International Journal of Molecular and Clinical Microbiology 9(2) (2019) 1220-1226

International Journal of
Molecular and Clinical Microbiology

Prevalence of Mycoplasma Infection in the Semen Sample from Infertile Men Referring to Royan Institute in Tehran by Cultural and Molecular Methods

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ARTICLE INFO

Article history:
Received 3 October 2019
Accepted 5 November 2019
Available online 1 December 2019

Keywords:
Infertility,
M. hominis,
U.urealyticum,
Mycofast rapid test

ABSTRACT

Genital Mycoplasma infections have a negative effect on men's reproductive system health and lead to infertility. The aim of this study was to investigate the presence of *Mycoplasma huminis* and *Ureaplasma urealyticum* in semen samples from infertile men using culture and molecular techniques and comparison the sensitivity and specifity of these methods. This descriptive-analytic and cross-sectional study carried out on infertile men referred to the Royan Institute of Infertility Treatment Center. The Rapid Test was done using the Mycofast Rapid kit. DNA extraction from semen was achieved, and then PCR test was performed by the IFG kit (Iranian technology gene). PCR products were transferred to agarose gel 1.5% and electrophoresed. The bands created were transferred to the photographic device and the result was observed. In this study, 147 samples of leukocytospermia were detected in *M. hominis* and *U.urealyticum* .Thirty seven samples (25.2%) were positive for *U.urealyticum* positive in the culture method, and positive samples were positive for *M. huminisis* Of 147 samples, 28 samples (19 Percent) was positive for *U.urealyticum* and 13 samples (8.8%) were positive for *M.hominis*. Considering that Iranian kits were used for PCR in the present study and there was no significant difference in *M.hominis*, it is believed that the Iranian PCR kit has a problem or to prove this French method and replace this method with rapid molecular culture with the method Hard and costly PCRs need more examples to prove this technique.

1. Introduction

Infertility, defined as the inability to achieve pregnancy after 1 year of regular intercourse, affects 15% of couples of reproductive age. As well as attempts to control population and development of contraceptive methods by the WHO in the past decades, it is essential to discover and dissolve the infertility problems. Bacterial infections (25%), hormonal, genetic, and environmental factors (50%), and unknown causes (25%) are the main cause of infertility in human (Khalili et al., 2000). Today, 13-18% of couples in the productive age are infertile all over the world. Also infertility and in correct non-scientific and non-realistic encounter to it, resulted in destruction of families as the community health columns. The 50% of infertile cases is related to men in some way, and 30% of them are absolutely related to them. The 50% of infertile men are curable (Safavifar et al., 2015). There are many causes of male infertility, and

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between 8 to 35% is associated with infection of the genital tract (Keck et al., 1998). Infectious organisms may have a direct negative effect on spermatozoa and a potentially negative impact on fertility (Bachir & Jarvi, 2014), and infertility caused by infection in the male reproductive system is an important topic in contemporary medicine. Infectious agents can affect the performance and abilities of the reproductive system by direct involvement of different parts of urogenital system, preventing the fertility or successful continue of pregnancy, or reducing fertility rate through systemic effects. The risk of infertility after infection directly depends on the patient's age, number of previous episodes of infection, disease severity at the beginning treatment, and the interval between the start of symptoms to initiation of treatment; the risk of infertility will increase with repeated infection (Golshani et al., 2006). Gram negative infections can results in occlusion of epididymis and cause to sperm motility impairments. Also, *Chlamydia trachomatis* and *mycoplasma* infections are involved in infertility and many researches have done to establish the role of these microorganisms. There are a number of pathogens known to contribute to male infertility; the two types that most commonly occur are genital *Ureaplasma* (*U.urealyticum*) and *mycoplasma* (*M.hominis*). They decrease the sperm count mobility and increase the percentage of abnormal sperms (Murtha & Edwards, 2014).

Mycoplasmas and *Ureaplasmas*, belonging to the Mycoplasmataceae family and Mollicutes class, are widely distributed in humans, mammals, birds, reptiles, fish and other vertebrates, as well as in plants (Yoshida et al., 2002). *Mycoplasmas* are which lack cell walls, and this characteristic along with their minute size separates them from other bacteria. These bacteria together with *Neisseria gonorrhoeae* and *C.trachomatis* are considered among the most prevalent sexually transmitted pathogens that have a global distribution (Krause, 2008, Gdoura et al., 2008). These bacteria are also associated with non-gonococcal urethritis, endometritis, bacterial vaginosis, preterm delivery, postpartum, or post-abortal fever, as well as perinatal disorders such as low birth weight and neonatal bacteremia/meningitis (Waites et al., 2009; Kataoka et al.,2006).

