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Investigation of Ureaplasma Infections of Semen in Fertile and Infertile Men of Bandar Abbas City

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ABSTRACT

Ureaplasma has been established as an infecting agent in non-gonococcal urethritis, prostatitis, epididymitis, and infertility. The infection by *Ureaplasma urealyticum* (*U. urealyticum)* has adversely affected reproductive health; also, it assists in infertility treatment but later on affects the outcome of pregnancies. This study aimed to assess the prevalence of *U. urealyticum* and *Ureaplasma parvum (U. parvum)* in the semen of infertile and healthy men using polymerase chain reaction (PCR) analysis. In this cross-sectional descriptive study, 100 male semen samples (50 fertile and 50 infertile) were collected from the OmeLeila specialized hospital, Bandar Abbas City. Semen specimens were taken from infertile patients and health controls, followed by routine andrological evaluation and PCR analysis. DNA extraction was carried out through the SimBioLab kit, and samples were analyzed using a PCR protocol with specific primers for targeted gene detection. PCR analysis identified Ureaplasma in 10% (5/50) of semen samples from infertile patients compared to 2% (1/50) in healthy controls, a statistically significant difference. Compared with healthy subjects, fertile urethral specimens showed decreased seminal volume, sperm concentration, and normal sperm morphology. The infertile patients with a positive PCR to *Ureaplasma* exhibited both significantly lower semen volume and sperm count and less normal sperm morphology than the PCR-negative infertile patients. Specifically, *U. urealyticum* was detected in 8 semen samples from infertile men compared to 2 samples from healthy controls, while *U. parvum* was detected in 2 samples of the infertile group. The findings indicate that *U. urealyticum* is more commonly found in the semen specimens of infertile males. Additionally, in the PCR-positive group for *U. urealyticum*, the percentage of normal sperm cells, semen volume, and sperm motility were significantly lower than those in the PCR-positive group for *U. parvum.*

Keywords: Infertility, PCR, Semen, *Ureaplasma urealyticum*, *Ureaplasma parvum*.

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INTRODUCTION

Infertility is a serious concern for public health worldwide. According to the epidemiological definition of the World Health Organization (WHO), the term infertility refers to the inability to conceive a child after one year of unprotected sexual activity before the age of thirty-five [1]. Infertility has a multifactorial substrate that is reflected by an impaired physiological environment [2]. Genital tract infections have been documented as essential factors in infertility, affecting 15-20% of couples of reproductive age globally [3,4], and male factors are the leading cause in 40% of infertility cases [5]. Many genital tract infections are asymptomatic and can become chronic, leading to several serious complications, including infertility. *Ureaplasma* species have been identified as a cause of male infertility since 1967 [6]. Friberg and Gnarpe reported a higher frequency of *Ureaplasma* species [7]. The prevalence of *Ureaplasma* in the genitourinary tract of infertile individuals varies significantly in regions, countries, and population groups based on factors such as age, ethnicity, and socioeconomic status [3,6]. This study aims to investigate the prevalence of *Ureaplasma* infections among men and their potential impact on male infertility in Bandar Abbas City. By examining the relationship between *Ureaplasma* colonization and infertility, this research seeks to contribute valuable insights into the role of these infections in male reproductive health within the region.

MATERIAL AND METHODS

In this cross-sectional descriptive study, 100 male semen samples (50 fertile and 50 infertile) were collected from the specialized hospital of OmeLeila Hospital in Bandar Abbas City. All participants were married and of reproductive age, ranging from 21 to 50 years old, and none had identifiable causes of infertility, including physiological, hormonal, or anatomical factors. Notably, all samples were obtained with written informed consent from the participants. This study was meticulously conducted according to stringent ethical guidelines and received approval from the Medical Ethics Committee (Code: IR.IAU.KERMAN.REC.1401.077).

