Original Article

Detection and Evaluation of Macrolide Resistance (Erythromycin) in *Mycoplasma hominis* Isolated from Endocervical Specimens of Patients Referring to Ibn Sina Infertility Treatment Centre, Tehran, Iran

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Abstract ————

Background: *Mycoplasma hominis (M. hominis)* is an important cause of bacterial infections of the genital tract. Macrolides are the first selective agents used to treat mycoplasma infections. However, widespread use of macrolides has led to a rapid and global emergence of macrolide-resistant strains. We evaluated macrolide resistance in *M. hominis* isolated from endocervical specimens of patients who referred to Ibn Sina Infertility Centre in Tehran, Iran.

Materials and Methods: In this cross-sectional descriptive-analytical study, 160 samples of Dacron endocervix swabs (80 infertile patient samples and 80 healthy controls) were collected and transferred to the laboratory. All samples were cultured in liquid pleuropneumonia-like organisms (PPLO) broth and PPLO agar solid media. After culturing and genome extraction, polymerase chain reaction (PCR) was performed using specific primers. Then, minimum inhibitory concentration (MIC) was obtained using the broth microdilution method. The MIC was recorded and reported for all samples positive for *M. hominis* against erythromycin.

Results: From the 160 endocervical specimens cultured in PPLO agar medium, 19 cases (23.75%) were positive. A total of 35 cases (42.5%) were positive using specific primers of *M. hominis* species. MIC results from all samples positive for *M. hominis* were measured against erythromycin. All of the *M. hominis* samples were resistant to erythromycin.

Conclusion: The results of the present study showed that a significant percentage of infertile women were infected with *M. hominis*. Also, MIC results from the broth microdilution method indicated that all strains positive for *M. hominis* were also resistant to erythromycin.

Keywords: Erythromycin, Infertility, *Mycoplasma hominis*, Resistance

Introduction

Mycoplasmas belong to an unconstrained class called Mollicutes and the Mycoplasmataceae family; this family includes Mycoplasma spp. *Mycoplasma hominis* (*M. hominis*), Mycoplasma genitalium and *Ureaplasma urealyticum* (1, 2). These species may cause oligosyptomatic genital infections, including urinary tract infection, chorioamnionitis, pelvic inflammatory disease, and sperm cell disorders in both reproductive age men and women. Complications of these infections can lead to infertility. *M. hominis* is among the smallest human pathogens responsible for a wide range of infections. However, knowledge regarding the genetic mechanisms and pathogenicity of *M. hominis* is limited (3-5).

M. hominis is located in the human lower genital tract as commensal flora, even though it is linked with development of non-gonococcal urethritis, infertility, chorioamnionitis, adverse pregnancy outcomes, and neonatal diseases. *M. hominis* infections can be found in asymptomatic individuals as well as symptomatic patients (6). Due to the lack of a cell wall, mycoplasmas are not affected by many of the common antibiotics that target cell wall synthesis, such as beta-lactam antibiotics (e.g., penicillin and cephalosporins). Mycoplasmas are sensitive to factors that interfere with protein synthesis, such as tetracyclines, macrolides, aminoglycosides, and chloramphenicol, as well as fluoroquinolones that act as inhibitors of topoisomerases (7-10). The increasing number of macrolide- and fluoroquinolone-resistant *M. hominis* strains can lead to drug resistance and the emergence of incurable infections (11). Macrolides are first-line treatment of *Mycoplasma* pneumoniae. Widespread use of macrolides has

led to rapid and global emergence of macrolide-resistant strains. The point mutation in domain V of the *23S rRNA* gene (nucleotide displacement at specific positions of domain V of *23S rRNA*) has been identified as the cause of macrolide resistance in mycoplasmas (11-13).

There is an increased prevalence of infertility in Iran. An elevated rate of genital infections caused by *M. hominis* in infertile women was reported by Seifoleslami et al. (14) Therefore, it is important to conduct observational studies on the prevalence and antimicrobial susceptibility of these bacterial species among infertile Iranian females. In this study, for the first time in Iran, we investigated the erythromycin macrolide resistance of *M. hominis* isolated from infertile women who referred to Ibn Sina Infertility Centre in Tehran, Iran. The objectives of this study were: i. Analysis of *M. hominis* prevalence by culture and molecular methods (e.g., presence of *23S rRNA* gene) from endocervical samples of the infertile women and ii. Evaluation of erythromycin macrolide resistance of *M. hominis* species isolated from these women.

