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ABSTRACT

Objective: This study aim to diagnosis the types of bacteria Ureaplasmas spp and Mycoplasma hominis by screening for multiple banding antigen (MBA) gene and 16S rRNA respectively in seminal fluid by using the multiplex-PCR technique.

Methodology: The study included, a total (106) semen fluid samples, were collected from 86 infertile men which have been diagnosed by specialized clinicians specialization in infertility and 20 other semen fluid samples from healthy men used as control through six months (November 2013 until April 2014). All these Date was analyzed by using spss(T-test) program. All these samples were obtained from subjects who attended to center infertility in medical city at Al-Sadar in Najaf province.

Results: The result of the multiplex-PCR revealed positive results in 13(15.1%) of the 86 seminal fluid samples from infertile patients which represented M.hominis (5.8%), U.urealyticum (5.8%) and U.parvum(3.5%).

Conclusion: in light of the results obtained this study we concludes that the efficiency of multiplex-PCR large and rapid in diagnosis of Ureaplasmas spp and Mycoplasma hominis in seminal fluid.

Recommendation: the study of detection of other microorganism (viruses, fungi, Chlamydia and Neisseria gonorrhoea) in men infertility.

Key words: Multiplex-PCR, Mycoplasma, Ureaplasma, Men, Infertility.

INTRODUCTION

Mycoplasmas and ureaplasmas, belonging to the family Mycoplasmataceae and Mollicutes class, are widely distributed in humans, mammals, birds, reptiles, fish, and other vertebrates as well as in plants. They are the smallest free living organism known on the planet able to multiply autonomously. Mycoplasmas do not have cell-wall, so can take many different forms by the absence of precursors of peptidoglycan which make them difficult to identify. There is an attachment organelle at the tip of filamentous. Fried-egg-shaped colonies are seen on agar and have a double-stranded DNA and divide by binary fission. Most interesting is the use of the universal stop codon UGA as tryptophan codon. It is so difficult to cultivate them in the laboratory and are often missed as pathogenic causes of disease. Mycoplasmas usually require cholesterol for growth and membrane function and are filterable through the usual bacteriological filters. Resistance of Mycoplasmal RNA
polymerase to rifampicin is another property distinguishing mycoplasmas from the conventional bacteria. Mycoplasmas constitute a large group of microorganisms but only a few species, i.e., Mycoplasma and Ureaplasma species, are pathogenic for humans, where they mainly inhabit the mucous membranes of the respiratory tract and genitourinary system. Three species have been isolated from the mucosal membrane of the genitourinary tract: Mycoplasma hominis, Ureaplasma urealyticum and recently discovered Mycoplasma genitalium (4). The role of mycoplasmas in the etiopathogenesis of inflammatory states of the genitourinary organ is still a subject of controversy. Their presence has been associated with non-gonococcal urethritis, vaginitis, cervicitis, pelvic inflammatory disease (PID) and pathology of pregnancy and newborns. M. hominis has been reported in 58-76% of women with bacterial vaginosis (BV) and is the only genital mycoplasma which is consistently more often isolated from vaginal swabs in women with BV than those without (5). Genital mycoplasmas and ureaplasmas seem to be widespread among the male partners of infertile couples in Iraq. The aim of this study is to determine of Mycoplasma hominis and Ureaplasma spp from infertile men in Al-Najaf province by using duplex primer assays.

MATERIAL AND METHOD

Sampling:
A total at 106 semen fluid samples were collected from 86 infertile men and 20 other semen fluid samples from healthy men used as control. All these samples were obtained from subjects who attended to center infertility in medical city at Al-sader Hospital in Najaf province through a period at 6 months (from November 2013 to April 2014). Swabs were inserted in tubes containing special transport medium to maintain the swabs. Each specimen was coated in ice bag until be taken to the laboratory for bacteriological analysis. Specimens were first incubated at 37°C for 30 minutes, then a loop-full from each sample was transferred to transport media (H-I broth) according to (5) for detection the Mycoplasma hominis, Ureaplasma urealyticum and Ureaplasma parvum respectively (7,8). After incubated this tube in anaerobic jar at 37°C for 24-72 hours in the laboratory.

Molecular assay: Molecular experiments included the extraction and amplification of Mycoplasma and Ureaplasma spp. DNA. For extracted DNA by G-spin™ total DNA extraction kit (intron) according manufacture company. Multiplex PCR was performed with primers specific for highly conserved regions in the multiple band antigen (MBA) gene of Ureaplasma spp and the 16S rRNA gene of M. hominis (9,10) show in table (1). Preparation of primers suspension the DNA primers were resuspended by dissolving the lyophilized primers provided by (Integrated DNA Technologies -USA) after spinning down with TBE buffer depending on manufacturer instruction as stock suspension as recommended by (11,12) show in table (1).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Size of amplified product (bp)</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. hominis</td>
<td>RNAH1 RNAH2</td>
<td>CAATGGCTAATGCCGGATACGC GTACCGTACAGTGCAT</td>
<td>334</td>
<td>16S rRNA</td>
</tr>
<tr>
<td></td>
<td>UMS125 UMA226</td>
<td>GTATTTGCAATCTTTATATGTTTTTCG CAGCTGATGTAAGTGCAGCATTAAAT TC</td>
<td>403 or 448</td>
<td>MB antigen gene</td>
</tr>
</tbody>
</table>