Sampling and transfer of samples to the laboratory: 100 male semen samples (50 fertile and 50 infertile individuals) were collected from OmeLeila Hospital in Bandar Abbas City, confirmed by a specialist doctor. All participants must abstain from sexual activity for at least 48 hours and refrain from taking antibiotics for one week before sampling. The participants' characteristics were recorded, including their age, type of infertility (primary or secondary), duration of infertility, smoking status, alcohol consumption, and any history of antibiotic use. Then, semen samples were taken in sterile containers, and semen analyses were performed according to the WHO guidelines in 2019. The samples were sent to Pasargad Research Microbial Laboratory for additional testing, including polymerase chain reaction (PCR) and DNA extraction.

Enrichment and isolation of bacteria

A PPLO broth culture medium was used to enrich the samples. The culture medium was prepared and autoclaved following the provided instructions. To prepare the media for the growth of *U. urealyticum*, respectively, it was enriched with the following components: 10% urea, 5% horse serum, penicillin G antibiotics (1000 IU/ml) to remove gram-positive bacteria, and polymyxin B (500 IU/mL) as Gram-negative inhibitors. The final pH of the medium was adjusted to 7. Finally, the prepared transport mediums were kept at 4°C. After transporting the mediums to the hospital, one milliliter of each semen sample was inoculated into the transport medium of pleuropneumonia-like organisms. Also, vaginal swabs were inoculated into the transport mediums and incubated at

37°C for 24 hours [8]. After 24 hours, all transport mediums of PPLO broth containing the sample were filtered into the second transport medium with the same materials, except for horse serum (20%) and phenol red (2%). This filtration was carried out using polyvinylidene difluoride (PVDF) needle filters with a diameter of 0.45 micrometers [9]. The mediums were then incubated at 37°C for 5 to 7 days. During this period, the daily change in color from yellow to purple, indicating bacterial growth, was investigated.

DNA extraction

DNA extraction was performed using the SimBioLab kit (Cat No. Sam007). Initially, the medium inoculated with bacteria was centrifuged for 5 minutes to pellet the cells. Following centrifugation, 20 µL of lysozyme solution (50 mg/ml) was added to each microtube, and the mixture was thoroughly vortexed to ensure homogeneity. The microtubes were then incubated at 30°C for 20 minutes, with gentle inversion of the tubes every 5 minutes to facilitate optimal lysis of the bacterial cells. 400 µL of buffer A was added to the samples and the tubes were inverted several times to ensure a homogeneous mixture. The samples were then incubated at room temperature for 3–5 minutes. After this, 300 µL of buffer B was added, and the tubes were again inverted multiple times before being incubated for 2 to 4 minutes at room temperature. The microtubes were subsequently centrifuged at 12000 g for 10 minutes, forming three distinct phases in the centrifuged samples. The samples were carefully placed on ice, and approximately 400 µL of the upper phase was transferred to a DNA spin column. 450 µL of cold isopropanol (-20°C) was added to the column, and the mixture was centrifuged at 10000 g for 1 minute. The liquid in the collection tube was discarded and reconnected for further processing. 500 µL of Buffer W was added to the DNA spin column, and the column was centrifuged at 10000 g for 1 minute. Following this step, the column was connected to a new 1.5 mL microtube, allowing time for the filter to dry completely. Subsequently, 50-100 µL of washing buffer was added to the column and incubated at room temperature for 1 minute. The tubes were centrifuged at 13000 g for 1 to 3 minutes to ensure thorough washing. Finally, the purified DNA sample was stored at -20°C for future analysis.

Polymerase chain reaction (PCR)

To perform PCR, DNA samples were taken out of the freezer and thawed at room temperature. The sequence of the primers was used to detect the genomes of *Ureaplasma* bacteria, specifically the *U. urealyticum* and *U. parvum* species.

Table 1

Nucleotide sequence and primers used in the research [10]

	Target	Sequence (5′-3′)	Length (bp)
***Ureaplasma* genus**	16S rRNA	F = ACGACGTCCATAAGCAACT R = CAATCTGCTCGTGAAGTATTAC	429
Ureaplasma urealyticum	16S rRNA	F = TTTGCAA AACTATAAATAGACAC R = TTTGT TGTTGCG TTTTCTG	363
Ureaplasma parvum	16S rRNA	F = AATAAATCTTAGTGTTCATATTTTTTTAC R = GTAAGTGCAGCATTAAATTCAATG	327

