Comparison of PCR and Cultivation Methods to Determine the Incidence of Infections due to *Mycoplasma Hominis* and *Mycoplasma Fermentans* in Women Genitourinary Tract

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Objective: **In this study, in order to compare PCR and cultivation methods to determine the incidence of infections due to *M. hominis* and *M. fermentans* in women genitourinary tract 100 genital swabs and 100 urine samples obtained from women with genitourinary tract (GUT) infection were studied.**

Method: **Genital swab and urine samples were inoculated and transported with a selective mycoplasma transport media. After incubation at 37°C for 18-24 hours 0.3 mL medium samples were transferred to the specific solid medium for mycoplasma. The agar plates were incubated at the same atmosphere conditions (5% CO_2 and 95% N_2 at 37°) for 48-72 hours. Characteristic mycoplasma colonies were determined by staining with Dienne's stain and examined by x10 microscope objective. The genital swab and urine samples were also analyzed by a nested PCR protocol with genus specific MCGpF11, R23-TR, R16-2 and MCGR21 primers. Another PCR protocol was also performed in order to confirm the samples which have compatible target sequences for *M. fermentans* by using RW004-RW005 primers. On the other hand, all other mycoplasma positive amplicons were also digested with *VspI* in order to determine two DNA fragments (123bp and 113bp) which were compatible for *M. hominis* in tested samples.**

Results: ***Mycoplasma*** **strains were isolated from 26 (26%) genital swabs and 11 (11%) urine samples by using a selective mycoplasma isolation media. Totally 40 samples were found to be positive for mycoplasmas which consisted of target genomic sequences of *M. hominis* and *M. fermentans* in 37(37%) and 3(3%) samples respectively.**

Conclusions: **We found that there could be an association with *M. hominis* (37%) and women with genital infection, also with *M. fermentans* (3%) and although the high specificity (100%) of cultivation, it has a low sensitivity (70.3%) and time consuming when compared with PCR . On the other hand, we concluded that, PCR is a sensitive and easily applicable protocol when genus specific primers are used for the diagnosis of mycoplasmas.**

Key words: *Mycoplasma hominis*, mycoplasma fermentans, *PCR, DNA, RFLP*

Both *M. hominis* and *M. fermentans* have been detected in the urogenital tract of adults and they revealed to have the potential of being sexually transmitted (1). *M. hominis* can be isolated from the urogenital tracts of up to 40% of asymptomatic males and females. However it is a proven cause of pelvic inflammatory disease, postpartum septicemia and endometritis, clinical amnionitis and pyelonephritis (2). It is also a major cause of pneumonia and central nervous system infection in newborns (3). Furthermore, *M. hominis* is increasingly being recognized as a common cause of septicemia, arthritis, surgical wound infection and peritonitis in immunocompromised patients (4). *M. fermentans* has recently been recognized as possible infectious pathogen in humans. Many patients with AIDS suffer a systemic infection caused by this agent (5). The role of this newly recognized mycoplasmal infection in AIDS, however, is still not understood (6). The agent found in the immunocompromised patients may simply represent another opportunistic infection playing a co-factor role of promoting disease progression in AIDS or actually producing major pathogenesis in the chronic debilitating AIDS (7).

Diagnosis of mycoplasmal infections is usually made by serological determination or in vitro isolation of the organism (8). However, serological procedures are often hampered by interspecies cross-reactions, while cultivation is time-consuming and hard to achieve for some fastidious mycoplasmas. Use of mycoplasma species-specific DNA probes made it possible to discriminate between different species, but although this method proved to be rapid and, specific, the sensitivity was rather low since only 10^4 organisms could be detected (9).

The need for an improved detection method for *M. hominis* and *M. fermentans* is evident. Polymerase chain reaction (PCR) for amplification of specific short segments of nucleic acid sequences is a promising rapid diagnostic test. This study compared the use of PCR with culture for the detection of both mycoplasmas in urogenital samples (urine and genital swabs).

Material and Method

Culture of Mycoplasma from Clinical Samples:

The genitourinary swabs and the urine samples were obtained from 100 females with vaginitis who attended to gynecology clinic of Cukurova University Hospital. The

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Table I. Sequences of oligonucleotides in the gene spacer region in 16S-23S rRNA of mycoplasmas (11).

Primers	Oligonucleotide sequences
MCGpF11	5'-ACA CCA TGG GAG (C/G) TGG TAA T-3'
R23-1R	5'-CTC CTA GTG CCA AG (C/G) CAT (C/T) C-3'
R16-2	5'-GTG (C/G) GG (A/C) TGG ATC ACC TCC T-3'
MCGpR21	5'-GCA TCC ACC A(A/T) A(A/T) AC(C/T) CTT-3'

Table II. Second step PCR products and restriction length polymorphisms after digestion by several restriction endonuclease (11).

