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**Original Articles**

# Detection of *Chlamydia trachomatis, Mycoplasma hominis* and *Ureaplasma urealyticum* by Multiplex PCR in Semen Sample of Infertile Men

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## Abstract

**Background:** The aim of this study was to detect *Chlamydia trachomatis*, *Mycoplasma hominis* and *Ureaplasma urealyticum* from semen samples of infertile men by Multiplex PCR and investigation of influence of bacteriospermia on semen parameters.

**Methods:** Semen samples of 200 infertile men were evaluated by Multiplex PCR. In addition, analysis of semen parameters was performed according to the WHO guidelines.

**Results***:* All the patients were without clinical symptoms of urogenital tract infection. Thirty three percent of cases showed at least one bacterium. We found a noticeable relation between the presence of bacteriospermia and the rate of non motile and morphologically abnormal sperms ($P< 0.0001$). In addition, sperm concentration was lower in positive cases ($P< 0.04$). There was no relation between leukocytospermia and bacteriospermia ($P> 0.05$).

**Conclusion:** Asymptomatic existence of *Chlamydia* and *Mycoplasmas* in urogenital tracts might play an important role in sperm impairment due to infertility. Bacteriospermia can influence sperm's motility, morphology and concentration.

**Keywords:** *Infertility, Male*, Chlamydia trachomatis, Mycoplasma hominis, Ureaplasma urealyticum.

## Introduction

Male urogenital tract infection is one of the most important causes of male infertility (1). Genital tract infection and inflammation have been associated to 8-35% of male infertility, worldwide (2). Asymptomatic bacteriospermia may play a major role (3). Male accessory sex gland infections (epididymitis, prostitis, etc) are major risk factors of infertility (4). The significance of pathophysiology of bacteriospermia has been discussed in recent years. *Chlamydia* and *Mycoplasmas* are discussed as the important etiological factors of male infertility (1). *C. trachomatis* is one of the most common sexually transmitted pathogens of humans, with an estimated 92 million new cases occurring worldwide each year (1, 5). *Chlamydial* infections in men include acute and chronic NGU (Non Gnococcal Urethritis), epididymitis and prostitis (5, 6). *U. urealyticum* and *M. hominis* are known as causes of NGU (Non Gnococcal Urethritis) and rarely prostitis (7, 8). *Chlamydial* and *Mycoplasmal* male urogenital infections are often asymptomatic. Asymptomatic presence of these microorganisms in urogenital tracts may have a negative influence on male's reproductive health (1, 8). Some possible pathomechanisms of the development of infertility linked with infection are considered as direct effect on sperm function, deterioration of spermatogenesis, autoimmune processes induced by inflammation and dysfunction of accessory sex glands (1, 9).

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The aim of this study was to detect *C .trachomatis*, *U. urealyticum* and *M. hominis* from semen sample of infertile men attending to an infertility clinic by Multiplex PCR method and evaluation of the influence of bacteriospermia on semen parameters.

## Materials and Methods

***Bacterial strains*** *Mycoplasma* strains used in this study were purchased from the Razi Type Culture Collection (RTCC, Iran); *Ureaplasma urealyticum* RTCC 1369 and *Mycoplasma hominis* RTCC 1365. *C. trachomatis* strain was purchased from the School of Medicine, Tehran University of Medical Sciences, Iran.

***Clinical specimens*** Semen samples of 200 infertile men were collected in the infertility clinic (Mahdeah Hospital, Tehran, Iran) after a 3 d abstinence period, by masturbation. Patients had not taken any antibiotic since one week before collecting the semen sample. Before collecting the sample, patients had to wash their hands and genital area with water and soap. Samples were collected in sterile plastic containers which were used for collecting of urine. All of the specimens were stored in -70 °C until DNA extraction.

***DNA extraction from standard strains*** The dried standard strains were suspended in 1cc DDW. 100 µl Lysis buffer (320 mM Sucrose, 10mM Tris (PH= 8), 5mM MgCl2, 1% SDS, 40 mg/ml proteinase K) was added to 100 µl of the strain's solution. After incubation at 55 °C for 2 h, the solution was mixed with 200 µl of equilibrated phenol and centrifuged at 8000 X g for 5 min. The aqueous phase was then mixed with 200 µl chloroform and centrifuged again at 8000 X g for 5 min. DNA was precipitated with 400 µl absolute ethanol and 2 M sodium acetate at -20 °C for 1 h. After drying the tubes, DNA was precipitated again with 100 µl 70% ethanol at -20 °C for 1 h and then dissolved in 50 µl TE buffer (10, 11).

