Archives of Razi Institute, Vol. 76, No. 1 (2021) 41-49

DOI: 10.22092/ari.2019.125966.1338

Original Article

Prevalence Determination of *m. Hominis* and *m. Genitalium* in the Semen Samples in the Northeast of Iran Using Culture and Multiplex Polymerase Chain Reaction

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Received 2 June 2019; Accepted 18 July 2019
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Abstract

Infertility has recently 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 present study aimed to determine the prevalence of *M. hominis* and *M. genitalium* in the semen samples in the northeast of Iran. During this cross-sectional study from February to May, 2018, 100 semen samples were collected from 100 infertile men in Mashhad, Khorasan Razavi province, northeast of Iran. The presence of M. hominis and M. genitalium was detected by cultivation, polymerase chain reaction (PCR), and Multiplex PCR assays. The colony of mycoplasma was confirmed by Diene's stain; moreover, arginine hydrolysis, glucose, and urea utilization were evaluated. The following semen indices were analyzed according to World Health Organization guidelines for semen analysis: 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. The gene of *16SrRNA* (GPO1& MGSO primers) was used as the target gene of *the Mycoplasma* genus in PCR assay. Multiplex-PCR was performed with a 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). According to the results, 9 (9%) samples were PCR-positive for *Mycoplasma spp*, while there were 7 (7%) cases isolated by cultivation. *M. hominis* was detected in 8 (8%) samples by Multiplex PCR, while there was no evidence for *M. genitalium*. The mean age scores of all infertile and infected men were obtained at 31 and 30 years, respectively. The study could not show any statistical correlation between *mycoplasma* infection and abnormal semen parameters. The heterogeneity of mycoplasma prevalence in the reports can be ascribed to differences in geographic areas, the sensitivity of the identification method, condition of the group (fertile/infertile), sample size, and operator proficiency. Various results have been reported in numerous studies conducted on the relationship between *mycoplasma* infection and abnormal semen parameters.

Keywords: Infertility, *Mycoplasma hominis*, *Mycoplasma genitalium*, semen, Multiplex-PCR

Détermination de la Prévalence de *m. Hominis* et *m. Genitalium* dans les Échantillons de Sperme dans le Nord-Est de L'Iran en Utilisant la Culture et la Réaction en Chaîne de Polymérase Multiplex

Résumé: L'infertilité est récemment devenue un problème mondial social et économique croissant. Les

mycoplasmes génitaux, tels que *Mycoplasma hominis* et *M. genitalium, sont le plus souvent associés à plusieurs effets indésirables sur la fertilité masculine. La présente étude visait à déterminer la prévalence de M. hominis et M. genitalium* dans les échantillons de sperme dans le nord-est de l'Iran. Au cours de cette étude transversale de février à mai 2018, 100 échantillons de sperme ont été prélevés sur 100 hommes stériles à Mashhad, dans la province de Khorasan Razavi, au nord-est de l'Iran. La présence de *M. hominis* et de *M.* genitalium a été détectée par culture, réaction en chaîne par polymérase (PCR) et tests de PCR multiplex. La colonie de mycoplasmes a été confirmée par la coloration de Diene; de plus, l'hydrolyse de l'arginine, l'utilisation du glucose et de l'urée ont été évaluées. Les indices de sperme suivants ont été analysés conformément aux directives de l'Organisation mondiale de la santé pour l'analyse du sperme: couleur, volume, apparence, liquéfaction, viscosité, concentration, pH, concentration de leucocytes, motilité progressive, normalité morphologique, concentration de spermatozoïdes mobiles, concentration de spermatozoïdes fonctionnels, indice de motilité des spermatozoïdes et le sperme fonctionnel. Le gène de *16SrRNA* (amorces GPO1 et MGSO) a été utilisé comme gène cible du genre *Mycoplasma* dans le test PCR. La multiplex-PCR a été réalisée avec une amorce spécifique pour les régions conservées dans le gène 16SrRNA pour *M. hominis* (amorces RNAH1 et RNAH2) et le gène de la protéine d'adhésion de 140 kDa pour *M. genitalium* (amorces MG1 et MG2). Selon les résultats, 9 (9%) échantillons étaient positifs à la PCR pour *Mycoplasma spp*, alors qu'il y avait 7 (7%) cas isolés par culture. *M. hominis* a été détecté dans 8 (8%) échantillons par PCR multiplex, alors qu'il n'y avait aucune preuve de *M. genitalium*. Les scores d'âge moyens de tous les hommes infertiles et infectés ont été obtenus à 31 et 30 ans, respectivement. L'étude n'a pas pu montrer de corrélation statistique entre l'infection à mycoplasme et les paramètres anormaux du sperme. L'hétérogénéité de la prévalence des mycoplasmes dans les rapports peut être attribuée à des différences dans les zones géographiques, à la sensibilité de la méthode d'identification, à l'état du groupe (fertile / stérile), à la taille de l'échantillon et à la compétence des opérateurs. Divers résultats ont été rapportés dans de nombreuses études menées sur la relation entre l'infection à mycoplasme et les paramètres anormaux du sperme.

