

Using the PCR and Blood Agar in Diagnosis of Semen Bacterial Contamination of Fertile and Infertile Men

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Abstract

Background: One of the causes of male infertility is Genital tract infections (GTI). Considering the importance of GTI, widespread recognition of them seems necessary. we aimed to characterize and compare semen microbial populations in fertile and infertile men who referred to an infertility clinic in Yazd, Iran.

Methods: Semen samples were collected from two groups of fertile (268) and infertile (210) men. Sperm analysis (concentration, morphology, viability and motility parameters) were performed according to the World Health Organization (WHO) 2010 guidelines laboratory manual. Bacterial isolation was performed in Sheep Blood Agar and Eosin Methylene Blue (EMB) agar plates. For PCR, samples were analyzed with genus specific primers.

Results: All semen characteristics were poor in the infertile group compared to those in the fertile men (p-value < 0.05). *Enterococcus* spp. (18.7%, 17.1%; p= 0.814), *E. coli* (7.9%, 11.4%; p= 0.486), *Staphylococcus aureus* (6.4%, 2.9%; p= 0.398) and *Proteus mirabilis* (0%, 2.9%; p= 0.002) were the most common agents, respectively. Also, the results obtained from PCR were confirmed using culture-base method.

Conclusions: *Proteus mirabilis* contamination was identified in the infertile group. While no significant association was observed between male infertility and semen microbial populations, *p. mirabilis* may be the leading cause of reproduction impairment in men.

Keywords: Infertility, Microbial contamination, culture, PCR.

Introduction

Male infertility is a worldwide health problem and its etiology is not well defined (1). Male infertility can be responsible for 20-30% of infertility cases that many parameters including anatomical defects, prostatitis, hormonal defects, varicocele, physiological and psychological disorders and immune defects can cause this problem (2-6). Genital tract infection (GTI) has been suggested as one of the harmful causes for male fertility (7, 8).

It is believed that contaminations may exert negative effects on spermatogenesis process or decrease semen quality and sperm function. Scientific reports suggests that the mechanism

of contamination process on male reproductive tract and fertility potential are related to sperm agglutination, sperm immobilization, sperm DNA fragmentation [by increasing in reactive oxygen species (ROS) level], induction of chronic inflammations which can lead to reductions in sperm parameters such as count, motility and morphology (9-11). However, to date the exact mechanism of microorganism actions and its effect on male fertility is not clear. Many studies showed some microorganisms including bacteria, viruses, and fungi have been isolated from seminal fluid of infertile men (12-14).

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Escherichia coli were seen in semen sample of infertile men as its prevalence was higher than those detected in fertile men. Also *E. coli* was observed in reproductive tract disorders such as prostatitis and epididymitis (15-17). *Staphylococcus aureus* is another microorganism that play an important role in male reproductive tract infection (18). Many studies showed *S. aureus* is one of the most common bacterium isolated from the seminal fluid of infertile men (12, 18, 19). Nabi *et al* in 2013 evaluated bacterial contamination in seminal fluid of male partners in recurrent abortion cases. They reported, *S. aureus* and *E. coli* were the two main bacteria in the seminal fluid of male partners of women with unexplained recurrent abortion (20). *Chlamydia trachomatis* is another bacterium found in prostatitis, epididymitis, and urethritis cases. A systematic review in 2016 by Ahmadi *et al*, pointed to *Chlamydia trachomatis* as a very important and prevalent pathogen in infertile men as its prevalence was significantly higher than those in fertile men (21).

Baud *et al* (2019) compared the bacterial composition in 26 normal and 68 abnormal spermogram parameters using PCR technique. They reported that overall sperm bacterial content didn't play a major role in male infertility (22). The novelty of our study compared to previous studies was to evaluate bacterial contamination semen with blood agar and PCR methods in the high population of fertile and infertile men. There are very limited studies regarding the microbial contaminations in seminal fluid of infertile men in Iran. Since geographic parameters, genetic history and hygiene conditions are contributing factors for prevalence of microorganisms in a population, we aimed to characterize and compare seminal microbial status in the fertile and infertile males referring to Yazd infertility research center and find out which of the two methods blood agar and PCR assay is more suitable semen bacterial contamination.