Mycoplasma species	2nd round PCR product	*VspI*	*HindIII*	*HincII*	*ClaI*	*PvuII*	*HaeIII*
M .pirum	323bp	169, 154	285, 38	196, 127	-	-	-
M. fermentans	365	270, 95	241, 124	-	-	-	-
M orale	290	151, 139	-	-	-	-	221, 69
M. arginini	236	134, 102	-	-	-	-	-
M. hominis	236	123, 113	-	-	-	-	-
M. genitalium	252	190, 62	-	-	-	-	-
M. hyorhinis	315	-	-	-	253, 62	-	-
M .pneumoniae	280	-	-	-	-	-	-
M. salivarium	269	-	-	-	-	-	-
A .laidlawii	430,223	-	-	-	-	-	-

Table III. Sequences of oligonucleotides in the IS-Like segment of *Mycoplasma fermentans (7)*

Primers	Oligonucleotide sequences
RW004	5'-GGA CTA TTG TCT AAA CAA TTT CCC-3'
RW005	5'-GGT TAT TCG ATT TCT AAA TCG CCT-3'

swabs were transported to the laboratory in mycoplasma transport medium containing inactivated horse serum (20%), 10 mL yeast extract (25%), 144 mL brain-heart infusion broth, 1 mL tallium acetate (1/80), 5 mL phenol red (0.2%), penicillin (1000U/mL) and 5 mL arginin (20%). The urine samples were collected and transported in sterilized tubes. The urine samples were centrifuged at 3000xg for 10 minutes and the pellet was transferred to the transport medium. All inoculated transport media (swabs and urine) were incubated in an atmosphere containing 5% CO_2 and 95% N_2 at 37°C for 18-24 hours. The color changes were taken as the criteria of growth. 0.3 mL of medium sample was transferred from color changed medium to the specific solid medium for mycoplasma containing brain-heart infusion broth, noble agar, inactivated horse serum (20%), 10 mL yeast extract (25%), penicillin (1000U/mL), 1mL phenol red (0.1%), 2 mL thallium acetate (1/80) and 10 mL arginin (20%). The agar plates were incubated at the same atmosphere condition (5% CO_2 and 95% N_2 at 37°C) for 48-72 hours. Characteristic mycoplasma colonies were determined by staining with Dienne's stain and examined by a microscope. These colonies were characterised by their haemolytic and glucose fermentation properties. We used horse blood instead of sheep or guinea pig erytrocytes for haemolytic activity. As known, recently *M. pneumoniae* has been isolated from genitourinary system (10). We cultured reference *M. pneumoniae* ATCC 15377 as control for haemolytic activity. This strain was grown better in horse blood containing mycoplasma medium than sheep erythrocyte containing mycoplasma medium with greenish β-haemolytic zones. None of our clinical isolates fermented glucose and showed haemolytic activity while reference *M. pneumoniae* ATCC 15377 made haemolysis on horse blood containing mycoplasma medium. Therefore, we partly characterised that those are not *M. fermentans* but highly probably *M. hominis.* We confirmed those findings by PCR and PCR-RFLP.

Sample Preparation for PCR

The swab samples were inoculated in the mycoplasma transport medium which is not containing any stain and antibiotics. The urine samples were centrifuged and the pellet was transferred to the same medium. The media were stored at –40°C until examining by PCR. When examined, they were thawed and centrifuged at 2000 G for 10

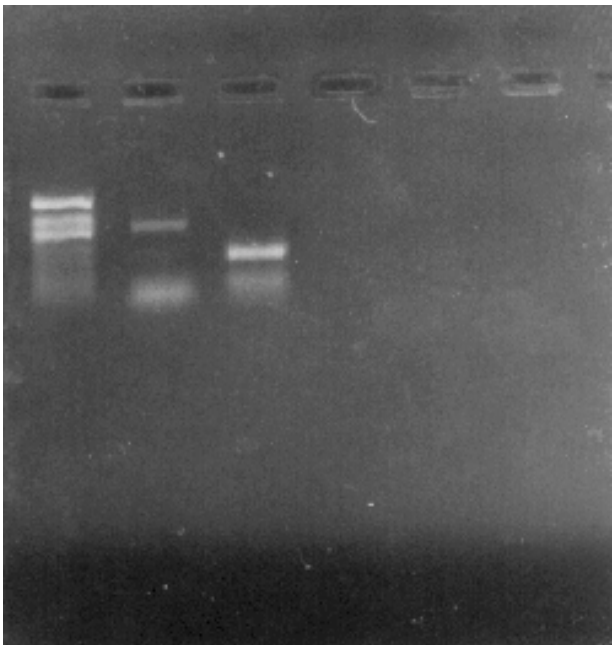

Figure 1. Lane 1 and Lane 2 show 365bp and 236bp PCR products which are genomic sequences of *M.fermentas and M.hominis respectively*.

