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Assessment of the Frequency and Molecular Identity of Mycoplasma Species in Women with a History of Idiopathic Recurrent Abortion: A Case Control Study

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ABSTRACT

Background: Abortion is the most common complication in the first trimester of pregnancy. Infections are reported as the etiology of abortions. Some species of Mycoplasmas are seen in the lower genital tract. The aim of this study was to investigate the frequency and the molecular identity of Mycoplasma species in women with a history of idiopathic recurrent abortion.

Methods: This study was carried out on 68 women with a history of recurrent abortion**.** Detecting 16S rRNA to identify the infection of Mycoplasma was done by polymerase chain reaction (PCR) on cervical samples. Furthermore, the sequencing of positive samples was performed to identify the species of Mycoplasma.

Results: The results showed that only one woman with history of three times abortions was infected by Mycoplasma. Her gestational age was six weeks and she had apparent signs of infection. After sequencing, *Mycoplasma genitalium* was diagnosed as the infectious agent. The prevalence rate of Mycoplasma was 1.47% and 0% in the case and control groups, respectively. There was no significant association between abortion and Mycoplasma infection.

Conclusion: It seems more studies are necessary to evaluate the relationship between Mycoplasma infection and abortion. Moreover the increase in the level of public health due to better education and the improvement of contraceptive methods compared to the past has created a significant reduction in Mycoplasma infection.

Introduction

Abortion is the most frequent complication in pregnancy. Approximately, 70% of the human fertilizations are not able to acquire life opportunity and nearly 50% of the human fertilizations would be failed before the next menstrual cycle. Recently, investigation about human chorionic gonadotropin (HCG) has been demonstrated that the rate of abortion is 31% after blastocyst nesting.[1] Also the abortion rate has been reported as 15% before the 20th week of pregnancy. A study, estimated the abortion rate between 10% and 50%.[2] Repeated pregnancy loss (RPL) is defined as the history of at least three abortions before the 20th week of gestational age. It has been estimated that the rate of RPL is 1 in 300 couples.[1] In addition, having a history of abortion, increases the rate of abortion in next pregnancies, so that it would be 24% after one abortion and it might increase to 32% - 53% after 3-6 abortions.[2] However, most of the time, the rate of a prosperous pregnancy is estimated 60% in these cases.[3] RPL is affected by various factors such as abnormal fetal chromosomes, teratogenic agents, reproductive problems and maternal age as well as the systematic factors such as endocrine problems, infections, immunologic factors, thrombophilia anatomic disorders and other factors.[4] Mostly, the etiology of abortions remains unknown.[5] Although infections are rare, they are considered as a primary cause of some abortions.[6] Infections of the reproductive organs, can theoretically cause inflammation and then blockage and scarring of the fallopian tubes.[6,7] A reason for premature abortion which is mediated by infectious agents is the activity of immune system in response to harmful microorganisms.[8] Parovirus B19, *Treponema pallidum*, Paramyxovirus, *Herpes simplex*, Rubella, Cytomegalovirus, *Chlamydia trachomatis*, Streptococcus and Mycoplasmas which are attributed to induce infection and inflammation, were the primary reasons of abortion in some infected women.[9] Although Mycoplasmas are usually well known as a normal flora of the body in the mucosal areas, some species of them are involved in respiratory and urinary system diseases.

Among the infectious agents, three species of Mycoplasma genus such as *Mycoplasma hominis*, *Ureaplasma urealyticum*, *and Mycoplasma genitalium* are the common bacteria which lead to urinary and genitourinary tract infections in both men and women.[7,10] The functional mechanism of *Mycoplasma hominis* is inhibiting the blastocyst implantation through producing neuraminidase and also making ovum inefficient.

Mycoplasmas are small bacteria (125-300 nm) with the genome size of 600-1350 kbp which are enclosed by a three-layer membrane (8 to 12 nm) without cell wall.[11] Lack of cell wall in these bacteria decreases the ability of bacteria to be stained, therefore, they cannot be detected through common bacteriological methods. Nucleic acid-based techniques such as polymerase chain reaction (PCR) are the best way to diagnose infection rather than culture-based methods and serological tests. Methods based on nucleic acid have several positive aspects including rapid results to improve treatment of the patients, low diagnostic limitations and specific diagnosis of the organism as well as designing protocols for antibiotic treatment.[12] The purpose of this study was to investigate the frequency and molecular identity of Mycoplasma species in women with a history of recurrent abortions.