Materials and Methods

Study population

In this case-control study, a total of 478 volunteer men who referred to Yazd infertility research center from September 2015 to July 2016 were divided into two groups of Fertile (n; 268) and Infertile (n; 210) males. Diagnosis of fertility or infertility were done by an urologist according to the World Health Organization (WHO) 2010 guidelines (23). Inclusion criteria included; Fertile and infertile men identified by the Andrology Laboratory. Exclusion criteria included infectious diseases sufferers and antibiotics usage of at least two weeks prior to semen collection, exposure to chemotherapeutics or radiation, testis trauma, smokers and varicoselectomy. The ethical committee based at the Yazd infertility research center approved the study protocol and use of patients' information. Also, all participants read and signed an informed consent.

Sperm collection and analysis

Semen specimens were collected in sterile container, the abstinence period for all participants was 2-5 days. Semen analysis was carried out according to the world health organization (WHO) laboratory manual for the examination and processing of human semen (Organization, 2010). Following masturbation induced ejaculation and were liquefied at 37 °C for 30 minutes. Sperm analysis including count and motility parameters (e.g., progressive, non-progressive and immotile) were assessed by Mackler chamber. Papanicolaou and Eosin-Nigrosin staining tests were done for sperm morphology and sperm viability evaluation, respectively (24).

Microorganism identification

Homogenized semen samples were cultured on the sheep blood agar and eosin methylene blue agar (EMB) (Condapronadisa, Spain) to isolation of gram positive and negative bacteria, respectively. Differential tests were done for phenotypic identification of the

species; Bile Esculin Agar (BEA), growth in 6.5% NaCl, catalase and hemolysis tests. Mannitol salt agar (MSA), DNase agar, coagulase and catalase tests were carried out for identification of *Staphylococcus* species. Differential tests were used for characterization of Gram-negative bacteria which included: catalase, oxidase, Simmons citrate agar, methyl red–voges-proskauer (MR-VP), lysine decarboxylase, triple sugar iron (TSI), and sulfide-indol-motility (SIM).

PCR reactions were performed for detection of various grown colonies (table 1). Chromosomal DNA was extracted from the pure colonies using the Qiagen extraction kit (QIAamp DNA mini kit, USA). The DNA

concentration and purity was assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, UK) and 1% gel electrophoresis, respectively. Template DNA was kept at -20 °C until more use. The primer sequences, PCR compounds and reactions are listed in Table 1. In general, PCR was performed in a final volume of 25 µl in an Eppendorf Master Cycle Gradient thermocycler (Eppendorf, Hamburg, Germany). PCR products were subjected to a 1% agarose/0.5×TBE gel electrophoresis (45 mM-Tris-borate, 1 mM-EDTA) stained with 0.1 µl/ml Gel Red™ (Biotium, USA), then photographed under an UV trans-illuminator (Tanon, China).

Table 1. The primer sequences, PCR compounds and reactions.

Gene target	Primer sequences	Reaction compounds	PCR program	Ref
<i>Staphylococcus spp.</i>	F: CAGGAGAAGTTAAAGAACAAGAAG R: GTGAACGAACTAATTGAGATACG	1.0 µL of template DNA, 12.1 µL of CinnaGen PCR Master Mix, 0.8 µL of each primer, and 10.3 µL of ddH ₂ O.	initial denaturation at 94 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 56 °C for 60 s, extension at 72 °C for 60 s and a final extension at 72 °C for 5 min.	
<i>Enterococcus spp.</i>	F: AGAGAGTAAGGTCCGATTGAAC R: GGTTGTTTCCCGTATTATGC	0.9 µL of template DNA, 10.6 µL of CinnaGen PCR Master Mix, 1.0 µL of each primer, and 11.5 µL of ddH ₂ O	initial denaturation at 95 °C for 5 min, denaturation at 94 °C for 45 s, annealing at 55 °C for 50 s, extension at 72 °C for 60 s and a final extension at 72 °C for 6 min.	
<i>Pseudomonas aeruginosa</i>	F: GCCTCTACCAGTACCTGCTAC R: AATAGAACAGCTCCAGCAGG	0.8 µL of template DNA, 11.6 µL of CinnaGen PCR Master Mix, 1.0 µL of each primer, and 10.6 µL of ddH ₂ O.	initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 55 s, annealing at 56 °C for 45 s, extension at 72 °C for 60 s and a final extension at 72 °C for 5 min.	(25)
16s rRNA	F: AGAGTTTGATCMTGGCTCAG R: ACGGCTACCTTGTTACGACTT	1.0 µL of template DNA, 12.5 µL of CinnaGen PCR Master Mix, 1.0 µL of each primer, and 9.5 µL of ddH ₂ O.	initial denaturation at 95 °C for 6 min, denaturation at 95 °C for 50 s, annealing at 53 °C for 45 s, extension at 72 °C for 50 s and a final extension at 72 °C for 10 min.	
<i>Streptococcus spp.</i>	F: AGA GTT TGA TCC TGG CTC AG R: GTA CCG TCA CAG TAT GAA CTT TCC	1.0 µL of template DNA, 12.3 µL of CinnaGen PCR Master Mix, 0.9 µL of each primer, and 9.9 µL of ddH ₂ O.	initial denaturation at 95 °C for 6 min, denaturation at 94 °C for 55 s, annealing at 57 °C for 50 s, extension at 72 °C for 60 s and a final extension at 72 °C for 5 min.	