***DNA extraction from specimens*** Semen samples were centrifuged at 3000X g for 10 min. Then 500 µl Lysis Buffer was added to pellets. After incubation at 55 °C for over night, DNA was extracted from the mixture of samplelysis buffer by the method described above for the standard strains. The entire extracted DNA was stored at -20 °C until PCR (10, 11).

***PCR*** The sequences of primers (MWG- Biotech, Germany), for each bacterium, are listed in Table1. Moreover internal control based on primers KM29 and KM38 for amplification of a 262 bp fragment of the β- Globin gene was used with each sample in an equal reaction (Table1). Multiplex amplification reaction was performed in a volume of 25 µl and contained: 0.2 mM dNTP, 2.5 mM MgCl2, 5o pmol of each primer, 1X PCR buffer, 3 µl extracted DNA and 1.6 u Taq polymerase. Reaction was run for 35 cycles, under the following condition: 45 s at 95 °C, 30 s at 50 °C and 45 s at 72 °C. After the last cycle, a final elongation step of 10 min at 72 °C was performed to complete the elongation. Amplification products were visualized and photographed under UV light after electrophoresis for 45 min at 100V through a 1% agarose gel, containing ethidium bromide. .

***Semen analysis*** Immediately following semen collection, the sample was placed in an incubator and allowed to liquefy at 37 °C for up to 30 min before the analysis commenced. Semen analysis was performed according the WHO guidelines (12) with all of the measures of semen quality, apart from sperm morphology and the quantification of leukocytes, being completed within 1 h. The identification and quantification of leukocytes was carried out by cytochemical peroxidase method.

***Questionnaire*** We used questionnaire to ask patients about their age, educational status, any symptoms of urogenital infection and history of infertility status.

***Statistical analysis*** Statistical analysis was performed by SPSS statistical software package version 12, using t-test (Post Hoc multiple comparison test) and Chi-square test. Statistical significance was assumed at the $P< 0.05$ level.

**Table 1:** Oligonucleotide primers used for multiplex PCR assay

| Pathogen | Oligonucleotides | Amplification Target | Amplification Size (bp) | Reference |
|---|---|---|---|---|
| CT | KL! 5' TCC GGA GCG AGT TAC GAGA<br>KL2 5'AAT CAT TGC CGG GGA TTG GT | ORF2 of 7.5-kb cryptic plasmid | 241 | 13 |
| UU | U9 5' GAG ATA ATG ATT ATA TGT CAG GAT CA<br>U8 5' GAT CCA ACT TGG ATA GGA CGG | Urease gene | 167 | 13 |
| MH | RNAH1 5' CAA TGG CTA ATG CTG GAT ACG C<br>RNAH2 5' GGT ACC GTC AGT CTG CAA T | 16s rRNA gene | 334 | 14 |
| β-globin | KM29 5' GGT TGG CCA ATC TAC TCC CCG G<br>KM38 5' TGG TCT CCT TAG ACC TGT CTT G | β – globin human gene | 262 | 15 |

## Results

All the positive patients were without clinical symptoms of urogenital tract infections. Among total 200 cases, 33% (66 cases) showed at least one pathogen (Table 2). Twenty two amplified products were visualized and photographed under UV light after electrophoresis is presented in Fig. 1.

Statistically analysis of semen by SPSS program showed that the percentage of non motile and morphologically abnormal sperms was significantly higher in positive cases ($P< 0.0001$). Moreover, the number of cases with low sperm concentration (< 20 mil/ml) was higher among positive cases (10 cases). There was no relation between leukocytospermia and bacteriospermia ($P> 0.05$). Among our positive patients, leukocytospermia (>1mil/ml) was detected in 24 positive cases (36.4%). There was no significant relation between bacteriospermia and other semen parameters (Table 3).

Investigation of filled questionnaires showed that the highest number of positive cases belonged to young patients (20-30 yr old) (Table 4) and patients who were under high school diploma (Table 5).

