**Mycoplasma hominis** and **M. genitalium** frequency of infertile men in the northeast of Iran by culture and Multiplex-PCR

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**Abstract**

**Background:**

Recently, infertility has become a growing social and economic world problem. Genital mycoplasmas such as *Mycoplasma hominis* and *M. genitalium* are most frequently associated with several adverse effects on men’s fertility. The study is going to determine the prevalence of *M. hominis* and *M. genitalium* in the semen samples in the northeast of Iran as the first such research in the area.

**Material and method:** During the cross-sectional study from February to May of 2018, one hundred semen samples were collected from 100 infertile men of Mashhad, Khorasan Razavi province, northeast of Iran. The presence of *M. hominis* and *M. genitalium* were detected by cultivation, PCR, and Multiplex PCR assays. By Diene’s stain, the colony of mycoplasma was confirmed, and arginine hydrolysis, glucose and urea utilization were evaluated. Some semen indexes like as color, volume, appearance, liquefaction, viscosity, concentration, pH, leukocyte concentration, progressive motility, morphological normality, motile sperm concentration, functional sperm concentration, sperm motility index, and functional sperm were analyzed according to WHO guideline for semen analysis.

The gene of 16SrRNA (GPO1& MGSO primers) was used as the target gene of *Mycoplasma* genus in PCR assay. Multiplex PCR was performed with specific primer for conserved regions in the
16SrRNA gene for *M. hominis* (RNAH1& RNAH2 primers) and the 140-kDa Adhesion Protein Gene for *M. genitalium* (MG1 & MG2 primers).

**Result:** By the results, nine samples (9%) were positive with *mycoplasma spp* in PCR, while there are seven (7%) isolated by cultivation. *M. hominis* was detected in eight samples (8%) by Multiplex PCR, while there’s no evidence for *M. genitalium*. The mean age of all infertile and infected men was 31 and 30 years respectively. The study could not show any statistical correlation between abnormality in semen parameters and mycoplasma infected.

**Conclusion:** The heterogeneity of mycoplasma prevalence in the reports can be caused by differences in the geographic areas, the sensitivity of the identification method, the condition of the group (fertile/infertile), the sample size, and the operator proficiency. There are many types of research with different results to show any relationship between *mycoplasma* infection and the abnormality in the semen parameters.

**Keyword:** Infertility, *Mycoplasma hominis*, *Mycoplasma genitalium*, semen, Multiplex PCR

**Introduction**

*Mollicutes* are the class of eubacteria that contain a group of cell wall-less bacteria with very small genome size (580-2200kb). They are widespread in nature as parasites of human, fish, plants, arthropods, and reptiles. The class are contained of eight genera; *Mycoplasma, Ureaplasma, Spiroplasma, Mesoplasma, Entoplasma, Asterolespasma, Acholeplasma* (Razin and Tully, 1995). The genus *Mycoplasma* inclusive more than 100 defined species of which sixteen are isolated from the man. Many species of human *Mycoplasma* are mainly commensal, but some of them like as *M. pneumonia, M. hominis, M. genitalium, Ureaplasma urealyticum*, and *U. parvum* are associated with a disease. Although as a transient colonized organism, some animal originated species such as *M. arginini* are occasionally detected in immunosuppressed human (Yoshida et al., 2002).

*M. genitalium* and *M. hominis* are common pathogens in male and female reproductive tract infections. It is also worth noting that these bacteria generally cause chronic and oligosymptomatic genital infections. They are the main causes of the fallopian tubes adhesion, change in endometrial mucosa, pelvic inflammatory disease (PID), chorioamnionitis, urethritis, defective sperm, epididymitis, and prostatitis (Ahmadi et al., 2010, Gdoura et al., 2007, Ona et al., 2016, Safavifar et al., 2015, Stojanov et al., 2018, Svenstrup et al., 2003). During pregnancy and birth, the bacteria can
also be associated with an increase in spontaneous abortion, preterm birth, meningitis or other neonatal infection (Direkvand-Moghadam et al., 2013, Jensen et al., 2016, Moghaddam et al., 2015).

