

BACTERIAL ANALYSIS FOR SEMINAL FLUID BEFORE IN-VITRO FERTILIZATION PROCEDURE

Abstract: *In vitro* fertilization (IVF) is the most advanced technique of assisted reproductive where the sperm reach the eggs for fertilization in a laboratory tube. Seminal fluid often contains bacterial contaminants that decrease the quality and quantity of seminal fluids. **Aim:** In this study we investigated the presence of bacteria in semen before IVF procedure. **Methodology:** All samples were identified according to the morphological, biochemical, molecular, and antimicrobial tests. **Results:** Microbial species were detected in 28% of semen samples, while 72% did not show any bacterial growth. The prevalent bacterial isolates were *Staphylococcus* spp., *Enterococcus* spp., *Catallicoccus* spp., and *Pseudomonas* spp. **Conclusion:** The use of microbiological analysis to identify resistant bacterial isolates is considered an effective method to reduce infertility in men by changing the contaminated semen.

Key words: Seminal fluid, *In vitro* fertilization, semen, and Infertility

INTRODUCTION

Seminal fluid is an organic liquid that may contain spermatozoa. It is secreted by the sexual glands of male or hermaphroditic animals and can fertilize female ovum. In humans, seminal fluid contains various components besides spermatozoa: proteolytic and other enzymes as well as fructose. These elements of seminal fluid enhance the survival of spermatozoa, and introduce an environment that allow them to swim (Dixson *et al*, 2004). Bacterial assessment of seminal fluid in the weeks preceding *in vitro* fertilization (IVF) procedure is requested to avoid low sperm quality. Contaminated semen by bacteria is the most frequent diagnosis of unsuccessful IVF. The quantity of the pathogenic bacterial flora in semen has been reported to be a serious medical threat as the low-quality sperm cannot fertilize eggs in a laboratory tube. Generally, the deficiency of seminal fluid accounts for 7% of Human infertility (Lotti and Maggi, 2014). Although bacteria isolated from this fluid account for a small proportion of the total microorganism number, they pose opportunistic behaviors that affect sperm quality, along with distortions (especially aberrant motility) loss of DNA integrity and deficient mitochondrial function (Merino *et al.*, 2009, Robertson and

Sharkey 2016). The most frequent bacterial isolates in seminal fluid are *Staphylococcus* spp., *Escherichia coli*, *Enterococcus* spp., *Neisseria gonorrhoea* and *Streptococcus* spp. (Ruggeri *et al.*, 2016). Personal habits such as sexual promiscuity and poor hygiene, increase the bacterial isolates number and change the basic characteristics of semen.

In a hospital, the laboratory protocol uses several antibiotics to treat pathogenic bacterial flora existing in seminal fluid in an effort to increase sperm quality. Moreover, bacterial antibiotics include ciprofloxacin, imipenem, ceftazidime, amikacin, and piperacillin were used (Rotimi *et al.*, 1998).

The aim of the current study is to isolate bacterial contaminants from seminal fluid and study their sensitivity to the antibiotics. Hopefully, by comparing the obtained results from different client's data, could help us to improve the sperm quality along with increasing pregnancy rates using IVF at fertility clinics.

MATERIAL AND METHODS

Study population: The ethical approval was obtained from the Biomedical Ethics Unit at King Abdulaziz University Hospital, Jeddah, Saudi Arabia. From October 2018 to April 2019,

a cross-sectional study conducted for 60 couples undergoing IVF procedure gave their written informed consent to participate in the study and permission for researchers to collect semen samples from males and molecular fluid from females and to access their information, test results, and reproductive history.

The inclusion criteria were fertile men who have an infertile female partner (female factor), and men who have various causes of infertility and unexplained infertility if the female and male underwent screening and not detected any abnormal results). Age between 25-50 years old. The exclusion criteria were men who used antimicrobials (oral or topical) within the previous one month prior to sampling and men with chronic diseases such as cancer and the human immunodeficiency virus (HIV). We collected semen samples from sixty male subjects. The subjects were attending the *in vitro* fertilization clinics of King Abdulaziz University Hospital from October, 2018 to October, 2019. Proper hygiene was ensured in the collection of the samples which was done in one of the private rooms in the laboratory.