Previous methods for detection of mycoplasma, which are involved in infertility, were: serological and microbe culture methods have no sufficient specificity and sensitivity. The PCR is the gold standard method for diagnosis of mycoplasma with high speed and accuracy. The developments of commercially available diagnostic assays, which are based on liquid broth cultures, offer a simpler alternative to conventional culture (Waites et al., 2012). Mycofast Revolution (EliTech Diagnostic, France) used assay for the detection of *M. hominis* and *U.urealyticum*, similar with regards to identification, antimicrobial susceptibility testing and turnaround time (Machado Ldel et al., 2014). There are few studies in Iran to detect these bacteria in semen samples. While identifying these bacteria in infertile men without clinical symptoms are very important. Our study aimed to evaluate this assay's performances for the detection of *M.hominis* and *U.urealyticum* in a hospital cohort.

2. Materials and Methods

2.1. Sampling

This descriptive analytical and cross sectional study was carried out during September 2017 to April 2018 and a total of 147 semen samples of infertile men were collected from patients referred to the Royan Institute in Tehran. Infertile men and WBC more than 1 000 000 in semen sample were the inclusion criteria of this study. Infertility of all specimens was confirmed by performing a spermogram as a common test in the infertility center. Entry criteria for non-exposure to ethoxylated substances and non-use of antibiotics up to three months before sampling and having sexual abstinence a for at least 48 hours and all men with leukocytospermia.

2.2. Mycofast kit

For Mycofast Revolution testing, 300 µl of the seeded saline suspension was dispensed on UMMt (Ureaplasma Mycoplasma medium transport) medium (EliTech Diagnostic, Puteaux, France); 100 µl were dispensed into wells 1 to 20 of the tray, as previously described (Redelinghuys et al., 2013). Fifty µl of Mueller Hinton supplement (S. Mh) (EliTech Diagnostic, Puteaux, France) were dispensed into wells 6 and 7. The wells were covered with two drops of

sterile mineral oil and the tray was incubatedat 37°C for 24-48 hours.

2.3. DNA Extraction

In order to extract the DNA, the frozen sperm samples were first placed at room temperature until the ice being melt. Then, 100 µl of the sample was transferred to a 1.5 ml microtube. The specimens were then placed at 100 °C for 10 minutes inside the bin Marie and, after leaving Ben-Marie, the time wasted to simulate the ambient temperature. In the next step, 400 µl of DNG^+ solution was added to the samples and vortexed for 30 seconds. Subsequently, 350 µL of isopropanol was added to the samples and well stirred by shaking. Completely mixed samples were placed in a centrifuge at 12000 rpm for 10 minutes. After centrifugation, the supernatant was discarded and added to the precipitated residue of 1000 µl of alcohol 70% and shaken well. Then, at 12000 rpm, it was centrifuged for 10 minutes and the fluid was removed again from the vial. The remaining sediment is placed in a 65 °C thermometer for 10 minutes to dry completely. After that, 30 µl of TE buffer was added to the microtube and the samples were well shaken and verified. The specimens were placed in a 65 °C temperature gauge for 5 minutes, and after 5 minutes if left at the end of the vial, it was re-centrifuged for 1 minute (if the sediment was observed again, the fluid transferred to another vial Data that does not have a precipitate). Finally, the extracted DNA sample was freeze for PCR.

2.4. Polymerase Chain Reaction

In this study, IGF kit (Company, model and country) was used to perform PCR reaction. *M.hominis* (PG21) and *U.urealyticum* were used as positive control and sterilized distilled water as negative control. The PCR reaction with a final volume of 25 µl containing 20 µl PCR Master mix containing the Taq DNA Polymerase (0.3 unit) and 5 µl DNA template was performed. Each set of microvials 8th, the first and second wells, respectively, with positive and negative controls, and the rest of the wells tested samples were allocated. continue the PCR reaction at a temperature of 5 minutes and start at 95 °C, then expel for 30 seconds at 94 °C and then continue for 40 cycles, including at 60 °C for 45 seconds, for prolongation at 72 °C for 45 seconds and the final elongation at 72 °C for 5 minutes. PCR products were resolved electrophoretically through agarose gel containing phenol dye. The bands were visualized with UV transilluminator, photographed with the gel documentation system (Doc Print DP-001-FDC, Vilber Loumate, France).