Mots-clés: Infertilité, *Mycoplasma hominis, Mycoplasma genitalium,* sperme, Multiplex-PCR

Introduction

Mollicutes is a class of bacteria characterized by the absence of a cell wall and a very small genome size (580-2200kb). They are widespread as parasites of humans, fish, plants, arthropods, and reptiles. This class contains eight genera: *Mycoplasma, Ureaplasma, Spiroplasma, Mesoplasma, Entoplasma, Asteroleplasma, Acholeplasma,* and *Anaeroplasma (Razin and Tully, 1995)*. The genus *Mycoplasma* includes more than 100 defined species, 16 of which are isolated from humans. Many species of human *Mycoplasma* are mainly commensal; nonetheless, some of them, such as *M. pneumonia, M. hominis, M. genitalium, Ureaplasma urealyticum,* and *U.parvum,* are associated with a disease. Some animal-originated species, such as *M. arginine*, are occasionally detected in immunosuppressed humans (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 are responsible for chronic and oligosymptomatic genital infections. They are the main causes of change in endometrial mucosa, adhesion of fallopian tubes, pelvic inflammatory disease, chorioamnionitis, urethritis, defective sperm, epididymitis, and prostatitis (Svenstrup et al., 2003; Gdoura et al., 2007; Ahmadi et al., 2010; Ona et al., 2016; Stojanov et al., 2018). During pregnancy and birth, 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).

Furthermore, infertility is a common complication caused by direct or indirect effects of infection. The World Health Organization (WHO) defines infertility as “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. *Mycoplasma* infection may lead to urinary tract inflammation and defective sperm,

which in turn, result in infection transmission. Therefore, semen samples can be of great help in the provision of valuable information. The clinicians require the correct report from microbiologists for effective treatment. Numerous cases of infertility and maternal-fetal diseases can be prevented by proper and prompt recognition and treatment of infected people. The current research is the first prevalence study of M. genitalium and M. hominis in the semen of infertile men in the northeast of Iran using molecular and cultivation methods.

Material and Methods

Clinical Specimens. The men who met the inclusion criteria (the age range of 18-50 and the failure to achieve their wives' pregnancy after one year or more of regular unprotected sexual intercourse) were randomly selected and informed. Self-semen sampling was performed upon their willingness. No data were available on the prevalence of *M. hominis* and *M. genitalium* in the semen of infertile men in the northeast of Iran. The sample size was estimated at 100 cases according to the prevalence report of another study in the country and the prevalence of cross-sectional studies formula with 95 % confidence and accuracy of 10%. Therefore, during the cross-sectional study from February to May, 2018, 100 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 the last intercourse, approximately 3 ml of self-collected semen specimen were placed in a sterile plastic container. The semen samples were liquefied at 37°C for 30 min. Thereafter, the following indices were assessed according to WHO guideline for semen analysis(Organization, 1999): 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. In brief, liquefaction is the duration of semen conversion from gel to liquid. Naturally, white blood cells should not be present in the semen. The concentration of semen refers to the number of live sperm in one ml of semen per ejaculation. Progressive motility signifies the percentage of motile sperm. Naturally, at least 30% of sperms should have a normal shape. The MSC is the concentration of progressive sperms per unit of volume (ml). The FSC refers to the concentration of sperm with normal morphology and progressive movement per unit of volume (ml). The sperm motility index is obtained from sperm number, movement, morphology, and the amount of sperm acrosome space. Motile sperm is the percentage of sperms that can move forward. The statistical tests included mean, standard deviation, standard error mean, Levene's test for equality of variances, t-test for equality of means, and Chi-Square test.