Materials and Methods

Sample collection

In this cross-sectional descriptive-analytical study, 160 endocervical swab samples were collected from women who referred to Ibn Sina Infertility Centre, Tehran Province, Iran. The samples were assigned to two groups, patient (n=80) and control (n=80). The inclusion criteria were: married women with clinical signs of vaginosis, green vaginal discharge with a foul fishy odour, history of infertility (infertility after one year of intercourse), history of abortion, history of preterm delivery, and no antibiotic use during the past month. All the clinical examinations were performed by a gynaecologist. All participants completed a questionnaire for patient eligibility, and a written informed consent was obtained before they were screened for sampling eligibility. The control group consisted the healthy and fertile individuals.

Culture

Sampling was performed using endocervical Dacron swabs. Two swabs were obtained from each patient, one for culture and the other for molecular assessment. All swabs were transferred to the laboratory in 2 ml of liquid transfer medium under sterile conditions. Afterwards, 1 ml of the transfer medium was filtered through 0.45 μm pore size filters and transferred to main pleuropneumonia-like organisms (PPLO) broth (pH=7.8 ± 0.2). The samples were then incubated in a 5%-10% CO_2 atmosphere at 37 °C for one week. After three subcultures in liquid medium, 100 μl of each sample was cultured in PPLO solid agar, and the samples were incubated in 5-10% CO_2 at 37°C for 3-5 days. In this study, we used the standard *M. hominis* (ATCC: 23114) strain, which was prepared at Baqiyatallah University, Tehran, Iran.

DNA extraction

We used the polymerase chain reaction (PCR) technique, which is a highly sensitive, specific test to confirm the presence of all pathogens, regardless of culture test results. A DNA Extraction Kit (Sinaclon, Iran) was used to extract DNA from the samples. After DNA extraction, all samples were kept at -20°C prior to PCR.

Molecular method

In this study, specific primers were used to identify the *M. hominis* gene, 23S rRNA, as shown in Table 1 (15, 16). Primer BLAST was performed, and sensitivity and specificity were confirmed at the NCBI site.

PCR was conducted with a final volume of 25 μl for each sample, which included 12.5 μl Master Mix 2X (Master Mix 2X, Pishgam, Iran), 5 μl DNA template, and 0.5 μl of each primer pair in a total volume of 25 μl. PCR was performed according to the protocol for different genes (Table 2). Finally, PCR amplification products were subjected to 1% gel agarose electrophoresis. DNA extracted from a standard strain of *M. hominis* (ATCC: 23114) was the control.

Table 1: Sequences of the study primers

Gene	Primer sequence (5´-3´)	Product size	Tm (°C)
M. hominis species gene	F: CAATGGCTAATGCCGGATACGC R: GGTACCGTCAGTCTGCAAT	334	58
23S rRNA	F: TAACTATAACGGTCCTAAGG R: CCGCTTAGATGCTTTCAGCG	793	52

M. hominis; Mycoplasma hominis and Tm; Melting temperature.

Table 2: Temperature protocol of PCR for *M. hominis* gene and the *23S rRNA* gene

Gene	Pre- denaturation	Denaturation	Annealing	Extension	Final extension
Gene species of *M. hominis*	95°C	95°C	56°C	72°C	72°C
	5 minutes	30 seconds	45 seconds	60 seconds	7 minutes
		←	35 cycles	←	
23S rRNA	94°C	95°C	55°C	72°C	72°C
	10 minutes	1 minute	1 minute	60 seconds	10 minutes
		←	40 cycles	←	

M. hominis; Mycoplasma hominis and PCR; Polymerase chain reaction.

Minimum inhibitory concentration determination using the microdilution method

We determined the minimum inhibitory concentration (MIC) of erythromycin for the samples that were positive for the species gene of *M. hominis* and the *23S rRNA* gene. We used microdilution of the arginine PPLO broth that was enriched with horse serum and L-arginine. The assessment was conducted in 96-well microplates following preparation of the macrolide suspension (erythromycin). The microplates prepared for the *M. hominis* culture were incubated at 37°C for 48-72 hours. The final results were interpreted based on previous studies (16, 17). Antimicrobial susceptibility was determined through PPLO broth microdilution for clinical strains, as described previously. The specific cut-off points (mg/litre) that indicated susceptibility (S) or resistance (R) to erythromycin were S ≤1 and R ≥4 (11). The MIC of erythromycin was measured and recorded for all *M. hominis* positive samples. The MIC for erythromycin was measured based on colour changes of the arginine PPLO broth enriched with horse serum and L-arginine. Arginine PPLO broth is a phenol red medium and growth of *M. hominis* in arginine PPLO broth changes the colour of the medium from purple to darker red.