Specimen Collection: Semen specimens were collected by masturbation after 3–7 days of abstinence. Before samples collection, instructions were given to the subjects on procedures to be followed to prevent sample contamination. The subject's hands were washed with soap two to three times. The penis, especially the glans and the coronal sulcus, was first cleaned with warm soapy water and then swabbed with 75% alcohol two to three times. The semen was ejaculated directly into a sterile glass receptacle, avoiding contact with the interior of the sterile wall of the container. The freshly collected seminal fluid was used for bacterial analysis, Gram staining, microscopy and molecular technique. The remainder of semen samples were transferred to sterile Eppendorf tubes and stored at -80°C within 2

hours of collection. The exclusion criteria are men using antibiotics for less than three months prior sampling and patients with chronic diseases such as prostate cancer and HIV. We were informed about the time of IVF procedure of each patient.

Cultures: The following bacterial culture media plates were used (chocolate, MacConkey, and blood agar) for aerobic incubation overnight at 37°C. The 24-hour growth was examined for size, shape, elevation, hemolysis and other cultural characteristics. Gram staining was obtained and pure growth was subjected to sensitivity to a range of antibiotics testing according to standard.

Bacterial isolation and identification: To identify the aerobic microorganisms, each semen sample was plated on blood agar, Mac-Conkey agar and chocolate agar and incubated at 37°C for 24-48 hours. The grown colonies were examined after Gram staining. Suspicious colonies were tested with antibiotics and submitted to DNA amplification and gene sequencing (Chesbrough, 2004).

Molecular identification of isolated bacteria

DNA extraction and PCR amplification: The bacterial isolates were individually inoculated in 5 ml of NB medium and incubated in shaking incubator (SI-100, Human Lab, Gyeonggi-do, South Korea) at 37°C to a density corresponding to 2.0 McFarland using spectrophotometer instrument (Genesys 10S UV-Vis, USA). The bacterial DNA was extracted using QiAMP mini DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Polymerase chain reaction (PCR) assay was performed to amplify the 16S ribosomal RNA (rRNA) using universal primer; the forward primer (27-f) (5-

AGAGTTTGATCCTGGCTCAG- 3) and the reverse primer (511-r) (5'-GCGGCTGCTGGCACRKAGT -3) in thermal cycler (Veriti Thermal Cycler, Applied Biosystems, USA) (Pomeroy, 2010). The PCR program was as follows: the initial cycle of 94 °C for 5 min, followed by 32 cycles of 45 s at 94°C, 60°C for 45 s, and 72°C for 90 s, with a final extension at 72°C for 10 min. The DNA concentration and purity were measured by using Nanodrop spectrophotometer. The PCR products were tested onto 1.5% agarose gel and visualized under UV light (versadoc imaging system, Bio- rad, USA) to evaluate the DNA quality. The samples were then sent to Macrogen Company, South Korea for sequencing.

DNA Sequencing: The sequence results for each PCR product were assembled using big dye terminator cycle sequencing kit (Applied Biosystems, UK). Sequence identities were characterized using the Basic Local Alignment search tool (BLAST general GenBank databases from the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>). Sequence alignment of constructions of neighbor analysis was done by MEGA-X. The sequencing data were submitted to GenBank and the obtained accession number was recorded for each isolated bacterial.

Antimicrobial susceptibility test: The susceptibility to seven antimicrobial agents was determined using the Kirby-Bauer agar disk diffusion method. The following antimicrobial agents were obtained from (Oxoid, Hampshire, U.K.), Metronidazole (MET) 5µg, and Clindamycin (DA) 10µg. Aliquots of 100µl from each bacterial suspension were spread-plated on Mueller hinton agar (MHA) (Himedia, Mumbai, India). The antibiotic disks were then applied on the top of plates using the Oxoid Disk Dispenser and the plates were incubated at 37 °C

for 18-24 hours. After incubation, the antibiotic inhibition zone diameters (IZD) were measured in millimeters (mm) (Hudzicki, *et al* 2009). Finally, the results obtained were used to classify isolates as being susceptible, intermediate, or resistant to a particular antimicrobial agent according to Clinical and Laboratory Standards Institute documents (CLSI, 2016).