Semen Bacterial Contamination in Males Infertility

<i>Escherchia. coli</i>	F: 5' TTGAAAATGGTCTGCTGCTG 3' R: 5' TATTGGCTTCATCCACCACA 3'	0.9 µL of template DNA, 12.2 µL of CinnaGen PCR Master Mix, 0.8 µL of each primer, and 10.3 µL of ddH ₂ O.	initial denaturation at 94 °C for 5 min, denaturation at 95 °C for 45 s, annealing at 55 °C for 60 s, extension at 72 °C for 60 s and a final extension at 72 °C for 10 min. (26)
<i>Klebsiella pneumonia</i>	F: ATTTGAAGAGGTTGCAAACGAT R: TTCACTCTGAAGTTTTCTTGTGTTTC	1.0 µL of template DNA, 13.2 µL of CinnaGen PCR Master Mix, 1.0 µL of each primer, and 8.8 µL of ddH ₂ O.	initial denaturation at 94 °C for 3 min, denaturation at 95 °C for 30 s, annealing at 56 °C for 60 s, extension at 72 °C for 60 s and a final extension at 72 °C for 9 min. (27)
<i>Citrobacter koseri</i>	F: 5-TTAATCATAGACTGCGAGTG-3 R: 5- ATGAGAAACGAGGAAGTCAT-3	1.5 µL of template DNA, 12.5 µL of CinnaGen PCR Master Mix, 1.0 µL of each primer, and 9.0 µL of ddH ₂ O.	initial denaturation at 94 °C for 3 min, denaturation at 95 °C for 30 s, annealing at 58 °C for 60 s, extension at 72 °C for 60 s and a final extension at 72 °C for 9 min. (28)
<i>Klebsiella oxytoca</i>	F: 5- GGACTACGCCGCTCTATCGTCAAG-3 R: 5- TAGCCTTTATCAAGCGGATACTGG -3	1.8 µL of template DNA, 13.5 µL of CinnaGen PCR Master Mix, 1.0 µL of each primer, and 7.7 µL of ddH ₂ O.	initial denaturation at 94 °C for 5 min, denaturation at 95 °C for 35 s, annealing at 56 °C for 45 s, extension at 72 °C for 60 s and a final extension at 72 °C for 9 min. (29)
<i>Diphtheroids</i>	F: 5- ACCGCACTTTAGTGTGTGTG-3 R: 5- TCTCTACGCCGATCTTGTAT-3	1.1 µL of template DNA, 12.3 µL of CinnaGen PCR Master Mix, 1.0 µL of each primer, and 9.3 µL of ddH ₂ O.	initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 50 s, extension at 72 °C for 60 s and a final extension at 72 °C for 9 min. (30)
<i>Yeasts</i>	F: 5- CTGGCTTGGTGTATGT -3 R: 5- CTGGCTTGCTACATAC-3	1.0 µL of template DNA, 13.2 µL of CinnaGen PCR Master Mix, 1.0 µL of each primer, and 8.8 µL of ddH ₂ O.	initial denaturation at 94 °C for 3 min, denaturation at 95 °C for 30 s, annealing at 56 °C for 60 s, extension at 72 °C for 60 s and a final extension at 72 °C for 9 min. (31)
<i>Proteus mirabilis</i>	F: 5-AGAGTTTGATCCTGGCTCAG-3 R: 5- CTACGGCTACCTTGTTACGA-3	0.8 µL of template DNA, 11.8 µL of CinnaGen PCR Master Mix, 0.9 µL of each primer, and 9.4 µL of ddH ₂ O.	initial denaturation at 94 °C for 6 min, denaturation at 95 °C for 33 s, annealing at 56 °C for 32 s, extension at 72 °C for 60 s and a final extension at 72 °C for 10 min. (32)

Statistical analysis

The statistical package for social science (SPSS) software version 20 (IBM Co., Illinois, USA) was used for statistical analysis of data. Student's t-test and proportional test were performed for numerical variables and categorical

variables, respectively. Results were presented as Mean±SD or frequency percentage. A statistically significant difference was considered when p value was less than 0.05.