Materials and Methods

Subjects: This case–control study consisted of 68 women with history of recurrent abortions (at least three times). All the patients were admitted to the infertility center in Yazd, Iran. Besides, 68 samples as control group were obtained from women with no history of abortion. We excluded subjects with anatomical problems of uterus,

endocrine disorders, diabetes, hypothyroidism and immunological disorders.

Both case and control groups were divided into sub-groups, according to the number of live births[1-3] and abortions[2,3], levels of education (illiterate/high school/diploma/more than a diploma), methods of contraception (natural/other methods) and clinical symptoms of infection (with/without symptoms).

Cervical sampling for Chlamydia was performed by specific cervical brush and samples were stored in phosphate buffered saline (PBS) at -80°C.

DNA extraction: Mycoplasma genomic DNA was extracted for PCR using RIBO-pre (K1-11-100-CE) kit (AmpliSens). Briefly, samples in PBS were centrifuged and followed by adding both 17 µL lysis reagent and 400 µL lysis buffer to sediment. Samples were then centrifuged after incubating at 65°C for 1 hour and several consecutive vortexing. Next, supernatant was collected and 25 µL Universal Sorbent was added to solution followed by several consecutive vortexing before being centrifuged again. In the next stage, in order to solve sediment, 300 µL washing solution was added. After several consecutive centrifuging and washing, samples were incubated at 65°C for 5 min. Following this, 50 µL TE-buffer was added to sediment. Finally, extracted DNA was obtained after incubating at 65°C for 5 min. Extracted DNA was stored at -20°C until usage for PCR. Quality and quantity of extracted DNA were detected by 1 % agarose gel and spectrophotometer.

Amplifying 16SrRNA gene: In current study, a fragment of Mycoplasma *16SrRNA* gene was used to detect Mycoplasma genus (Figure 1). *16 SrRNA* gene was amplified to form fragment of 222bp by using primers 5'-GGGAGCAAACAGGATTAGATACC3' and 5'TGCACCATCTGTCAYTCTGTTAACCT-3' as forward and reverse primers, respectively. Nucleotide Y in reverse primer shows either nucleotide C or nucleotide T. It was used as a mix of both primers. Primers were designed by Perprimer and then approved by Gene Runner software (Pishgam. Co).

>KY064173.1 Uncultured Mycoplasma sp. clone Rubyspira_204C_D6 16S ribosomal RNA gene, partial sequence
TCCCGGAATTATTGGGCGTAAAGCGTTCGTAGGTGGTTTGTTAAGTCTGAAGTTAAAGCCTGGGGCTCAA
CCCCAGCCCGCTTCGGATACTGGCAGACTAGAGTTATAGAGAGGTCGGTAGAACTCCATGTGAAGCGGTG
GAATGCGTAGATATATGGAAGAATACCAATGGCGAAGGCAGCCGACTGGATATATACTGACACTGAGGGA
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGATCATTAGCTGG
TGGATTTAATCACCGGCGCAGCTAACGCGTTAAATGATCCGCCTGAGTAGTATGCTCGCAAGAGTGAAAC
TTAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTTGAAGATACGCGTAGAACC
TTACCCACTCTTGACATCCTCTGCAACGCTATAGAGATATAGTTGAGGTTAACAGAGTGACAGATGGTGC
ATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTAGGTTAAGTCCTGCAACGAGCGCAACCCCTGTCTTTAG
TTACCATCATTAAGTTGGGGACTCTAGAGAGACTGCCTGGGTAACCAGGAGGAAGGTGGGGACGACGTCA
AATCATCATGCCTCTTACGAGTGGGGCAACACACGTGCTACAATGGCTGATACAAAGGGATGCGAAATGG
CAACATTTAGCTAACCTCAAAAAATCAGTCTCAGTTCGGATTGGAGTCTGCAACTCGACTCCATGAAGTT
GGAATCGCTAGTAATCGTAGGTCAGCTACACTACGGTGAATACGTTCTCGGGTCTTGTACACACCGCCCG
TCACACCACGGGAGTTGGTAATGCCCGAAGCCGG

Figure 1. A Fragment of Mycoplasma 16S rRNA Gene Used to Detect Mycoplasma Genus

The PCR mixture for 20µL volume of reaction was 25 ng DNA sample, 1.5 mM MgCl2, 0.8 mM dNTP, 0.2 µM forward and reverse primer, reaction buffer 1X and 1U Taq DNA polymerase. Table 1 presents the PCR condition for amplifying *16S rRNA* gene.