The reaction mixture: Amplification of DNA was carried out in a final volume of 25 μl containing the contents according (9). All reactions were performed in a Gene amplification PCR System 9600 Thermocycler (Perkin-Elmer, Norwalk, Conn.) under the following
conditions: 1 cycle of 10 min at 95°C, followed by 35, two-step cycles of 95°C for 15 s and 60°C for 60 s, followed by 5 min at 72°C.

Detection M. hominis and U. urealyticum by Multiplex-PCR: Amplification of specific gene 16S rRNA for M. hominis and multiple banding antigen (MBA) gene for U. urealyticum made according to method recommended by \(^{(13)}\) show in table (1).

Detection of amplified products by agarose gel electrophoresis: Successful PCR amplification was confirmed by agarose gel electrophoresis as mentioned by \(^{(14)}\). Agarose gel was prepared by dissolving 2 gm of agarose powder in 100 ml of TBE buffer 1X(pH:8) in boiling water bath, allowed to cool to 45°C and ethidium bromide at the concentration of 0.5mg/ml was added. The comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber filled with TBE buffer that covered the surface of the gel, and 10µl of each DNA sample was transferred into the wells in agarose gel, and in one well we put the 10µl of DNA ladder. The electric current was allowed at 65 volt for 90 min. UV transilluminater was used for the observation of DNA bands, and gel was photographed using a digital camera.

**Statistical analysis**

T-Test recommended by \(^{(15)}\) was used for statistical analysis to show if there is any significant differences between results.

**RESULTS:**

The PCR amplified assay revealed positive results in 13(15.1%) of the 86 semen samples from infertile patients which represented M. hominis 5(5.8%) samples, U. urealyticum 5(5.8%) samples and U.parvum 3(3.5%) as shown in table (2).

**Table (2) percentage of isolated mycoplasmas by using multiplex PCR**

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Single isolates (n)</th>
<th>Mixed isolates (n)</th>
<th>Total isolates (n) (%)</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycoplasma hominis</td>
<td>4</td>
<td>1*</td>
<td>5(5.8%)</td>
<td>334bp</td>
</tr>
<tr>
<td>Ureaplasma urealyticum</td>
<td>4</td>
<td>1*</td>
<td>5(5.8%)</td>
<td>448bp</td>
</tr>
<tr>
<td>Ureaplasma pavum</td>
<td>1</td>
<td></td>
<td>3(3.5%)</td>
<td>403bp</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>9</td>
<td>4</td>
<td>13(15.1%)</td>
<td></td>
</tr>
</tbody>
</table>

Table (2) shows that two isolates of U.pavum were mixed with one isolate of U.urealyticum and one isolated M.hominis.
Table (3) Means ± standard deviations (SD) parameter semen of considered variables in each group of patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>M. hominis</th>
<th>U. Urealyticum</th>
<th>U. Parvum</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count</td>
<td>21.0±25.3***</td>
<td>51.80±56.6***</td>
<td>35.0±49.4***</td>
<td>85.0±11.8</td>
</tr>
<tr>
<td>Agglutination</td>
<td>6.0±9.2***</td>
<td>90.0±13.41***</td>
<td>5.0±7.07***</td>
<td>0</td>
</tr>
<tr>
<td>Leukocyte</td>
<td>17.0±8.45***</td>
<td>23.80±13.7***</td>
<td>25.0±7.07***</td>
<td>0</td>
</tr>
<tr>
<td>Motile</td>
<td>8.67±10.32</td>
<td>17.0±21.67***</td>
<td>5.0±7.07***</td>
<td>68.95±5.08</td>
</tr>
<tr>
<td>Sluggish</td>
<td>10.4±8.64*</td>
<td>13.0±17.17*</td>
<td>10.0±14.14*</td>
<td>14.30±6.91</td>
</tr>
<tr>
<td>Immotile</td>
<td>47.3±37.3***</td>
<td>50.0±38.56**</td>
<td>35.0±49.4**</td>
<td>16.75±6.83</td>
</tr>
<tr>
<td>Normal</td>
<td>17.8±14.6***</td>
<td>30.80±24.8***</td>
<td>7.5±70.6***</td>
<td>95.55±4.47</td>
</tr>
<tr>
<td>Abnormal</td>
<td>48.8±38.2***</td>
<td>49.2±32.19***</td>
<td>42.5±60.1**</td>
<td>4.45±4.47</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, ***P<0.005.

This table explains the different variables in each group with those of the control group (20 fertile individuals). The number of patients considered for each group is reported in parentheses.