Statistical Analyses: All the results were analyzed using SPSS version 25 software to determine the significant difference among the data (Armonk, NY: IBM Corp). The data were tested for normality using Kolmogorov–Smirnov test, Shapiro–Wilk tests. According to the type of data qualitative represented as number and percentage, quantitative continues group represented by mean ± SD (for parametric data) median and range (for non-parametric data). T-test: was used for comparison between two groups having quantitative variables with a normal distribution (for parametric data). The differences between three or more groups having quantitative variables were analyzed using ANOVA (f) test. A P-value of < 0.05 was considered statistically significant.

RESULTS

1- Microbial isolation: Overall, 17 (28%) of the 60 semen specimens were positive for bacterial growth, while 43 (72%) were negative for bacterial growth, on the other hand no growth on (Mack) media was reported. Under microscopic test the most frequent bacteria were Gram-positive bacteria; *Enterococcus* spp. (56%) followed by, *Staphylococcus* spp. (31%), and *Catelliococcus* spp. (6%). The presence of Gram-negative was 7% with only one genus (*Pseudomonas* spp.) as shown in Fig. (1). All colonies were counted

using counting forming unit (CFU/ml) as shown in table (1).

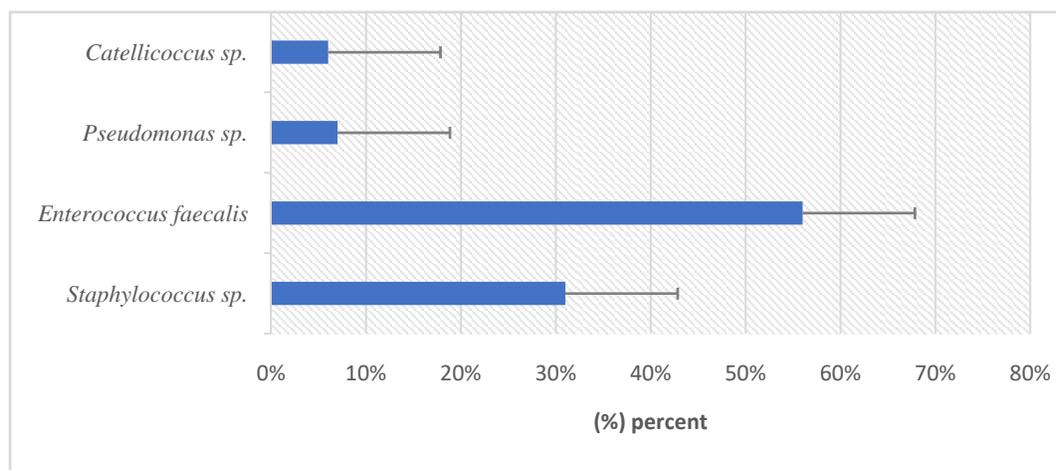


Fig.1:The percentage of bacterial species detected within the seminal fluid samples. Graph showing that the predominant species was *Enterococcus spp.* (56%), *Staphylococcus spp.* (31%), *Catellicoccus spp.* (6%), and *Pseudomonas spp.* (7%).