Results

The mean age for the fertile and infertile groups was 36.3 ± 4.2 and 34.6 ± 3.8 years, respectively. Sperm analysis in both groups were shown in Table 2. The mean semen volume and sperm count as well as mean frequencies of sperm normal morphology, viability and motility (progressive and non-progressive motility) were significantly lower in infertile individuals compared to fertile (for all, p -value ≤ 0.0001).

Bacterial culture showed contamination with various species in 36.5% and 34.3% of fertile and infertile samples, respectively (Table 3). *Enterococcus* and *E. coli* were respectively the most common agents identified in both groups. Also, other isolated microorganisms including *Klebsiella pneumonia* (1.4%), *Citrobacter koseri* (0.6%), *Streptococcus spp.* (0.6%), *Klebsiella oxytoca* (0.3%), *Diphtheroids* (0.3%) and yeasts (0.3%), had very low

prevalence in the semen of fertile men.

Proteus mirabilis (n; 6 of 210, 2.8%) were identified only in infertile patients. There was no significant difference in distribution of different microorganisms between the fertile and infertile groups. PCR using specific primers revealed various microorganisms in 36.6% and 45.7% of the fertile and infertile groups, respectively (Table 4).

In line with culture-based method, PCR showed that *Enterococcus* and *E. coli* were the most common agents in the semen collected from both fertile and infertile men. Molecular characterization showed no significant difference in distribution of different microorganisms between the fertile and infertile groups. According to the culture results, all 6 samples were positive for *P. mirabilis* in PCR (p -value = 0.002). *Candida spp.* (0.3%) was isolated only from semen samples of fertile men.

Table 2. Comparisons of the sperm characteristics in the fertile and infertile groups.

Groups	Semen Volume (ml)	Sperm Count (millions/ml)	Sperm Normal Morphology (%)	Progressive Motility (%)	Non-progressive Motility (%)	Immotile (%)	Viability (%)
Fertile (no. 268)	3.0 ± 1.1	72.0 ± 45.2	4.9 ± 3.9	42.1 ± 9.4	10.4 ± 3.9	47.5 ± 8.9	67.2 ± 7.6
Infertile (no. 210)	2.0 ± 1.0	10.2 ± 0.4	2.1 ± 1.8	12.8 ± 8.5	6.3 ± 4.7	80.9 ± 9.4	36.4 ± 8.2
p value	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*

The data were presented as mean \pm SD, * p -value ≤ 0.05 was considered as significant between fertile and infertile groups.

Table 3. Phenotypic identification of semen microbial contaminations in fertile and infertile groups.

Bacterium	Fertile (no. 268)	Infertile (no. 210)	p value
None	169 (63.5%)	138 (65.7%)	0.813
Enterococcus spp.	50 (18.7%)	36 (17.1%)	0.814
<i>E. coli</i>	21 (7.9%)	24 (11.4%)	0.486
<i>Staphylococcus aureus</i>	17 (6.4%)	6 (2.9%)	0.398
<i>Klebsiella pneumonia</i>	4 (1.4%)	0 (0%)	0.460
<i>Citrobacter koseri</i>	2 (0.6%)	0 (0%)	0.642
<i>Streptococcus spp.</i>	2 (0.6%)	0 (0%)	0.642
Yeast	1 (0.3%)	0 (0%)	0.742
Diphtheroid	1 (0.3%)	0 (0%)	0.742
<i>Klebsiella oxytoca</i>	1 (0.3%)	0 (0%)	0.742
<i>Proteus mirabilis</i>	0 (0%)	6 (2.9%)	0.002*

* p -value ≤ 0.05 was considered as significant between fertile and infertile groups.

Table 4. Molecular diagnosis of semen microbial load in fertile and infertile groups.