In addition, infertility is the common complications which is caused by the direct or indirect effects of the infection. The World Health Organization (WHO) definition of infertility is “the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse”. Semen assessment is the first-line evaluation of male fertility. As the *mycoplasma* infection may cause urinary tract inflammation and defective sperm which may lead to transmission of infection, so the semen sample makes worthy information. The clinician requires the correct report from microbiologists for effective treatment. Many cases of infertility and maternal-fetal diseases can be prevented by proper and prompt recognition and treatment of infected people. The present study is the first prevalence study of *M. genitalium* and *M. hominis* in the semen of infertile men by molecular and cultivation methods in the northeast of Iran.

**Materials and Method**

**Clinical specimens**

Men who had the criteria (age 18-50, the failure to achieve his wife pregnancy after one year or more of regular unprotected sexual intercourse) were randomly selected and informed. Self-semen sampling was done after their satisfaction. Although there was no data about the prevalence of *M. hominis* and *M. genitalium* in semen of infertile men in the northeast of Iran. The sample size according to the prevalence report of the other study in the country and on the base of the prevalence of cross-sectional studies formula with 95% confidence and accuracy of 10% estimated 100 samples. Therefore, during the cross-sectional study from February to May of 2018, one hundred semen samples were collected from infertile men who were referred to the laboratory of Mashhad Jahad Daneshgahi in the northeast of Iran.

**Semen assay**

Three to five days after last intercourse, approximately 3 ml of self-collected semen specimen were placed in a sterile plastic container. semen samples were liquefied for 30 min at 37°C. Then some indexes include age, color, volume, appearance, liquefaction, viscosity, concentration, pH, WBC/hpf (leukocyte concentration), progressive motility, morphological normality, MSC (motile sperm concentration), FSC (functional sperm concentration), SMI (sperm motility index), motile
sperm, and functional sperm were performed according to WHO guideline for semen analysis (Organisation, 1999). Briefly, liquefaction is the duration of semen conversion from gel to liquid. Naturally, white blood cells should not be present in the semen. Concentration of semen refer to the number of live sperm in one ml of semen per ejaculation. Progressive motility is the percentage of the motile sperm. Naturally, at least 30% of the sperm should have a normal shape. MSC is the concentration of progressive sperms per unit of volume (ml). FSC refer to concentration of sperm with normal morphology and progressive movement per unit of volume (ml). The sperm motility index is taking from the sperm number, movement, morphology, and the amount of sperm acrosome space. Motile sperm is the percentage of the sperm that can move forward.

The mean, standard deviation, standard error mean, Levene’s test for equality of variances, t-test for equality of means and Chi-Square tests were used statistically.

**Culture**

One ml of each semen specimen was inoculated to the pleuropneumonia-like organisms (PPLO) transport medium. The medium was enriched with the decomplemented normal horse serum (7%), yeast extract (12 gr/lit), along with penicillin (1000 IU/ml), and polymyxin B (500 IU/ml) as inhibitors for Gram-positive and Gram-negative bacteria. Transport medium was incubated in 4°C for 48 hours. After cold enrichment, the samples were inoculated from transport medium to PPLO broth with the same ingredients except the extra 20% normal horse serum. As their small cellular dimensions, *Mycoplasmas* cannot usually produce turbidity during growth in the broth media. However, the growth rate is sometimes determined with faint turbidity by an expert practitioner, but it is not reliable. Thus phenol red as a pH indicator is applied to reveal the growth (pH=7). PPLO broth tubes were incubated at 37 °C for 48 hours. As soon as observing any color change in the specific pH indicator, 200µl of the broth was inoculated on the PPLO agar. The plates were incubated at 37 °C in a 5% CO2 incubator (Jahl co., Iran) for ten days. As well as a sterile cotton ball moistened with sterile water containing sodium propionate was also placed at the bottom of the jar so as to prevent drying and fungal growth. During the incubation period, the plates were daily evaluated with a microscope (×40) to find the typically fried egg small colonies. Finally, Diene’s stain was done to confirm the *Mycoplasma* colonies on the PPLO agar and differentiated from L-form bacteria. In the staining, *Mycoplasma* colony was colored like a dark blue granular center surrounded by the light blue zone.
Arginine, urea and glucose utilization tests were used on the specific tiny bacteria which pass through 220 nm Millipore filter with penicillin and polymyxin resistance pattern to specifically biochemical identification.