Table 1. The PCR Condition for Amplifying 16S rRNA Gene

Program PCR		Time	Temperature (C°)
Primary denaturation		5 min	95
(32 cycles)	Denaturation	60 s	95
	Annealing	60 s	56.7
	Extension	60 s	72
Final extension		4 min	72

Then, the PCR products were separated on a 1.5% agarose gel with TBE reaction buffer 0.5X.

DNA sequencing: The PCR products and forward/reverse primers were sequenced to evaluate the accuracy of the products and to determine the species of Mycoplasma (Macrogen. Co).

Statistical analysis: Statistical analysis was performed by SPSS for Windows (SPSS Inc., Chicago, IL). One-way ANOVA was used to evaluate statistical differences between case and control groups. *P* values less than 0.05 were considered as statistical significant differences.

Table 2. Demographic Data of the Patient and Control Groups

Demographic data	Control group (%)	Patient group (%)	P
Number of participation	68	68	
Infection with Mycoplasma	0	1 (1.47)	
Age	30.8 ± 4.1	28 ± 5.4	
Age of abortion (Week)	-	11.4 ± 6.3	
Number of births		-	
2	43 (63.2)		
3	21 (30.9)		
4	4 (5.9)		
Number of abortions	-		0.57
3		46 (67.6)	
4		16 (23.5)	
5		4 (5.9)	
6		1 (1.5)	
7		1 (1.5)	
Level of education			0.95
Illiterate	1 (1.4)	0 (0)	
High school	5 (7.3)	3 (4.4)	
Diploma	12 (17.6)	4 (5.9)	
More than a diploma	50 (73.5)	61 (89.7)	
Method of contraception			0.61
Natural	26 (38.2)	14 (20.6)	
Other methods	42 (61.8)	3 (4.4)	
Missing	0 (0)	51 (75)	
Clinical symptoms of infection			0.096
With symptoms	14 (20.6)	18 (26.5)	
Without symptoms	41 (60.3)	50 (73.5)	
Missing	13 (19.1)	0 (0)	

Results

General characteristics of the cases and controls have been given in Table 2. The mean age of the cases and the controls were 28 ± 5.4 (range: 19 to 45) and 30.8 ± 4.1 years (range: 29 to 39), respectively. Also, the mean gestational age of abortion in case group was 11.4 ± 6.3 weeks (range: 1 to 32 weeks).

In the control group, 63.2% and 36.8% of women had two and three/four live births without any abortions, respectively. Moreover, in the case group, women had 3-7 abortions in which nearly 68% of them had 3 abortions with no live birth.

Results from PCR and gel electrophoresis indicated that only one woman in the case group has been infected by Mycoplasma, whereas none of the women in control group showed contamination (Figure 2). It demonstrated that the frequency of Mycoplasma in case and control groups were 1.47% and 0%, respectively.

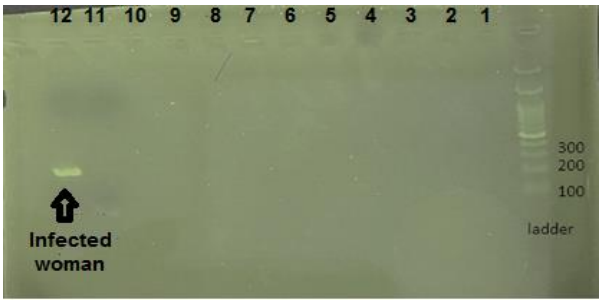

Figure 2. PCR Data on Electrophoresis gel for 16S rRNA Gene

The PCR product from the infected sample was sequenced to determine the species of Mycoplasma (Macrogene Co). Results from sequencing and blasting indicated that it had the highest identity (97%) with *Mycoplasma genitalium* among other species of Mycoplasma genus (Figure 3).

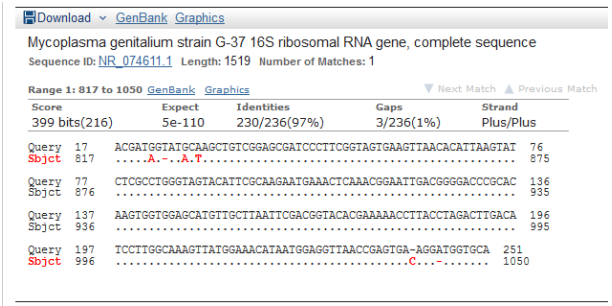

Figure 3. Sequence Blasting for Infected Sample

Based on the statistical analysis, there was no significant association between recurrent abortion and Mycoplasma infection ($P = 0.57$).