First, polymerase chain reaction was performed on the extracted DNA of each sample to identify the genus of *Ureaplasma*. Then, the study was conducted to determine the *U.*

urealyticum and *U. parvum* species. To perform PCR, we used a 2X Master Mix (Cinagen, Iran) containing Taq polymerase enzyme (0.5 IU/L), $MgCl_2$ (4 mmol/L), and dNTPs (4 mmol/L). The standard strain of *Ureaplasma*, prepared by the National Center of Genetic and Biological Resources of Iran, was used as a positive control, and distilled water without DNA was used as a negative control. In this study, each PCR reaction sample had a volume of 20 microliters divided among microtubes in the specified volume. Then, based on the reaction mixture written in the table (Table 2), 15 microliters were added to each microtube, followed by 5 microliters of the specific template DNA. This brought the final volume of the PCR reaction mixture to 20 microliters. After vortexing and spinning the samples, the microtubes were placed in a thermocycler.

Table 2

Polymerase chain reaction mixture

Material	Amount in microliters	Concentration
DNA	5	50ng
Forward Primer	1	*10pmol*
Reverse Primer	1	*10pmol*
Master Mix (2x)	10	*2x*
Deionized distilled water	3	-
Total volume	20	-

After the sample was prepared, the resulting mixture, in a final volume of 20 microliters, was transferred to the thermocycler (Germany, Eppendorf Mastercycler Gradient), which was already turned on. Its temperature and time were set for different stages according to Table [3].

Table 3

PCR temperature program for amplifying the desired gene [11]

Cycle	40 cycles				
Stage	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
Temperature	95	94	54	72	72
Time	5 min	30 sec	45 sec	45 sec	5 min

Performing electrophoresis and detecting the PCR product

After preparing a 1.5% agarose gel and reaching a temperature of about 45°C, 5 microliters of SYBR Green dye (Sinaclon, Iran) were added to every 10 ml of gel. After cooling the gel, it was placed inside the electrophoresis tank. Then, 8 microliters of the PCR product were carefully poured into the wells created in the gel. Also, about 3 microliters of the DNA ladder (100bp-Fermentas) were added inside one of the wells. The standard strain served as a positive control, while distilled water was utilized as a negative control. An electric current was applied at a voltage of 180 V, and the amplified PCR products were meticulously monitored using electrophoresis through agarose gel. DNA bands were observed and photographed with a transilluminator device (UV-transilluminator-Red) [12].

Statistical analysis

After collecting the data, statistical analysis was done using SPSS, Excel software, and the Chi-square test.

RESULTS

Enrichment results

After 5 to 7 days of incubation, enriched culture mediums were examined for turbidity and color change, a sign of bacterial growth. Among the 100 analyzed samples (50 fertile and 50 healthy), 10 semen samples of infertile people and 2 samples of healthy people were evaluated positively for the presence of *Ureaplasma*, which was used for DNA extraction and PCR testing in the next step was selected.

Polymerase chain reaction results

The results of the PCR test using the *16S rRNA* primer showed that 12 selected samples from the previous stage were positive for the presence of *Ureaplasma*. Figure 1 shows the electrophoresis of PCR-amplified products. A fragment of 429 base pairs of the urease gene was amplified to detect *Ureaplasma*.

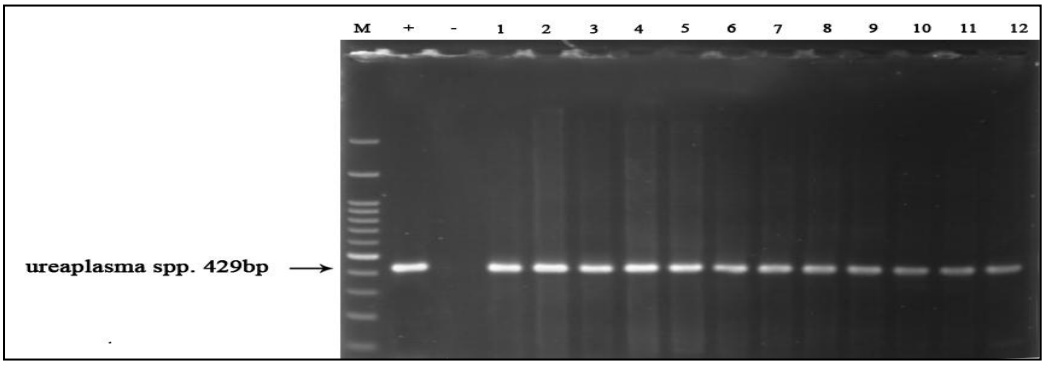

Fig. 1 *Ureaplasma* genus detection, from left to right: DNA Ladder (100bp- Fermentas), positive control, negative control, and sample number 1-12