2.5. Statistical analysis

SPSS software version 24.0 was used for data analysis. The results were presented as mean values with deviations (±SDs). Significance of the differences was performed using t-test for equality of means, ANOVA correlation, Descriptive, Frequency and chi-squared test. A p-value of <0.05 was considered.

3. Results

In this study, from 147 cases of leukocytospermia, 37 samples (25.2%) were positive by mycofast revolution (Elitech) method (Fig. 3). By using PCR method, 119 samples (81%) were negative for *U. urealyticum* negative and 28 (19%) were positive.

The presence of bands of 429 and 334 base pair in the samples was shown. These specific bands represent the species of *U. urealyticum* and *M.humicis*, respectively (Figures 1 and 2).

By evaluating the total number of samples, positive and negative number in both methods, there was a statistically significant difference in the two methods ($P <0.001$) (Table 1).

Of 147 patients with leukocytospermia, 146 samples *M. hominis* (99.3%) were negative and 1 positive for mycofast revolution (Elitech) culture, and 147 samples were analyzed by molecular PCR method, 134 samples (91.2%) were negative and 13 samples (8.8%) were positive. There was no significant difference in the two methods in evaluating the total number of samples in both methods and positive and negative numbers in both methods ($P > 0.001$) (Table 2).

In table 2, chi-square test has been performed and the results revealed that there is not any significant difference between two methods.

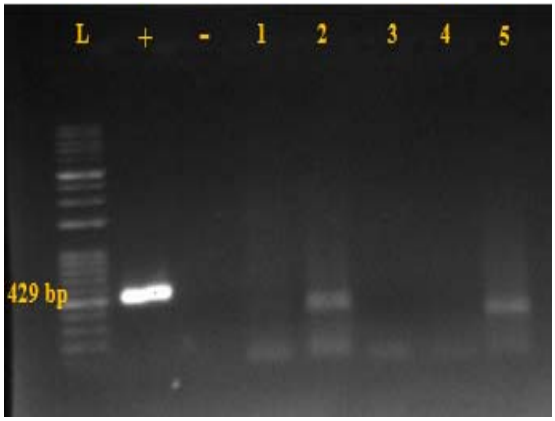

Figure 1. Electrophoresis of the PCR product of *U.urealyticum*. L: Leader 1bp. (+): Positive control. (-): negative control. Columns 1 to 5: *U.urealyticum* DNA samples (429 bp).

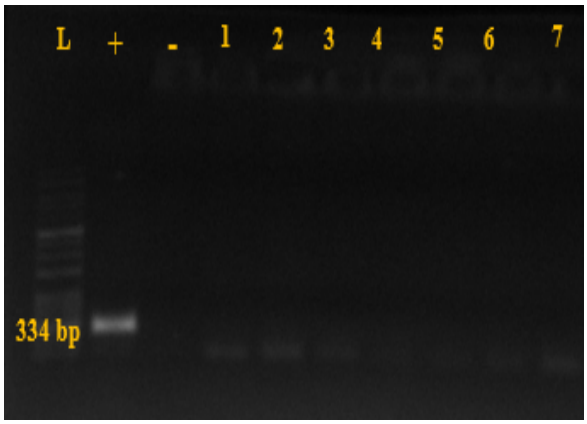

Figure 2. PCR product electrophoresis *M.hominis*. L: Leader bp 1. (+): Positive control. (-): negative control. Columns 1 through 7: *M.hominis* DNA samples (334 bp).

Figure 3. Positive results of *U.urealyticum* on the mycofast revolution kit

Table 1. the rate of positive sample in diagnosis of *U.urealyticum* and *M.humicis* in semen sample of infertile men using Mycofast Rapid and PCR techniques.

	Mycofast Rapid	PCR
U.urealyticum	37 (25.2%)	28 (19.0%)
M.humicis	1 (0.68%)	13 (8.8%)

Table 2. Chi-Pearson test using PCR method and mycofast kit method on *U. urealyticum*

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	13.391	1	.000		
Continuity Correction	11.640	1	.001		
Likelihood Ratio	11.927	1	.001		
Fisher's Exact Test				.001	.001
Linear-by-Linear Association	13.300	1	.000		
N of Valid Cases	147				

Table 3. Chi-Pearson test using PCR method and mycofast kit method on *M.hominis*

