pairs and their set-up in a single PCR reaction mixture is rather tedious and cannot be effectively performed in every clinical laboratory.^{24,25}

In summary, the PCR assay developed in this study using only one primer pair has proven to be a simple, rapid, and more sensitive method for detection of genital mycoplasmas (*M. hominis*, *M. genitalium*, and *U. urealyticum*). Rapid detection of genital mycoplasmas is clinically very significant, particularly

in the management of low-birth-weight infants in whom these bacteria are a major cause of meningitis, respiratory disease, and death. We therefore recommend that this PCR assay may be used, instead of bacterial culture or conventional PCR, for rapid diagnosis of genital mycoplasma in clinical samples.

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