Ethical considerations

This study was approved by the Ethics Committee of Shahed University School of Medicine, Tehran, Iran (IR. SHAHED.REC.1398.014).

Statistical analysis

The data were analysed using IBM SPSS statistical software, version 20.0 (SPSS Inc., Chicago, IL) and presented as statistical tables. $P<0.05$ were considered significant according to the Chi-square test.

Results

In this study, 160 endocervical swab samples were collected from participants, of which 80 samples were collected from infertile women (experimental group) and 80 samples from healthy women (control group). The study participants ranged in age from 20 years to 49 years. As shown in Table 3, *M. hominis* was most prevalent in women 30-39 years of age in both groups.

Table 3: Frequency distribution of M. hominis in endocervical samples by age group

Age (Y)	Number of patients (n=160)		Patient group (n=80)		Control group (n=80)	
	Patient group	Control group	Culture	PCR	Culture	PCR
20-29	21	19	3	4	0	0
30-39	47	45	11	17	1	3
40-49	12	16	4	8	0	2
Total	80	80	18	29	1	5

M. hominis: *Mycoplasma hominis* and PCR; Polymerase chain reaction.

Among the investigated endocervical specimens, 19 samples grew *M. hominis* colonies on PPLO agar, 18 (22.5%) were from the experimental group, and 1 (1.25%) from the control group (Table 4).

**Table 4:** Detection of *M. hominis* by culture on PPLO agar and PCR

Number	Age (Y)	Cultivation	PCR	Sample type
		Patient group		
1	20	+	+	Endocervical mucosa
2	25	+	+	Endocervical mucosa
3	27	-	+	Endocervical mucosa
4	29	+	+	Endocervical mucosa
5	30	+	+	Endocervical mucosa
6	31	+	+	Endocervical mucosa
7	31	-	+	Endocervical mucosa
8	32	+	+	Endocervical mucosa
9	32	-	+	Endocervical mucosa
10	33	+	+	Endocervical mucosa
11	33	+	+	Endocervical mucosa
12	34	-	+	Endocervical mucosa
13	35	-	+	Endocervical mucosa
14	35	+	+	Endocervical mucosa
15	36	-	+	Endocervical mucosa
16	36	-	+	Endocervical mucosa
17	37	+	+	Endocervical mucosa
18	37	+	+	Endocervical mucosa
19	38	+	+	Endocervical mucosa
20	39	+	+	Endocervical mucosa
21	39	+	+	Endocervical mucosa
22	40	-	+	Endocervical mucosa
23	42	+	+	Endocervical mucosa
24	44	+	+	Endocervical mucosa
25	47	-	+	Endocervical mucosa
26	47	+	+	Endocervical mucosa
27	48	-	+	Endocervical mucosa
28	48	-	+	Endocervical mucosa
29	49	+	+	Endocervical mucosa
		Control group		
1	29	-	+	Endocervical mucosa
2	36	+	+	Endocervical mucosa
3	37	-	+	Endocervical mucosa
4	39	-	+	Endocervical mucosa
5	48	-	+	Endocervical mucosa

PPLO; Pleuropneumonia-like organisms broth, PCR; Polymerase chain reaction, and *M. hominis*; *Mycoplasma hominis*.

Results of polymerase chain reaction detection of Mycoplasma hominis (*M. hominis*) species gene and 23S rRNA gene

Results of PCR on 160 Dacron endocervical swabs showed that 34 samples (42.5%) were positive for *M. hominis* by using first primers for the *M. hominis*