**Polymerase chain reaction (PCR) assay**

Bacterial DNA from one ml of the broth media was extracted by phenol-chloroform protocol. After a spin, DNA was suspended in 20µl of RNase-DNase free sterile deionized water. The PCR was done for *16S rRNA* gene as the target of *Mycoplasma* genus (GPO1; forward primer: 5′-ACTCCTACGGGAGGCAGCATAG-3′ & MGSO; reverse primer: 5′-TGCACCATCTGTCACTCTGTTAACCTC-3′)(Kong et al.,2001, Tabatabaei-Qomi et al.,2014). The PCR reaction consisted of 500 ng of template DNA, 10µl of master mix (Ampliqon), 1 µl of each one of forward (GPO1) and reverse (MGSO) primers, and 3µl of sterile deionized distilled water. The Astek thermocycler machine (Hollywood co. Thailand) makes planned first for 5 min at 95°C and then 40 cycles at 93°C for 20 s, 60°C for 20 s, and finally 72°C for 30 s. The agarose gel (1.5%) was made with Green Viewer (Pars Tous co.) as a nucleic acid stain. The PCR products were electrophoresed for detection of 715 bp bands beside the 100 bp ladder (Pars Tous co.).

**Multiplex PCR assay**

Multiplex PCR was performed with the specific primer for conserved regions in the *16S rRNA* gene of *M. hominis* (RNAH1; forward primer: CAATGGCTAATGCCGGATACGC& RNAH2; reverse primer: GGTACCCTAGTGTGCAAT) and the *140-KDa Adhesion Protein* gene of *M. genitalium* (MG1; forward primer: AGTTGATGAAACCTTAACCCCTTGG & MG2; reverse primer: CCGTGGGAGGGTTTTCCATTGG)(Stellrecht et al.,2004). Multiplex PCR was performed on the equivalent of 5 µl of each sample in 20µl reactions. Each reaction contained 10µl of master mix (Ampliqon), 1µl of RNAH1 forward primer (10pmol), 1µl of RNAH2 reverse primer (10pmol), 1µl of MG1 forward primer (10pmol), 1µl of MG2 reverse primer (10pmol) and 1µl of distilled water. After preparing and mixing the microtubes, Multiplex PCR was performed under the following condition: in the first round one cycle at 95°C for 10 min; in the second round, 35 cycles in two-step at 95°C for 15s and at 60°C for the 60s; and the third round in 5 min at 72°C(Stellrecht et al.,2004). Amplified products were electrophoresed beside a 100 bp ladder (Pars Tous co.) for 25 min at 110 V through a 1.5% gel agarose containing DNA Green Viewer (Pars Tous co.) and evaluated for the specific band under gel documentation system (Bio-Rad). The PCR
product of 334bp and 282bp bands demonstrated of 16SrRNA gene of *M. hominis*, and 140-KDa Adhesion Protein gene of *M. genitalium* respectively.

**Result**

The results of the PCR assay on the 16SrRNA gene for genus *mycoplasma* revealed 9% of semen samples were infected with *Mycoplasma* spp. By multiplex PCR, *M. hominis* and *M. genitalium* were detected observing the 334 and 282 bp amplifying segments respectively. *M. hominis* was detected in 8% and *M. genitalium* in none of the semen samples collected from infertile men in multiplex PCR. While cultural and biochemical test results showed 7% of samples were positive for *M. hominis* and zero for *M. genitalium*. All of the positive culture samples were positive in PCR assay for a conserved region of the 16SrRNA gene of *mycoplasma* genus, too.