Bacterium	Fertile (no. 268)	Infertile (no. 210)	p value
None	168 (63.4%)	114 (54.3%)	0.291
Enterococcus spp.	51 (19.4%)	42 (20.0%)	0.930
<i>E. coli</i>	23 (8.9%)	36 (17.1%)	0.119
<i>Staphylococcus aureus</i>	13 (4.9%)	6 (2.9%)	0.584
<i>Klebsiella pneumonia</i>	4 (1.5%)	6 (2.9%)	0.563
<i>Citrobacterkoseri</i>	2 (0.6%)	0 (0%)	0.642
Streptococcus spp.	3 (0.9%)	0 (0%)	0.568
Yeast	1 (0.3%)	0 (0%)	0.742
Diphtheroid	1 (0.3%)	0 (0%)	0.742
<i>Klebsiellaoxytoca</i>	1 (0.3%)	0 (0%)	0.742
<i>Proteus mirabilis</i>	0 (0%)	6 (2.9%)	0.002*
Candida spp.	1 (0.3%)	0 (0%)	0.742

* p-value ≤ 0.05 was considered as significant between fertile and infertile groups.

Discussion

In the preset study, we isolated different microorganisms from seminal fluid of fertile and infertile men. Our results similar to Baud *et al*, study indicated that there was no significant difference in the prevalence of identified microorganisms between the fertile and infertile males (1). This data was contrast with other studies that shown the prevalence of microorganism in the semen of the infertile was significantly higher than the fertile group and it may be correlated with sperm quality in infertile men (33).

Enterococcus faecalis was commonly isolated from the seminal fluids as it was the most abundant bacterium. However, the prevalence of *E. faecalis* was not significantly different between fertile and infertile men. Similar to our funding, Vilvanathan *et al* in 2016 assessed the presence of bacteria in the seminal sample of infertile men. They reported isolation of *E. faecalis* from 30 % of seminal fluid samples which was the first common organism in the infertile group (12). Also, Machen *et al* in 2018 and Filipiak *et al*

in 2015 reported *E. faecalis* as the second most common bacterium isolated from infertile human semen samples (19, 34). Recently in 2019, Carda-Dieguez *et al* proposed that the source of *Enterococcus* contamination in seminal fluid may well be infection in female vagina or urethra (35).

Escherichia coli is another common bacterium isolated from semen samples (36, 37). There is controversy in scientific reports regarding relation between the presence of *E. coli* in the semen sample and prevalence of male infertility (38, 39). However, there seems to be a positive relation between presence of *E. coli* and acute epididymitis and prostatitis (40, 41).

In a recent study (2018), assessment of microorganisms such as *E. coli* was evaluated in infertile Nigerian patients. The investigators reported that *E. coli* was not only a prevalent organism in the seminal fluid of infertile men, but there seems to be a significant relation between its presence and decrease in sperm parameters including sperm count, motility and morphology (14). Although, *E. coli* was the

second most prevalent bacteria in the semen of infertile men, its prevalence was not significantly different compared to the fertile group. Similar to our finding, Filipiak *et al* reported no statistically significant association between *E.coli* and other facultative anaerobic bacteria with male infertility (42).

Nabi *et al.* in 2013 reported *S. Aureus* to be the main microorganism in the semen culture of men with history of at least two recurrent miscarriages (20). Additionally, Momoh *et al* noted *S. Aureus* as the most prevalent bacterium isolated from 75% of infertile seminal fluid (43). Kaur *et al* in 2010 demonstrated that co-incubation of human spermatozoa with *S. aureus* led to decreased sperm parameters including count, motility, morphology and increased agglutination (44). In the present study, we found this bacterium to be the third most common microorganism in the semen of both fertile and infertile men. Importantly, the frequency distribution of *S. aureus* in the infertile group was insignificantly lower than that in the fertile group. This observation casts doubt on the role of *S. aureus* in male infertility.

We also isolated other microorganisms including *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Citrobacter koseri*, *Streptococcus* spp., Diphtheroids, and yeasts, with very low incidence in the semen of fertile

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men. Due to lack of supporting evidence, the possibility of implication of these microorganisms in male infertility cannot be discussed at the present time. However, *Proteus mirabilis* was isolated in six cases of semen culture from infertile men. Similar to our results, Elgozali *et al* in 2015 also isolated *Proteus* species from semen of infertile man (45). Mehta *et al* observed *Proteus* species in the semen of men with Oligo asthenoterato spermia (46). Therefore, *p. mirabilis* might be implicated in male infertility, though this hypothesis needs further confirmatory studies.

The results of present study didn't show any significant association between male infertility and microbial populations isolated from semen samples. *E. faecalis*, *E. coli*, *S. aureus* and *Klebsiella pneumoniae* were the most prevalent bacteria in the semen of fertile and infertile men, respectively. However, *P. mirabilis* was detected in the semen of infertile men which may be responsible for lack of reproductive ability. However, much more studies are essential to confirm this inference.

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The authors declare that they have no conflicts of interests.

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Semen Bacterial Contamination in Males Infertility

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