Culture. One ml of each semen specimen was inoculated to the pleuropneumonia-like organisms (PPLO) transport medium. The medium was enriched with 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. The transport medium was incubated at 4ºC for 48 h. After cold enrichment, the samples were inoculated from transport medium to PPLO broth with the same ingredients, except an extra 20% normal horse serum. *Mycoplasmas* cannot usually produce turbidity during growth in the broth media due to their small cellular dimensions. Nevertheless, the growth rate is sometimes determined with faint turbidity by an expert practitioner, however, it is not reliable. Therefore, phenol red as a pH indicator is applied to reveal the growth (pH=7). PPLO broth tubes were incubated at 37°C for 48 h. In total, 200µl of the broth was inoculated on the PPLO agar immediately after observing any color change in the specific pH indicator. The plates were incubated at

37°C in a 5% CO_2 incubator (Jahl co., Iran) for ten days. Moreover, a sterile cotton ball moistened with sterile water containing sodium propionate was also placed at the bottom of the jar to prevent drying and fungal growth. During the incubation period, the plates were daily examined with a microscope (×40) to find the typically fried egg small colonies. Finally, Diene's stain was performed to confirm the existence of *Mycoplasma* colonies on the PPLO agar and differentiate them from L-form bacteria. In the staining, *the Mycoplasma* colony was colored like a dark blue granular center surrounded by the light blue zone.

For specific biochemical identification, 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.

Polymerase Chain Reaction Assay. Bacterial DNA from 1 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 polymerase chain reaction (PCR) was carried out for *the 16SrRNA* 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 mg 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 sec, 60°C for 20 sec, and finally 72°C for 30 sec. The agarose gel (1.5%) was made with Green Viewer (Pars Tous co.) as a nucleic acid stain. The PCR products were electrophoresed for the detection of 715 bp bands beside the 100 bp ladder (Pars Tous co.).

Multiplex Polymerase Chain Reaction Assay. Multiplex PCR was performed with the specific primer for conserved regions in the *16SrRNA* gene of *M. hominis* (RNAH1; forward primer: CAATGGCTAATGCCGGATACGC& RNAH2; reverse primer: GGTACCGTCAGTCTGCAAT) and the *140-KDa Adhesion Protein* gene of *M. genitalium* (MG1; forward primer: AGTTGATGAAACCTTAACCCCTTGG & MG2; reverse primer: CCGTTGAGGGGTTTTCCATTTTTGC) (Stellrecht et al., 2004). Multiplex PCR was performed on an 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: one cycle at 95ºC for 10 min in the first round, 35 cycles in two-step at 95ºC for 15 sec and at 60ºC for 60 sec in the second round, and 5 min at 72ºC in the third round (Stellrecht et al., 2004). The amplified products were electrophoresed next to 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 *16SrRNA* gene of *M. hominis* and *140-KDa Adhesion Protein* gene of *M. genitalium*, respectively.

Results

The results of the PCR assay on the *16SrRNA* gene for genus *mycoplasma* revealed that 9% of semen samples were infected with M*ycoplasma* spp. *M. hominis* and *M. genitalium* were detected by multiplex PCR, observing 334 and 282 bp amplifying segments, respectively. *M. hominis* and *M. genitalium* were detected in 8% and 0% of the semen samples collected from infertile men in multiplex PCR. Cultural and biochemical test results indicated that 7% and 0% of samples were PCR-positive for *M. hominis* and *M. genitalium*, respectively. All of the positive culture

samples were also positive in PCR assay for a conserved region of *16SrRNA* gene of *Mycoplasma* genus.