The mean age of all infertile men entering the study was 31 years old and the mean age of the infected men was 30. In the comparison of the mean semen samples analysis with the same in PCR positive for *mycoplasma* to demonstrate the effects of these microorganisms on the sperm cells, it wasn’t seen any statistical changes for abnormality generation. With 95% confidence interval of the difference, the present study could not show any statistical correlation between two groups (*mycoplasma*+/−) in the mean of semen quantitative and qualitative variable parameters like as color, volume, appearance, liquefaction, viscosity, pH, W.B.C /hp, concentration, progressive motility, morphological normality, MSC, FSC, SMI, motility, and function of sperm (P>0.05).

**Discussion**

One of seven couples is affected by infertility due to problems of female, male, or both of them in the world. In couples seeking infertility treatment, approximately 50% are found with abnormal semen parameters (Borght, 2018, Stojanov et al., 2018). In male fertility, a decreased chance of conception can be associated with abnormal semen parameters such as concentration and motility. Therefore, male infertility is always evaluated through a semen assessment. In the infections, the presence of bacteria in the semen may effect on quality of sperm, there is a hypothesis of induction of apoptosis and necrosis by the bacteria, which may reduce sperm motility and concentration. *Mycoplasmas* can be colonized in the male urethra and transferred through ejaculation to a sexual partner. Defective sperm, prostatitis, acute urethritis (4-42%), epididymitis, urethral discharge, dysuria, and sexually acquired reactive arthritis which be caused to semen damaged can be seen
in genital mycoplasma infection (Ahmadi et al., 2010, Jensen et al., 2016). *M. hominis* be isolated from 35% of men without any sign of urinary tract infection (Moghaddam et al., 2015). While 10-35% of nongonococcal-nonchlamydia urethritis (NGNCU) be caused by *M. genitalium* (Horner et al., 1993, Sabo et al., 2013, Tsai and Li, 2013). Some interventional experimental studies show that *M. hominis* and *M. genitalium* adhere to human spermatozoa and lead to the sperm agglutination and immobility (Moretti et al., 2009, Svenstrup et al., 2003). As it known the higher incidence of *M. hominis* causes abnormal semen patterns as like as *M. genitalium* in infertile men (Al-Sweih et al., 2012).

*Mycoplasmas* involvement in male infertility can be suspected. Gdoura et al. failed to demonstrate a relationship between altered semen parameters and the presence of mycoplasma in the semen in infertile males (Gdoura et al., 2007, Stojanov et al., 2018). In Gdoura study, there is no statistical relationship between the present of genital mycoplasmas and the abnormality in seminal volume, motility, vitality, pH and leukocyte count in the infertile men. Although a negative correlation was seen between sperm concentration and *M. genitalium* infection whereas there were low sperm concentration and abnormal sperm morphology in the presence of *M. hominis* (Gdoura et al., 2007).

The present study result compare to Gdoura result shows that in both studies there isn’t any relationship between mycoplasma infection and the abnormality in semen analysis parameter except in Gdoura result there is a statistical correlation between *M. hominis* infection and low sperm concentration, and abnormal sperm morphology. However, in our study, it isn’t shown any correlation between *M. hominis* infection and the abnormality of the two semen variable factors like other tested parameters (P>0.05).

There are different reports about the prevalence of *M. hominis* and *M. genitalium* in Iran. By PCR technique, Soleimani Rahbar indicated *M. hominis* in 3% of semen specimens of infertile men in Tehran (Rahbar et al., 2007). In the same year, Golshani detected *M. hominis* by multiplex PCR in 11% of infertile men in Tehran (Golshani et al., 2007). In a study, Ahmadi isolated *M. hominis* from 15% of infertile men in Tehran (Ahmadi et al., 2010). Vosooghi recognized *M. hominis* in 22% of the semen sample of infertile men in Kerman (Vosooghi et al., 2013), but Bahaabadi could detect *M. hominis* in 7% of the semen specimen of infertile men in Kerman (Bahaabadi et al., 2014). Whereas Asgari demonstrated *M. hominis* in 39% of infertile men in Qom (Asgari et al., 2018), which is the highest prevalence among the other Iranian reports. While in our study, seven isolates
(7%) were isolated by the cultivation method and confirmed in Diense staining in the northeast of Iran. None of the isolated strains could not ferment glucose and urea while all of them hydrolyzed arginine, which more often reflects the presence of *M. hominis* but *M. genitalium*. Our PCR data on a conserved gene that is specific for *mycoplasma* genus showed which 9% of samples were positive for *mycoplasma* infection. Also, *M. hominis* was present in 8% of the semen specimens of infertile men using the multiplex PCR which confirmed the PCR and cultivation results.