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.107	1	.293		
Continuity Correction	.635	1	.425		
Likelihood Ratio	1.194	1	.275		
Fisher's Exact Test				.444	.216
Linear-by-Linear Association	1.100	1	.294		
N of Valid Cases	147				

4. Discussion

Semen infections, especially mycoplasma, are one of the most important causes of infertility in men. Mycoplasma bacteria are very sensitive to environmental conditions such as pH, temperature and compounds in the culture medium due to lack of cell wall. Therefore, when sampling and transferring to the laboratory, the bacteria may be weakened, destroyed or in the environment cultivars cannot be retrieved (Najar Pirayeh and Al Yasin, 2005). Therefore, bacterial culture method is expensive, time consuming, difficult and low sensitivity in comparison with molecular methods such as PCR. However, the PCR method does not require a live bacterium. Therefore, the bacteria are less affected by the sampling and transfer steps. In this study, 147 samples of leukocytospermia were used for diagnosis of *M. hominis* and urea plasma urea lithium by bacterial culture of mycofasts and PCR methods. Considering the high number of *U. urealyticum* samples in the culture and molecular culture, there was a significant difference for this bacterium. Due to the high number of negative samples for *M. hominis* in bacterial culture method and more positive samples for this species in the molecular method, there was not a significant difference for this bacterium with these two methods.

In the study of Amir Mozafari et al (2008), in line with the present study, the results showed that a high proportion of infertile men were infected with *M. hominis* bacteria and urea plasma urea lithium, and given that in the case

of Failure to diagnose and treat these infections can lead to PID and infertility, detection and isolation of these bacteria through culture in infertile men without clinical symptoms seems necessary (Mozafari et al., 2006).

Regarding the prevalence of genital mycoplasmas, there are different reports and statistics, in which the prevalence of Urea lithium urea plasma in semen samples of infertile men varies from 5% to 42% in various articles (Wang et al. 2006). This wide range is related to different diagnostic methods and tests used for the study of different populations (Gdoura et al., 2008). In the present study, the prevalence of *U. urealyticum* in the infertile men's semen was 25.2% and 25% by PCR method, respectively, which was within the scope of the above studies.

This study was similar to the study done in Ahvaz in 2003, and the positive results of this study were in this age group. But these studies are contrary to studies conducted in the United States, Greece and Canada (Schlicht et al., 2004, Petrikkos et al., 2007) the total separation of these organisms by cultivation was higher than 50%. This difference can be due to differences in laboratory methods, geographical and cultural characteristics of these countries compared to Iran, and also because the prevalence of these organisms is high in people with multiple sexual partners (Amirmozafari et al., 2008).

Also, in the study of Dhawan et al (2006), The isolation of *M. hominis* and *U.urealyticum* was 31.18% and 7.76%, respectively, which is similar to the studies conducted in India and Turkey, which may be due to the close proximity of their culture With Iran (Dhawan et al., 2006). However, the prevalence of these organisms in these studies, contrary to studies by Schlicht et al (year) in the United States, was 26% for *M. hominis* and 54% for *U. urealyticum*, and in Greece *M. hominis* was 18% and *U. urealyticum* was 48%. The percentage of these differences can also be due to the above reasons (Schlicht et al., 2004).

In another study conducted by Niaakan et al in the Royan Research Institute on semen samples, the prevalence *of M.hominis* in infertile men was 17.5% in comparison to the control group (5%) (Niakan et al., 2009), which was Compared to the results of the present study (0.7%), the rapid mycofast culture method showed a higher rate; of course, in the study, the number of subjects was limited (40 infertile men and 40 fertilized men) and thus cannot accurately measure the prevalence of these microorganisms.

In a study by Wang et al. In China, the prevalence of *U. urealyticum* was 39.31% higher in comparison with our findings (Wang et al., 2005). Microbial screening is necessary for infertile men, especially at younger ages. Also, given that in some clinics, microbial studies are performed only on samples that have high leukocyte levels, while. Studies have shown that the presence of leukocytes in seminal fluid is not a reliable indicator for the prediction of mycoplasma infections. Therefore, microbial screening for all infertile men should be performed as a routine test in the presence or absence of leukocytes in seminal fluid, and in case of contamination with these bacteria, the treatment of couples should be carried out simultaneously. Therefore, useful rapid mycofast culture method, which is a cheaper and more specific method. Also, informing people, especially young people, about genital and urinary tract infections and ways to protect sexual health is inevitable and can be an important part of the program for controlling sexually transmitted diseases.

Refereces

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