Furthermore, data analysis showed no significant difference between either level of education ($P = 0.95$) or method of contraception ($P = 0.61$) with Mycoplasma infection.

Discussion

Age, levels of education and methods of contraception were considered as critical variables in our study. Increasing the age raises the risk of developing congenital diseases such as trisomy in the fetus. The level of education was associated with better personal hygiene and lower risk of infections.[13]

Beside of internal factors such as endocrine problems, immunologic factors and anatomic disorders, external factors such as infectious agents also affect maternal reproductive system and lead to inflammatory reactions in the fetus.[14] Abortions are the most common complication in a pregnancy. Among all recognized reasons of abortion, infections which are caused by bacteria, viruses and fungi are considered as a significant cause of some abortions. One principle issue with this complication is contamination with Mycoplasma genus. Some species such as *Mycoplasma hominis, Ureaplasma urealyticum, and Mycoplasma genitalium* are involved in the genitourinary system.[10] Infectious agents affect the abortions through blockage and scarring of the fallopian tubes.[6,15]

There are several ways to detect Mycoplasmas including culture methods, serological and molecular techniques.[16] Sampling from cervix is the best way to get the samples in comparing with blood, semen, umbilical cord and placenta. Mycoplasma does not enter the bloodstream and does not cause bacteremia unless the immune system is defective or suppressed by certain drugs. On the other hand, mostly, the placenta and umbilical cord are not infected due to the resistance of the maternal and fetal immune system.[17]

Restricted biosynthetic and metabolic activity of Mycoplasma has made culturing as difficult, expensive and time consuming detecting method. Mycoplasma culturing outside the body requires complex, specialized factors and specific nutritional supplements.[13] On the other hand, antibodies which are secreted through Mycoplasma infection, are detectable by various serological tests such as complement fixation, haemagglutination inhibition, hemolysis, indirect immunofluorescence, DNA-Probe, micro immunofluorescence or Elisa. One of the most important problems with detecting by serological tests is creating a false positive result due to occurrence of cross-reactions.[18] Moreover, lack of cell wall in these bacteria decreases the ability of bacteria to be stained; therefore, Mycoplasmas cannot be detected through common bacteriological methods. In this study, PCR was used for diagnosing Mycoplasma genus. Nucleic acid-based techniques such as PCR are the best way to diagnose Mycoplasmas in laboratory rather than culture-based or serological methods.[12]

Considered as a conserved gene in Mycoplasmas, 16S rRNA was used to detect species of Mycoplasma genus.[19] Data from sequencing and blasting detected *Mycoplasma genitalium* in only one woman among case group, whereas none of the woman in control group was infected by mycoplasma (Figure 1). Although the effects of *Mycoplasma genitalium* on abortions are being investigated, some recent studies have reported negative effects of *Mycoplasma genitalium* on pregnancy.[20]

In our study, the frequency rate of Mycoplasma was 1.47%. It was a long with a

recent study which reported the prevalence rate of 1.5% for *Mycoplasma genitalium*as through real-time PCR.[21] In the other study, *Mycoplasma genitalium* was detected in 6.2% of women with recurrent abortions. It indicated that contamination with *Mycoplasma genitalium* was not the only cause of abortion.[22] Also, there wasn't significant association between number of abortions and infection. In our study, there was no Mycoplasma infection in control group; however some of the Mycoplasmas are normal flora of the body in the mucosal areas. It seemed that irregular and arbitrary usage of antibiotics in our study population and other conditions such as changes in vaginal pH might have negative effect on the bacterial population in the cervixes of our study population. A study conducted in Iran showed a Mycoplasma prevalence rate of 43% among women with low levels of education. The study also showed the importance of education in personal hygiene. There was an indirect association between level of education and infection.[23] Developing science and technology has created methods of contraception healthier, therefore, people who used healthier methods of contraception rather than traditional ones became less vaginally infected.[24]

However, people involved in the present study had high levels of education; it might be a reason for low rate of Mycoplasma prevalence in this population.

Conclusion

Infection with *Mycoplasma genitalium* was not a critical factor in recurrent abortions, by itself. It can be considered as an abortion etiology along with other bacteria and microorganisms such as other species of Mycoplasmas, Chlamydia, Ureaplasma, viruses and fungi. It seems more studies are necessary to evaluate the relationship between Mycoplasma infection and abortion. Moreover, the increase in the level of public health due to better education and the improvement of contraceptive methods compared to the past has created a significant reduction in Mycoplasma infection.

Conflict of Interests

Authors have no conflict of interests.

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