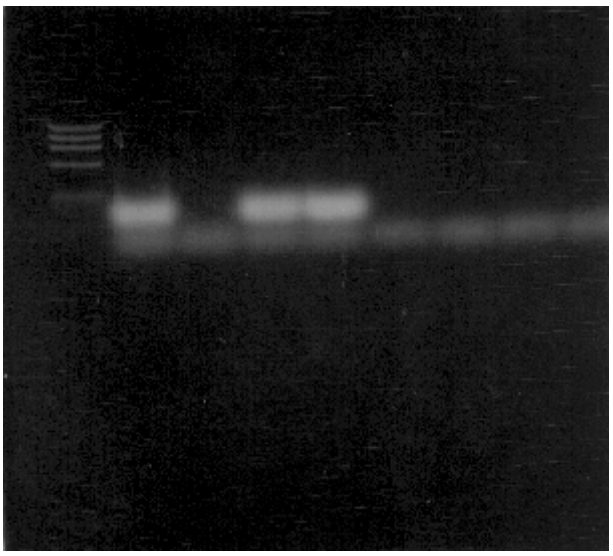

Figure 3. Lane 1, 3 and 4 show 206bp PCR products which are compatible for *M.fermentans*

minutes. The pellet was transferred to a microfuge tube and the same volume of sterilised distilled water was added to the pellet. This mixture was incubated at 37°C for 20 minutes and the final mixture was used as DNA sample for PCR.

PCR Amplification

Oligonucleotide primers were chosen from the published nucleotide sequences of conserved intergenic spacer region in 16S-23S rRNA of mycoplasmas (11). We have used a protocol reported for the diagnosis of mycoplasmas in cell cultures by Harasawa et al (11) and modified for diagnosis of mycoplasmas in clinical samples (11). This is a nested PCR protocol with the oligonucleotides MCGpF11, R23-1R and internal R16-2, MCGpR21 using (Table I). The restriction length

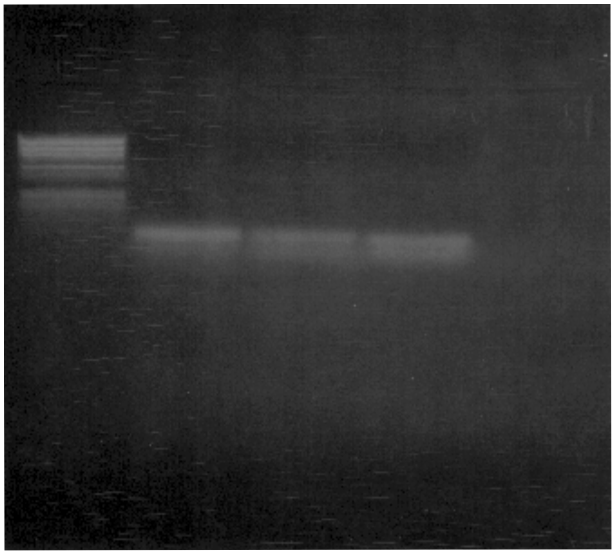

Figure 2. Lanes show resulted restricted products after digest ion by *VspI* which are 123bp and 13bp.

polymorphisms of the end product of second step have a significant diagnostic value (Table II).

The restriction length polymorphisms of the end product of second step have a significant diagnostic value (Table II).

Amplification of samples (10mL) was performed in a final volume of 50mL. The reaction mixture consisted of 2.5 units of *Taq* DNA polymerase (Stratagene 600131); 200mM each of dATP, dCTP, dTTP and dGTP; 100pM primers MCGpF11 and r23-1R and 1x assay buffer (50mM KCI; 10mM Tris-HCI [pH 8.8]; 1.5 mM $MgCI_2$; 0.01% gelatine) supplied with the enzyme by the manufacturer. Two drops of light mineral oil were added to the each tube and the amplification reaction was performed after pre-heating at 94°C for 30 seconds, annealing at 72°C for 2 minutes and elongation at 72°C for 2 minutes was performed. After the first step of amplification, 1mL of amplificated product was transferred to a new reaction tube and amplified in the same reaction mixture as used in the first amplification step.