The mean age of all infertile men under the study was reported as 31 years, and the mean age of infected men was obtained at 30 years. A comparison was made between semen samples negative for *mycoplasma* and those positive for *mycoplasma* to demonstrate the effects of these microorganisms on sperm cells. The results did not show any statistical changes for abnormality generation. With a 95% confidence interval, the present study could not demonstrate any statistical correlation between the two groups (*mycoplasma*+/-) in the mean of semen quantitative and qualitative variable parameters, such as color, volume, appearance, liquefaction, viscosity, pH, W.B.C /hpf, concentration, progressive motility, morphological normality, MSC, FSC, SMI, motility, and function of sperm (P>0.05).

Discussion

Around one in seven couples worldwide struggles with infertility due to the factors related to females, males, or both. Abnormal semen parameters were observed in approximately 50% of couples who seek infertility treatment (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 case of infections, the presence of bacteria in the semen may affect the quality of sperm. Moreover, it is hypothesized that bacteria induce apoptosis and necrosis which may reduce sperm motility and concentration. *Mycoplasmas* can be colonized in the male urethra and transferred to the sexual partner through ejaculation. Defective sperm, prostatitis, acute urethritis (4%-42%), epididymitis, urethral discharge, dysuria, and sexually acquired reactive arthritis can be observed in genital *mycoplasma* infection (Ahmadi et al., 2010; Jensen et al., 2016). *M. hominis* is isolated from 35% of men without any sign of urinary tract infection (Moghaddam et al., 2015). On the other hand, 10%-35% of nongonococcal-nonchlamydia urethritis (NGNCU) are caused by *M. genitalium* (Horner et al., 1993; Sabo et al., 2013; Tsai and Li, 2013). Some interventional experimental studies showed that *M. hominis* and *M. genitalium* adhere to human spermatozoa and lead to sperm agglutination and immobility (Svenstrup et al., 2003; Moretti et al., 2009). The higher incidence of *M. hominis* causes abnormal semen patterns in infertile men as *M. genitalium* does (Al-Sweih et al., 2012).

The involvement of *Mycoplasmas* 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 men (Gdoura et al., 2007; Stojanov et al., 2018). In the referred study, there was no statistical relationship between the presence of genital mycoplasmas and abnormality in seminal volume, motility, vitality, pH, and leukocyte count in infertile men. Although a negative correlation was detected between sperm concentration and *M. genitalium* infection, low sperm concentration and abnormal sperm morphology were reported in the presence of *M. hominis*.

The comparison between the result of the present study and those reported by Gdoura demonstrates that there is no relationship between *mycoplasmas* infection and abnormality in semen analysis in both studies. Nonetheless, the study by Gdoura pointed to the statistical correlation of *M. hominis* infection with low sperm concentration and abnormal sperm morphology. However, in the current study, no correlation was found between *M. hominis* infection and the abnormality of the two semen variable factors the same as other tested parameters (P>0.05).

There are different reports about the prevalence of *M. hominis* and *M. genitalium* in Iran. For instance, Soleimani Rahbar indicated *M. hominis* in 3% of semen specimens of infertile men in Tehran using PCR technique, (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 another study, Ahmadi isolated M. *hominis* from 15% of infertile men in Tehran (Ahmadi et al., 2010). Vosooghi et al. (2013) recognized *M. hominis* in 22% of semen sample of infertile men in Kerman; nonetheless, Bahaabadi et al. (2014) could detect *M. hominis* in 7% of semen specimen of infertile men in this city. Along the same lines, Asgari et al. (2018) demonstrated *M. hominis* in 39% of infertile men in Qom, and it was the highest prevalence among other Iranian reports. In the present study, 7 (7%) isolates were separated by the cultivation method and confirmed in Dienes' staining in the northeast of Iran. None of the isolated strains could ferment glucose and urea, while all of them hydrolyzed arginine, which more often reflects the presence of *M. hominis*, rather than *M. genitalium.* In the present study, the PCR data on a conserved gene that is specific for the *mycoplasma* genus showed that 9% of the samples were positive for *mycoplasma* infection. Moreover, *M. hominis* was present in 8% of semen specimens of infertile men using the multiplex PCR, confirming the PCR and cultivation results.