species gene and primers for the *23S rRNA* (Fig.1). Of 80 endocervical samples in the experimental group, 29 (36.25%) were positive for *M. hominis*, and from 80 endocervical samples in the control group, 5 (6.25%) were positive for *M. hominis* (Table 4).
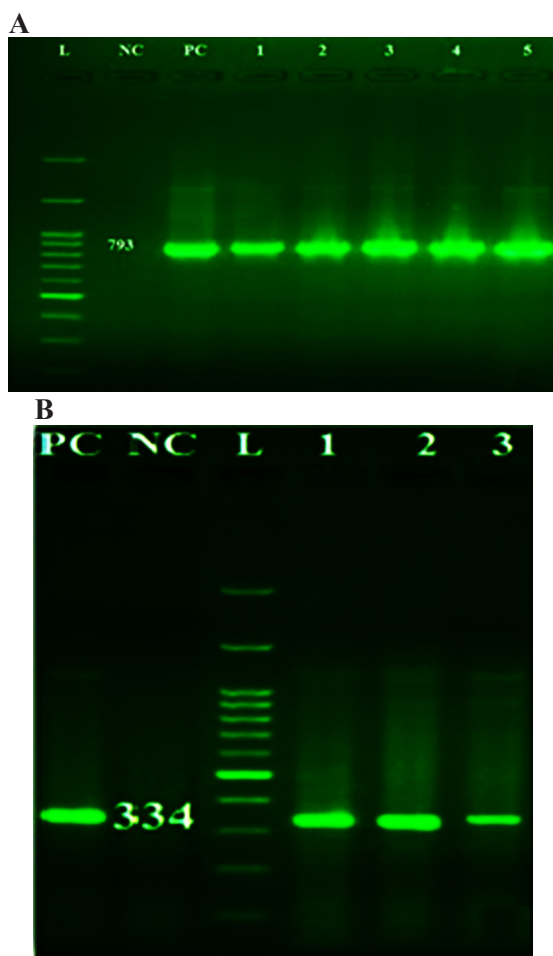
Fig.1: Results of 1% gel agarose electrophoresis. **A.** PCR results for the *23S rRNA* gene, L; DNA ladder (100 bp DNA Ladder, Pishgam, Iran), PC; Positive control, NC; Negative control, Lanes 1-5 are positive for *23S rRNA* gene. **B.** PCR results for the *M. hominis* species gene, L; DNA ladder (100 bp DNA Ladder, Pishgam, Iran), PC; Positive control, NC; Negative control, Lanes 1-3 are positive for species gene of *M. hominis*, and PCR; Polymerase chain reaction.

The frequency and distribution of *M. hominis* in both patient and control groups according to the culture and PCR methods indicated that positive culture of *M. hominis* in the infertile patients (P=0.004) and diagnosis by molecular PCR (P=0.003) were much higher compared to the control group. PCR results had a very high sensitivity compared to the culture method for detection of *M. hominis* (P=0.037).

Minimum inhibitory concentration of *Mycoplasma hominis (M. hominis)* positive specimens

We performed MIC on the positive samples to determine the susceptibility or resistance of erythromycin on these samples. According to the findings of our study, all of the *M. hominis* samples were resistant to erythromycin (Table 5).

Table 5: MIC ranges (µg/ml) for erythromycin against *M. hominis*

Patient group	MIC_{50}	MIC_{90}	Range	S (%)	I (%)	R (%)
1	256	256	≤1-≥512	0	100	0
2	128	128	≤1-≥512	0	100	0
3	128	256	≤1-≥512	0	100	0
4	128	128	≤1-≥512	0	100	0
5	128	256	≤1-≥512	0	100	0
6	256	256	≤1-≥512	0	100	0
7	128	128	≤1-≥512	0	100	0
8	256	256	≤1-≥512	0	100	0
9	128	256	≤1-≥512	0	100	0
10	256	256	≤1-≥512	0	100	0
11	64	128	≤1-≥512	0	100	0
12	256	256	≤1-≥512	0	100	0
13	128	128	≤1-≥512	0	100	0
14	128	256	≤1-≥512	0	100	0
15	256	256	≤1-≥512	0	100	0
16	128	128	≤1-≥512	0	100	0
17	256	256	≤1-≥512	0	100	0
18	128	128	≤1-≥512	0	100	0
19	256	256	≤1-≥512	0	100	0
20	128	128	≤1-≥512	0	100	0
21	256	256	≤1-≥512	0	100	0
22	128	256	≤1-≥512	0	100	0
23	256	256	≤1-≥512	0	100	0
24	256	256	≤1-≥512	0	100	0
25	128	128	≤1-≥512	0	100	0
26	64	64	≤1-≥512	0	100	0
27	128	256	≤1-≥512	0	100	0
28	128	128	≤1-≥512	0	100	0
29	256	256	≤1-≥512	0	100	0
Control group						
1	128	256	≤1-≥512	0	100	0
2	256	256	≤1-≥512	0	100	0
3	128	128	≤1-≥512	0	100	0
4	64	128	≤1-≥512	0	100	0
5	128	128	≤1-≥512	0	100	0

MIC; Minimum inhibitory concentration, *M. hominis*; Mycoplasma homini, S; Susceptible, I; Intermediate, and R; Resistant.