Analysis of Amplified Samples

The products of PCR were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. Samples containing a band of the expected size for *M. hominis* (236bp) subjected to digestion with a restriction endonuclease enzyme. Amplified DNA from *M. hominis* was digested with *VspI* in order to confirm. Other samples containing expected size of DNA band for *M. fermentans* (365 bp) were subjected to amplification with primers RW004 and RW005 (Table III) which are very sensitive and spesific for IS-Like segment of *M. fermentans* reported by Wang et al (7).

The second PCR was performed for confirmation of the first PCR products consisting of expected size bands samples. For this reason, 10mL of sample DNA was amplified in a final volume of 50mL reaction mixture consisting of 2.5 units of taq DNA polymerase (Stratagene

600131), 200mM each dNTP (Stratagene 200415), 0.5 mM primers RW004 and RW005 and 1 x assay buffer (50mM KCI, 10mM Tris-HCI [pH8.8];1.5 mM $MgCI_2$; 0.001% gelatine). After addition of two drops of light mineral oil and pre-heating at 94°C for 2 minutes, PCR was performed totally 45 cycles of denaturation at 94°C for 35 seconds, annealing at 55°C for 45 seconds and elongation at 72°C for 50 seconds for incubation. The analysis of the products was also performed by 2% agarose gel electrophoresis and ethidium bromide staining.

Results

M. hominis strains were isolated from 26 (26%) genital swabs and 11 (11%) urine samples by using specific mycoplasma isolation media. *M. fermentans* strains were isolated from neither genital swabs nor urine samples by cultivation. But we have also found 40 samples positive for mycoplasmas which consisted of target genomic sequences (236bp and 365bp) of *M. hominis* and *M. fermentans* in 37 (37%) and 3 (3%) samples respectively by PCR which used genus specific MCGpF11, R23-TR, R16-2 and MCGR21 primers (Figure 1).

Amplified *M. hominis* DNA (236bp) from 37 samples were digested by *VspI* and confirmed with the presence of resulted 123bp and 113bp compatible for *M. hominis* in tested samples (Figure 2). Another PCR protocol was also performed for *M. fermentans* compatible sequences which is 206bp consisting samples by using RW004 and RW005 primers, and 3 samples were found positive by this second PCR (Figure 3).

In conclusion, a possible relation was found between colonisation of *M. hominis* (37%) or *M. fermentans* (3%) and women with genital infection. Additionally, we determined that despite the high specifity (100%) of cultivation, it has a low sensitivity (70.3%) and is time consuming when compared with PCR.

Discussion

The role of mycoplasmas in the genital and extragenital systems is speculative and depends on epidemiologic data. Experimental infection and colonisation attempts in the genital region were unsuccessful by mycoplasmas. Clinical results showed that mycoplasma incidence is raised in the presence of an anaerobic primer pathogen bacterium such as *T. vaginalis*, *C. trachomatis* or *N. gonorrhoea*. These findings were frequently obtained from studies which used microbiologic cultivation methods. According to these findings mycoplasmas are either non-pathogen microorganisms or satellite microorganisms which are growing in the stress environment created by primary pathogen. On the other hand, it could be thought that these microorganisms colonise numerously in a sexually active woman but could not be detected due to less sensitivity of microbiological cultivation methods. However after an infection, their colonisation ratio raises up and can be detected by conventional cultivation methods. Out of this thesis, if they are really pathogen and can lead to a chronic infection, it is known that pathological damage would lead to genital cell metaplasia occurred by cytokines secreted by inflammatory cells.

In order to answer all these questions, a sensitive, specific, fast, cheap and easily applicable, diagnostic method is necessary. PCR is recommended for mycoplasma infections like many other infections as an extremely sensitive and spesific method. In addition to PCR, Harasawa (11) has reported that PCR-RFLP is fast, specific, and sensitive method for the diagnosis of human originated mycoplasma.

In this study, we used Harasawa's (11) PCR-RFLP and conventional cultivation methods in order to detect the incidence and species of mycoplasma in patients with genitourinary tract infection. Another PCR protocol was also performed which is reported by Wang et all (7) in order to confirm the samples which have compatible target sequences for *M. fermentans* by using of RW004-RW005 primers. We concluded, that PCR method is a sensitive and easily applicable protocol when genus specific primers, *VspI* and Wang's primers, is used for the diagnosis of genital mycoplasmas.

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