Figure (1) A photograph of Ethidium bromide stained by 2% agarose gel showing in gel electrophoresis for multiplex PCR product (334bp-M. hominis-403-U. parvum,448bp-U. urealyticum.)

Figure (1) Show M: 100 bp standard size reference marker. Lane 1, lane 4 and lane 6, M. hominis positive results, Lane 2, lane 5 and lane 7 and lane 8 Ureaplasma urealyticum positive results and Lane 3, lane 4 and lane 5 Ureaplasma parvum positive results. Lane 4 are mixed between M. hominis and Ureaplasma parvum and lane 5 are mixed between Ureaplasma urealyticum and Ureaplasma parvum.
DISCUSSION

Other fastidious, non-cultivable bacteria are also included in this study using the multiplex polymerase chain reaction (Multiplex-PCR) technique for the detection of these bacteria accordingly Mycoplasma hominis represents, Ureaplasmaurealyticum and Ureaplasmapavum. However, this reliability is limited by PCR technique which has the advantages of being easy and rapid in detection these organisms, but the matter is, there are no commercially available molecular tests, therefore, efforts for new techniques to detect Mycoplasma and Ureaplasma are ongoing at present time since various procedures based on DNA amplification have been developed for detection of M. pneumonia.

Various phenotypic and molecular methods have been described by other research to distinguish the two main groups of human urea plasmas (formerly two biovars of U. urealyticum, now proposed species U. parvum and U. urealyticum). Our results showed that there is a homology between sequences of the multiple binding antigen genes(MBA) subunits of biovars within species was used subtyping two biovars by using universal primer which distinguished between them depending on the specific product 403bp for U. parvum and 448 bp for U. urealyticum and 338bp for M.hominis that show in figure (1). Accordingly, it can be concluded that the results be achieved by this study, can be a confirmation, for other studies(14).

The comparison of semen characteristics between infected and non-infected men showed that motile spermatozoa and viability were lower when present the genital Mycoplasma and genital urea plasma. M. hominis could cause extra genital and systemic infections in people suffering from immunodeficiency syndromes (16). Studies in human reproduction and in vitro fertilization showed that M. hominis adheres to or invades the human sperm cells, showing no apparent damage and significant effects on sperm count, motility and fertilization. With respect to women, mycoplasmas cause infertility by impairing normal sperm function in the cervix and causing endometritis and tubal damage. M.hominis invades human sperms and result in non-apparent or subtle damage and might have implication for long-term infertility (17). U. urealyticum can adhere to the sperm membrane, thereby potentially causing gamete dysfunction and enhance the adverse effects of superoxide and hydrogen peroxide produced by the organism, with subsequent spermatozoan hyper production of reactive oxygen species(ROS)(18). The ROS induce lipid peroxidation, which reduces membrane fluidity and sperm fertilization capability, and may be the mechanism by which U.urealyticum impairs sperm function(19). Other works have reported that the presence of U. urealyticumin semen was related to a decrease in sperm concentration (20). Show in table (2).

Other study(21) have investigated the occurrence of M. hominisin first-voided urine specimens from young men. They found that the occurrence of M. hominis was 4%. They concluded that there is a need to use PCR to determine the of Mycoplasmas role in STDs. More over Yoshida and coworker, stated that U.urealyticum strongly associated with males urethritis by using PCR technique. Recently, PCR technique was successfully used in Iraq by our team work(22). Other study the most common bacterial types isolated from patients with bacteriospermia were C. trachomatis (41.4%), U. urealyticum(15.5%) and M. hominis(10.3%) (23).

Enhanced sensitivity for genital Mycoplasma detection with PCR is consistent with the literature (24,25). This finding is not surprising given the fact that mycoplasmas are labile organisms lacking a cell wall. PCR has an advantage in that it can still detect nonviable organisms.
Another advantage of the multiplex PCR is that the presence of other microorganisms does not interfere with testing. Indeed, the specimen excluded from study due to bacterial overgrowth was actually positive for *Ureaplasma* by PCR. Finally, the multiplex PCR is a relatively rapid assay that can be performed in less than 8 h. Isolation by culture may take 2 to 5 days to obtain a result for *Ureaplasma spp.* and *M. hominis* The rapid detection of genital mycoplasmas and *Ureaplasma spp.* is very important, particularly in the management of pelvic inflammation disease, infertility, in whom these organisms are a significant cause of meningitis, respiratory disease, in and death.

**CONCLUSION**

Both, *Ureaplasmaspp* and *Mycoplasma hominis* are frequently infect genitourinary tract of men. *U. urealyticum* seems to be more frequent. Multiplex- PCR assay is fully efficient in detection of genital mycoplasmas and/or *Ureaplasmaspp*, consequently, rapid detection, confirmed identification and reliable diagnosis of these organisms.

**RECOMMENDATIONS:**

1- Further study can be designed to detect the role of other organism (viruses, fungi, Chlamydia and Neisseria gonorrhea) in men infertility.
2- A achievement further study on Mycoplasma and Urea plasma to determine the exact effect on spermatozoa.

**REFERENCES:**