The MIC of erythromycin was measured and recorded for all *M. hominis* positive samples. We determined the MIC for erythromycin was based on colour changes of the arginine PPLO broth enriched with horse serum and L-arginine from the growth of *M. hominis*.

Discussion

Infertility is one of the most important issues in medical science, which is commonly defined as a biological reproductive disability (10, 18). Some infections cause infertility amongst women, and some interfere with embryo

implantation in the uterus, causing miscarriage. Bacterial infections can also cause infertility, and *M. hominis* is one of the most prevalent bacterial pathogens associated with infertility over the past few years. *M. hominis* generally colonizes in the urogenital system and can be seen as a natural flora in the genital tract of many sexually active men and women. This pathogen has been isolated from vaginal secretions, amniotic fluid, placental tissue and umbilical cord blood during pregnancy (18, 19).

In this study, we recruited 160 women from which 80 infertile women comprised the experimental group and 80 fertile women were in the control group. From the 160 endocervical samples, 19 cases were positive for this pathogen when assessed by the culture method; 18 (22.5%) belonged to the patient group and 1 (1.25%) to the control group. The PCR test results from the 160 endocervical samples showed that 34 samples (42.5%) were positive for *M. hominis*. Out of the 34 positive samples, 29 (36.25%) were from the patient group and 5 (6.25%) from the control group. Both the culture and PCR method results showed a significant difference in frequency of the study microorganism in the patient group compared to the control group which indicated two main, important issues. First, *M. hominis* species were more prevalent in the patient group than the control group. Therefore, the presence of infectious bacterial species, such as *M. hominis*, could be associated with infertility. Second, the PCR method had much higher sensitivity than the culture method for detection of *M. hominis*.

Petrikkos and colleagues investigated and compared diagnostic methods of routine culture versus PCR. They found that 13 *M. hominis* negative samples by culture were positive by PCR, which indicated the higher sensitivity of PCR compared to the culture method (20).

Macrolides are the first selective agents for the treatment of mycoplasma infections. Extensive use of macrolides has led to the rapid and global emergence of macrolide resistance species. The presence of macrolide-resistant strains among infections caused by *M. hominis* has led to genetic mutations associated with macrolide resistance in these species and emergence of incurable infections.

In the present paper, the MIC of all the *M. hominis* positive samples with the *23S rRNA* gene against erythromycin was recorded and reported by using the broth microdilution method in 96-well microtiter plates. We found that all of the samples of *M. hominis* were resistant to erythromycin. MIC results by the microdilution technique was positive for *M. hominis* against erythromycin in all of the samples. The MIC was determined based on colour changes in the arginine PPLO broth medium, which was caused by the growth of *M. hominis*. The highest rate of *M. hominis* infection in the patient and control groups was observed in women who were 30-39 years of age.

Wang et al. conducted a study in China and found that all strains of *M. hominis* were 100% resistant to erythromycin due to the antimicrobial sensitivity of *M. hominis* to macrolides. *M. hominis* infection was most prevalent in 30-39 year-old age group; both findings were consistent with the results of the present study (21).

Pereyre and colleagues detected *23S rRNA* mutations associated with resistance against macrolides, including erythromycin and azithromycin. A point mutation at position 2057 (G2057A) was obtained in domain V of *23S rRNA* (16). These results were consistent with the results of our study.

Bayraktar et al. (6) conducted a study with 100 pregnant women; they reported that 50 patients were symptomatic and 50 were asymptomatic. Also, 29 subjects (29%) were positive for *M. hominis* and *Ureaplasma urealyticum*. Of these, 27 females were in the patient group (54%) and two were from the control group (4%). They successfully cultured *M. hominis* from five women (5%) and *Ureaplasma urealyticum* from 27 women (27%), which was consistent with the results of the present study. *M. hominis* and *Ureaplasma urealyticum* were 100% sensitive to doxycycline, tetracycline and pristinamycin; 90% to josamycin; 84% to clarithromycin; and 50% to erythromycin. The results of antibiotic susceptibility testing were significantly inconsistent with the results of the present study.