The foreign studies conducted on *M. hominis* prevalence have reported various results. For example, Zinzendorf detected *M. hominis* in 23.8% of infertile men in Africa using the strip method (Zinzendorf et al., 2008). In another study, Taken (2016) determined *M. hominis* in 3% of infertile men in Turkey using PCR.

It is widely known that *mycoplasmas* are relatively slow growth and fastidious. Therefore, most studies in the field of *mycoplasma* detection have focused on molecular techniques. In fact, molecular methods are developed for the identification of *mycoplasmas* with higher specificity and sensitivity, compared to traditional cultural procedures. Several studies compared the result of cultivation and the PCR method. Stellrecht recognized *M. hominis* in 6% and 8% of semen samples of infertile men using culture and PCR methods (Stellrecht et al., 2004). Furthermore, the results of the present study showed 7% positive *mycoplasma* in cultivation, compared 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 due to the lack of proper procedure for culture (Baseman et al., 2004; Korte et al., 2006). Moghadam et al. (2014) detected *M. genitalium* by the molecular method in 13% of infertile men in Kerman. Safavifar et al. (2015) showed the prevalence of *M. genitalium* by PCR in about 40% of infertile men in Tehran. Nevertheless, in the current 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 merely conducted on infertile men, whereas in some other reports, the prevalence of *M. genitalium* and *M. hominis* are compared between infertile and fertile groups. Liu et al. (2014) indicated the prevalence of *M. hominis* in 11% of infertile men and 9% of fertile men by cultivation method in China. Moreover, Lee et al. (2013) in Korea displayed *M. hominis* in 14% and 6.3% of infertile and fertile men, respectively. In a similar vein, Abusarah et al. (2013) detected *M. genitalium* by PCR in 3.2% and 1.4% of infertile and fertile men in Jordan. (Jensen et al., 1993). Jensen et al. (1996) determined *M. genitalium* in 17% of 99 urogenital tract samples of male patients using *140-kDa Adhesin Protein* gene in PCR and 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., 1993; Jensen et al., 1996). In a study by Horner et al. (1993), 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). There are rare reports with contradictory results. For instance, Al-Sweih et al. (2012) in Kuwait identified *M. hominis* and *M. genitalium* by PCR in 17.1% and 4.7% of infertile men, while these values were reported as 32.4% and 3.2% infertile men.

The heterogeneity of prevalence in the reports can be ascribed to differences in geographic areas, the sensitivity of the identification method, condition of the group (fertile/infertile), sample size, and operator proficiency. Moreover, it can be caused by the transient and unstable nature of mycoplasma infection in the population. However, genital *mycoplasmas* are surface cellular parasites (cell-independent), and the bacteria cannot live long out of the body. However, the prevalence of *M. genitalium* is lower than *M. hominis* in other countries, such as Iran.

It is suggested that in future studies on genital *mycoplasma*, two groups of fertile (healthy) and infertile couples be simultaneously assessed over several years.

Authors' Contribution

Study concept and design: Gh. K., Kh. M. and M. H.
Acquisition of data: Kh. M., H. H. and M. T.
Analysis and interpretation of data: Kh. M., M. H. and K. Gh.
Drafting of the manuscript: Kh. M. and M. H.
Critical revision of the manuscript for important intellectual content: H. M. and Kh. M.
Statistical analysis: M. H. F. M.
Administrative, technical, and material support: M. H., Kh. M. and K. Gh.

Ethics

It is declared that all ethical standards have been respected in the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of the present study.

Grant Support

This study was financially supported by Razi Vaccine and Serum Research Institute, Mashhad, Iran, as well as the Department of Microbiology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

Acknowledgment

We gratefully acknowledge the help and financial support provided by Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Mashhad, Iran, as well as the Department of Microbiology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

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