**Table 2:** Multiplex PCR results from semen sample of infertile men

| Percentage (%) | Number | PCR result |
|---|---|---|
| 9 | 18 | *Chlamydia trachomatis* |
| 11 | 22 | *Mycoplasma hominis* |
| 3 | 6 | *Ureaplasma urealyticum* |
| 7 | 14 | *Mycoplasma hominis+ Chlamydia trachomatis* |
| 1 | 2 | *Mycoplasma hominis+ Ureaplasma urealyticum* |
| 1 | 2 | *Ureaplasma urealyticum+ Chlamydia trachomatis* |
| 1 | 2 | *Chlamydia trachomatis+ Mycoplasma hominis+ Ureaplasma urealyticum* |
| 67 | 134 | Negative |
| - | 200 | Total |

**Table 3** Relationship of PCR results and semen quality

| Semen analysis [a] | PCR results | | |
|---|---|---|---|
| | **Positive** | **Negative** | ***P*** |
| Volume(ml) | 3.3 (1-7. 5) | 3.5 (1.4-11) | NS [c] |
| pH | 7.8 (7- 8. 5) | 7.8 (7-8.5) | NS |
| Sperm concentration ($\times 10^6$/ml) | 52 (1-135) | 76 (17-178) | S |
| **Motility** [a] | | | |
| Rapid progressive motility (%) | 4 (0-10) | 11 (0-40) | S |
| Slow progressive motility (%) | 14 (5-25) | 20 (10-70) | S |
| Non progressive motility (%) | 37 (10-48) | 25 (5-34) | S |
| Non motile (%) | 59 (35-97) | 43 (15-70) | S |
| **Morphology a** | | | |
| Abnormal (%) | 50 (39-79) | 34 (14-63) | S |
| Leukocytospermia ($>1\times 10^6$/ml) [b] | 36.4% (24/66) | 24% (32/134) | NS |

[a] Values are medians per group (with ranges in parentheses)
[b] Values are percentages per group (with the number of patients/total number of patients per group in parentheses)
[c] Not significant

**Table 4:** Classification of PCR positive and negative patients according to their age

| | PCR Results | |
|---|---|---|
| **Age (yr.)** [a] | **Positive** | **Negative** |
| 20-30 | 48.5 (32/66) | 43.3 (58/134) |
| 31-40 | 39.2 (26/66) | 44.8 (60/134) |
| 41-50 | 12.12 (8/66) | 12 (16/134) |
| >50 | 0 | 0 |

[a] Values are percentages per group (with the number of patients/total number of patients per group in parentheses)

**Table 5:** Educational status of PCR positive and negative patients

| | PCR Results | |
|---|---|---|
| **Educational status** [a] | **Positive** | **Negative** |
| <High school diploma | 64(42/66) | 51(68/134) |
| High school diploma | 24.2(16/66) | 28.3(38/134) |
| Academicals education | 12.1(8/66) | 21(28/134) |

[a] Values are percentages per group (with the number of patients/ total number of patients per group in parentheses)

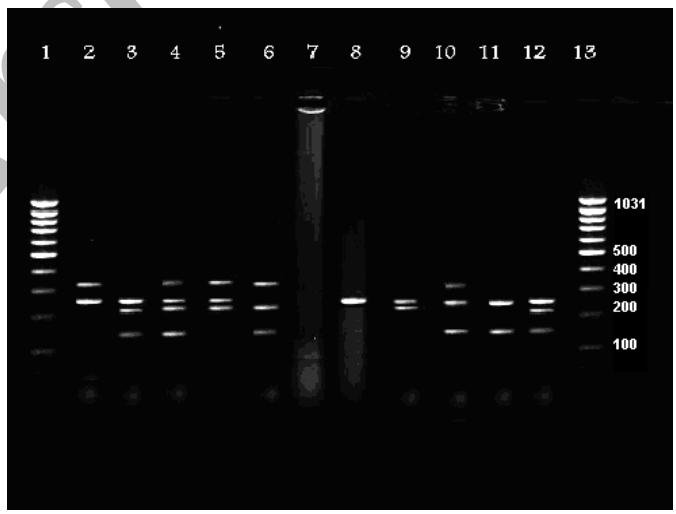


**Fig. 1:** Detection of CT, MH and UU from Clinical Samples by Multiplex PCR. Lane 2,3,4,5, 8,9,10,11,12 clinical samples: Lane 2 MH, Lane 3,12 CT+UU, Lane 4 MH+CT+UU, Lane5 MH+CT, Lane 8 negative sample (β- globin), Lane 9 CT, Lane 10 MH+UU, Lane 11 UU, Lane 1,13 100bp DNA Ladder, Lane 6 standard bacterial strains, Lane 7 negative control