Zhou et al. (22) recruited a total of 5016 infertile males and 412 healthy males from 2011 to 2016. Culture, identification and antimicrobial susceptibility of *Ureaplasma uralticum* and *M. hominis* were evaluated in their study. A total of 30%-55% of the infertile men had *M. hominis* infections in their genital tracts. Two age groups were identified as high-risk for mycoplasmas: 26-30 years (37.8%) and 31-35 years (30.7%). The results of this study were in line with the results of our study.

Yang et al. (4) investigated 492 species of Ureaplasma and 13 strains of *M. hominis* in Hangzhou, China. They stated that the levels of resistance to levofloxacin, moxifloxacin and erythromycin were 84.69, 51.44, and 3.59%, respectively, in *Ureaplasma parvum* and 82.43, 62.16% and 5.40, respectively, in *Ureaplasma uralticum*. Among 13 *M. hominis* strains, 11 were resistant to levofloxacin and moxifloxacin, and five strains were resistant to clindamycin. The results of this study showed that new S21A mutations in the L4, G2654T and T2245C proteins were detected in the *23S rRNA* and *ermB* genes in the *erythromycin*-resistant Ureaplasma species. Fluoroquinolone resistance in the Ureaplasma and *M. hominis* species is relatively high in China; thus, the use of these antibiotics should be further controlled and limited. The results of this study were consistent with our study.

Ozturk and colleagues (5) isolated 72 strains of *M. hominis* from 220 clinical specimens. They observed that all of the selected strains were mostly resistant against pristinamycin (100%), followed by tetracycline (80%) and josamycin. (75%), and *M. hominis* was mostly resistant against levofloxacin (100%), clindamycin

(97%), roxithromycin (97%), and ciprofloxacin (94%). This study also reported resistance to erythromycin (81%) and clarithromycin (89%). The results of this study were almost similar to the results of our study, which indicated a high level of resistance against macrolide antibiotics.

Maldonado-Arriaga and colleagues (1) enrolled 37 infertile couples (74 samples) to diagnose genital mycoplasmas and evaluate antibiotic resistance. They observed that the prevalence of *Ureaplasma urealyticum*, *M. hominis* and coinfections were 71.4, 9.5 and 19%, respectively. In this study, most mycoplasmas showed significant resistance against fluoroquinolones, macrolides and tetracycline, with the highest resistance to macrolides in all strains. The results of this study were almost similar to the results of our study, which indicated a high level of resistance against the macrolide antibiotics.

Doroftei et al. reported that women aged 30-35 years were the most affected group, followed by 25-30 year-old women in their study. Cumulatively, the prevalence of *Ureaplasma urealyticum, M. hominis*, and coinfection with both, were 28.46% (n=117), 0.48% (n=2), and 2.91% (n=12), respectively. Drug susceptibility was evaluated in this study. Pristinamycin (100 vs. 100%) and Josamycin (100 vs. 98.00%) were the most efficient antibiotics in eradicating *Ureaplasma urealyticum* and *M. hominis*. High efficiency was observed with doxycycline (98.23%), minocycline (96.00%), tetracycline (96.48 vs. 68.00%), and erythromycin (70.17 vs. 92.00%). Based on antibiograms, clarithromycin (88.00%), roxithromycin (88.00%), levofloxacin (82.00%), and azithromycin (78.94%) could be used to treat these infections (23).

Conclusion

The results of the present study showed that a significant percentage of infertile women were infected with *M. hominis*. Also, all samples that were positive for *M. hominis* showed resistance against erythromycin according to MIC assessment by the broth microdilution method. This is the first study that investigated erythromycin macrolide resistance of *M. hominis* isolated from infertile women in Iran. Although this study examined a small population of infertile women, due to the increasing trend in infertility, further research is needed in Iran to achieve precise results in relation to increased antibiotic resistance in larger populations, especially macrolide antibiotics such as erythromycin. According to the MIC results obtained by the broth microdilution method, all of the strains that were positive for *M. hominis* and the *23S rRNA* gene were resistant to erythromycin. Since the target site for erythromycin is the S50 subunit of the bacterial ribosome, many cases of macrolide resistance in clinical isolates can be associated with specific nucleotide changes in domain V of the 23S rRNA gene in the ribosomal S50 subunit.

Acknowledgements

Authors' Contributions

M.N., M.H.A.; Contributed to conception and design. F.N., S.A., I.P.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. M.N., M.H.A.; Were responsible for overall supervision. F.N.; Drafted the manuscript, which was revised by M.H.A., M.N. All authors read and approved the final manuscript.

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