Multiplex PCR products with species-specific primers are shown in Figure 2. Out of 10 positive samples of *Ureaplasma* in infertile men, 8 samples were *U. urealyticum*, and 2 samples were *U. parvum*. Out of 2 positive *Ureaplasma* PCR samples in the healthy group, both belonged to the *U. urealyticum*.

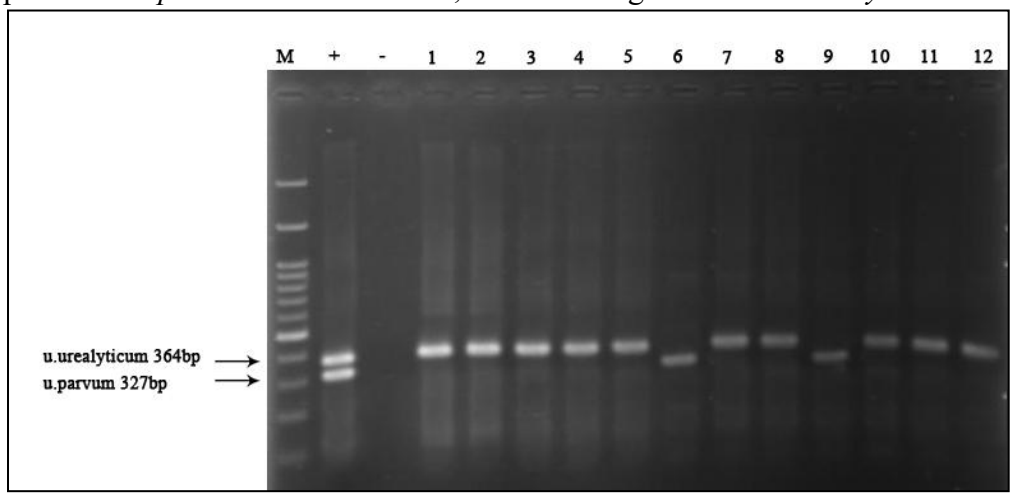

Fig. 2 Detection of *U. urealyticum* (364bp) and *U. parvum* (327bp) from left to right: DNA Ladder (100bp- Fermentas), positive control, negative control and sample number 1-12

Results of statistical tests

After statistical analysis and using SPSS software and the Chi-square test for variables, the results showed a significant difference between the two investigated groups (infertile and healthy) regarding *Ureaplasma*. The age of patients and healthy people was between 27 and 46 years, and no significant difference was observed between the ages of people with the presence of *Ureaplasma*. Infertile men with the traditional age range of 30-40 years had more bacterial contamination than other traditional groups. The Chi-square test showed a statistically significant relationship between the consumption of cigarettes, alcohol, and tobacco with infertility ($P<0.05$). It was also shown that the variables *U. urealyticum* and *U. parvum* also have a statistically significant relationship with the health status of people and increase the chance of infertility ($P<0.05$).

DISCUSSION

Concerning the WHO guidelines, infertility refers to the inability to conceive a child after one year of unprotected sexual activity before the age of thirty-five [2]. Infertility is a growing social and economic problem in today's society worldwide, which affects couples emotionally, psychologically, and economically. It is estimated that 8-12% of couples in the world may have problems conceiving at some point in their reproductive lives [13], and male factors are the leading cause in 40% of infertility cases [5]. Although the cause of male infertility is often unknown, infectious agents such as bacteria, viruses, and fungi can disrupt various human functions, including reproduction and pregnancy [14]. Among them, *U. parvum* and *U. urealyticum* are pathogens that have a potential etiological role in genital infections and male infertility [15]. The prevalence of *Ureaplasmas* in the semen samples of infertile men reported between 5 and 42% in different articles, is a significant concern [11]. However, with the use of the highly accurate and sensitive PCR method in this study, we were able to evaluate the prevalence of *Ureaplasma spp.* (*U. urealyticum* and *U. parvum*) in the semen of infertile men, and the findings showed a prevalence of 12%. The same as the present study, similar results have been obtained in previous studies by the researchers:

According to the study of Zeighami et al. (2009), research was conducted on sperm samples from infertile patients and healthy individuals, using routine andrology analysis and PCR. They detected *Ureaplasma* in 12% of samples from infertile men (12 out of 100) compared to 3% from healthy men (3 out of 100), with significant detection by PCR. Infertile men also had lower semen volume, sperm concentration, and normal sperm morphology. The results generally indicate that *U. urealyticum* is more prevalent among infertile men. The percentage of normal sperm, seminal fluid volume, and motility rate of sperm cells were lower in the PCR-positive group for *U. urealyticum* compared to those for *U. parvum*. This shows that genital *Ureaplasma* infections play an important pathogenic role in male infertility [10]. These findings align with our own regarding the PCR method and the role of *Ureaplasma* in male infertility.

Yun He Zhou's 2018 research estimated the prevalence and antimicrobial resistance of *Ureaplasma* and *Mycoplasma hominis* (*M. hominis*) in semen samples from infertile men in Shanghai, China, collected between 2011 and 2016. A total of 5016 infertile men and 412 healthy men were examined for *Ureaplasma* and *M. hominis* through cultivation, identification, and antimicrobial susceptibility testing using a kit and selective solid agar culture. Between 2011 and 2016, the positive rate of genital *Mycoplasma* infections in infertile men ranged from 30-55%, initially decreasing in the first four years before rising in the last two years. Two high-risk age groups for genital

Mycoplasma infection were identified: 26-30 (37.8%) and 31-35 (30.7%) [16]. These findings align with the current research, which also utilized PCR methods and considered the average age of participants.

Michael L. Beeton's 2019 research highlighted the presence of two genital bacteria, *U. urealyticum* and *U. parvum*, in humans. These bacteria have been subject to controversy regarding their pathogenic role in non-gonococcal urethritis and male infertility, mainly due to their high isolation rate from the urethra of apparently healthy men. The available literature has discussed the role of these bacteria in the infertility of men with idiopathic appearance [17]. The results of this study also show the importance of the pathological role of these organisms in male infertility.

Minh Tam Le's research was conducted in 2022 to determine the presence of *U. urealyticum* and *Mycoplasma genitalium* (*M. genitalium*) in semen and investigate their effect on sperm quality. This cross-sectional study was conducted on 380 men from infertile couples at a university hospital from July 2017 to June 2018. Semen quality was assessed by the 2010 WHO guidelines, and the presence of *U. urealyticum* and *M. genitalium* in semen samples was determined using PCR techniques. The findings showed that 338 men (88.9%) presented with at least one abnormal sperm parameter. The detection rates for *U. urealyticum* and *M. genitalium* were 16.05% and 0.79%, respectively. Notably, there was no significant difference in sperm characteristics between the ureaplasma-positive and ureaplasma-negative groups. However, sperm motility and vitality were significantly lower in the mycoplasma-positive group compared to the mycoplasma-negative group. The presence of *U. urealyticum* in the semen of infertile men did not affect sperm characteristics. Despite the low positivity rate for *M. genitalium*, its colonization was associated with a detrimental impact on sperm quality [5]. The role of *Ureaplasma* in male infertility has been a considerable subject over the past few decades. Some studies have failed to demonstrate a significant impact of sperm fertilizing ability on positive cultures, pregnancy rates, or overall pregnancy outcomes. However, other research has indicated that *Ureaplasma* infections may adversely affect various parameters of semen quality, including sperm concentration, motility, activity, and morphology [18]. However, due to the increasing rate of infertility, more research is needed to investigate the mechanism, as well as appropriate criteria to identify patients with infertility caused by *Ureaplasma* species.

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Transparency declaration

There is no conflict of interests.

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