## Discussion

Male urogenital tract infections are discussed as one of the significant etiological factors for male's infertility, worldwide (1). Infectious processes may lead to deterioration of spermatogenesis, impairment of sperm function or obstruction of the seminal tract (9). In vitro studies show that overnight incubation of sperm with *Chlamydia* or *Mycoplasmas* impairs sperm physiology; especially it has a negative influence on sperm motility, morphology and vitality (16-19).

In our study, we found a high number of infertile men with asymptomatic bacteriospermia (66/ 200). In addition, the number of cases with mixed infection was noticeable (20/66, 30.3%). Our results show that there are a high number of patients with mixed infection of *M.hominis* + *C. trachomatis*. We think that the presence of *M.hominis* in urogenital tracts may increase the colonization of C. *trachomatis*. In comparison to our study, other authors have mentioned the prevalence of *C. trachomatis*, *M. hominis* and *U. urealyticum* about 10-28%, 5-13% and 11-35%, respectively depend on a type of diagnostic method, specimen and population that they have worked (20-25). It seems that studies are frequently incomparable because different diagnostic methods and specimens have been used to different populations. It is believed that presence of *Chlamydia* and *Mycoplasmas* in urogenital tracts of infertile men is often asymptomatic and there are likely fewer bacteria in asymptomatic patients, therefore these cases usually remain unrecognizable by routine serological and culture methods (5, 26-28).

Statistically analysis of semen samples of our patients show that there is a noticeable relation between the presence of *Chlamydia* and *Mycoplasmas* in urogenital tracts and the percentage of non motile, abnormal sperms ($P< 0.0001$) and low sperm concentration ($P< 0.04$). The rate of non motile sperms, morphologically abnormal sperms and samples with low sperm concentration is higher in PCR positive cases. We could not find any significant relation between bacteriospermia and other semen parameters. Results obtained by other authors show that presence of *Chlamydia* and *Mycoplasmas* in urogenital tracts of infertile men may have a negative effect on semen volume, pH and sperm motility, morphology, concentration and vitality (17, 29-32). However some authors could not find any noticeable relation between bacteriospermia and poorer semen quality (18, 33, 34).

Another statistical finding of our study is about the lack of concordance between leukocytospermia and infection. Among our patients, leukocytospermia was found in 28% (56/ 200). However not all samples that were PCR positive were leukocytospermic and. Although a possible etiology of leukocytospermia is sub clinical genital infection, this study and some others claim that leukocytospermia is not a reliable marker for predicting of infection (35-37). Because in some clinics microbiological investigations are performed only in case of leukocytospermia, it should be noticed that performing of microbeological investigations seems to be necessary to all infertile men, as a routine test, in presence or absence of leukocytospermia, especially to patients under-going Assisted Reproductive Techniques (ART). Presence of bacteria in semen is associated with unsuccessful ART. Genital bacteria may attach to sperm and some of them can not be removed even during the washing processes in IVF (In Vitro Fertilization) method. On the other hand, antibiotics that are used in IVF culture system do not have any effect on bacteria such as *C. trachomatis*. So, semen may have a potential to contaminate IVF culture (23, 26, 38, 39).

Moreover to the results presented above, results obtained from questionnaire show that, the highest number of positive cases belonged to young patients (20-30 yr old). Furthermore, there is a meaningful relation between the level of education and infection. Patients who are under high school diploma are more likely to have infection. With regard to these facts, it seems that people's knowledge and culture have a direct effect on their perception toward sexual health.

Lack of knowledge of genital infections protection ways, involving unprotected sex repeatedly and not following the treatment process, in case of infection, are factors which may raise the probability of persistent presence of bacteria in urogenital tracts.

Conclusively, because of the significant destructive effects of bacteriospermia toward male's reproductive health, informing people (especially young people) about urogenital infections and protection ways, screening and treating of asymptomatic cases seem to be indispensable. It should be noticed that existence of bacteria and inflammation in urogenital tracts should be eradicated by suitable antibiotic and anti- inflammatory treatment. Antibiotictherapy must be performed to couple, simultaneously (2, 40).

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