The results of *M. hominis* prevalence studies are different as well in the foreign of the country. Zinzendorf investigated *M. hominis* in 23.8% of infertile men by strip method in Africa (Zinzendorf et al., 2008). In another study, Kerem Taken could determine *M. hominis* in 8% of infertile men by PCR in Turkey (Taken, 2016). As is well known, *mycoplasmas* are relatively slow growth and fastidious. Therefore, most studies in the field of *mycoplasma* detection have focused on molecular technique. Indeed, molecular methods are developed with higher specificity and sensitivity than traditional cultural procedures for identification of *mycoplasmas*. Some studies compared the result of cultivation with the PCR method. Stellrecht recognized *M. hominis* in 6% of semen samples by culture and 8% by PCR method in infertile men (Stellrecht et al., 2004). Also, our study results showed 7% positive *mycoplasma* in cultivation compare to 9% in the molecular method.

*M. genitalium* is one of the important agents in men genital tract infections and several previous attempts failed to isolate this organism, probably due to the lack of proper procedure for culture (Baseman et al., 2004; Korte et al., 2006). Mohseni Moghadam detected *M. genitalium* in 13% of infertile men by the molecular method in Kerman (Moghadam et al., 2014), and Safavifar showed the prevalence of *M. genitalium* about 40% in infertile men in Tehran by PCR (Safavifar et al., 2015). While in our study neither molecular (PCR and multiplex PCR) nor biochemical identification methods showed any *M. genitalium* in the semen samples of the study.

The present study was done just on infertile men, whereas in some other reports the prevalence of *M. genitalium* and *M. hominis* are compared in infertile and fertile groups. Liu indicated the prevalence of *M. hominis* in 11% of infertile men and 9% of fertile men by cultivation method in China (Liu et al., 2014); as well as Lee in Korea displayed *M. hominis* in 14% and 6.3% of infertile and fertile men respectively (Lee et al., 2013). Similarly, Abusarah detected *M. genitalium* in 3.2% and 1.4% of infertile and fertile men by PCR in Jordan (Abusarah et al., 2013).
M. genitalium in 17% of the 99 urogenital tract samples of the male patient by using 140-kDa Adhesin Protein gene in PCR; whereas they could isolate only four strains of M. genitalium from 17% PCR-positive samples. Their attempts to isolate M. genitalium from all PCR-positive samples were failed(Jensen et al., 1996, Jensen et al., 1993). In Horner study, 23% of men with acute NGNCU and 6% of men without NGNCU were PCR-positive for M. genitalium. They concluded that in many cases of NGNCU, M. genitalium was the unique etiologic agent(Horner et al., 1993). Although, some assay by other researchers with a reverse result is rare; as like Al- Sweih that distinguished M. hominis and M. genitalium in 17.1% and 4.7% of infertile men; while their value was 32.4% and 3.2% in the fertile men by PCR in Kuwait (Al-Sweih et al., 2012).

The heterogeneity of prevalence in the reports can be caused by differences in the geographic areas, the sensitivity of the identification method, the condition of the group (fertile/infertile), the sample size, and the operator proficiency. Also, it is may be caused by the nature of the transient and unstable of the mycoplasma infection in the population. However, the genital mycoplasmas are surface cellular parasites (cell independent) and the bacteria cannot be alive in the out of the body for a long time. However, the prevalence of M. genitalium also is lower than M. hominis in the other country like Iran.

It is suggested that in future studies on genital mycoplasma, evaluation and investigation should be done on two groups of fertile (healthy) and infertile couples simultaneously in the period of years.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that there is no conflict of interest in this study.

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