Isolate no.	BA	CA
	CFU/ml Mean*± SD**	CFU/ml Mean*± SD**
SF1B	11.6×10 ± 1.24	4×10 ±2.1
SF2B	18×10 ±2.14	16.3×10 ±3.08
SF3B	24.6×10 ±2.86	9.3×10 ±1.2
SF4B	9.6×10 ±2.62	2×10 ±.7
SF5B	223×10 ±3.55	282×10 ±3.4
SF6B	4.33×10 ±.2	3.3×10 ±.5

Table 1. The CFU/ml of bacterial colonies isolated on (BA, CA) media at 37°

2- Molecular analysis: The amplification of DNA extracted from the bacterial strains by PCR was done with the 16S universal primer pair. The intense sharp bands on agarose gel appeared with product size of nearly 500 bp for all bacterial isolates. Purification and sequencing were done by Macrogen Company in South Korea. The obtained sequences were aligned and the isolates were identified based on the 82 – 100% similarity of their sequences with that of the known species already published in NCBI databases. All of bacterial isolates could be confidently identified to six different species, SF1B showed 90% similarity with the type strain *Staphylococcus spp.*, SF2B shared 90%

similarities with *Enterococcus faecalis*, SF3B was similar to *Pseudomonas spp.* by 92% similarity for the, SF4B showed 86% similarity with the *Catelllicoccus spp.*, SF5B was 82% similar to *Staphylococcus warneri*, and SF6B was similar to *Enterococcus hiraey* by 88% as shown in table (2).

3- Antimicrobial test: all bacterial isolates were found to be resistant to the mentioned antibiotics: (10µg) clindamycin and (5µg) metronidazole, with the exception of *S. warneri*, which gave inhibition zone with diameter of 11mm, when tested with clindamycin as shown in table (3).

Table 2. Identify of bacterial isolates to the species level and the identity percentage found in the NCBI (National Center for Biotechnology Information) website

Bacterial species	Clindamycin	Metronidazole
	Zone diameter interpretive standards (mm)	Zone diameter interpretive standards (mm)
<i>S. spp.</i>	- (-)	- (-)
<i>E. faecalis</i>	- (-)	- (-)
<i>P. spp.</i>	- (-)	- (-)
<i>Catelicoccus</i> spp.	- (-)	- (-)
<i>S. warneri</i>	11 (s)	- (-)
<i>E. hirae</i>	- (-)	- (-)

Isolate code	Closet related species	Gene bank no.	Similarity (%)
SF1B	<i>Staphylococcus spp.</i>	HM076758.1	90%
SF2B	<i>Enterococcus faecalis</i>	AY942560.1	90%
SF3B	<i>Pseudomonas spp.</i>	GQ243735.1	92%
SF4B	<i>Catelicoccus spp.</i>	KF250929.1	86%
SF5B	<i>Staphylococcus warneri</i>	KM282104.1	82%
SF6B	<i>Enterococcus hirae</i>	KX752826.1	88%

Table 3. Antimicrobial taste results for bacterial isolates from seminal fluid samples using the Kirby-Bauer agar disk diffusion method

DISCUSSION

Contaminating microbiota could be a potential cause for male infertility that reduce the quality and quantity of seminal fluids, whereas normal levels of bacterial isolates are associated with an increased pregnancy rate (Vignera *et al.*, 2013). Many studies have viewed the effectiveness of bacterial semen contamination in male fertility; however, the assumed detrimental effect of bacteria on the sperm quality is still debated (Haidl, 1990). Bacteria can affect the male reproductive ability, causing the agglutination of motile sperm, decreasing the causing alterations in cell morphology and indirectly, ability of acrosome reaction and through the production of reactive oxygen species generated by the inflammatory response to the infection (Tremellen, 2008).

In the present study, four genera and six bacterial species were identified from 17 semen specimens. The bacterial isolates were present in 28% of semen, while there were 72% of semen did not show any bacterial growth. However, there is not clear agreement on the detrimental role of the presence of bacteria in the semen (Maciejewska *et al.*, 2005). We concluded that seminal fluid has different genera of bacteria (*E. faecalis*, *E. hirae*, *P. spp.*, *C. spp.*, and *S. warneri*), which could affect the quality of the sperms. In agreement with our findings, previous studies have investigated the most common pathogenic bacterial isolated from semen were *P. aeruginosa*, *Streptococcus spp.*, *Staphylococcus spp.*, *Proteus spp.* and *Bacillus spp.* Ruggeri *et al.* (2016) evaluated the most four frequent bacterial isolates in seminal fluid including *Escherichia coli*, *Enterococcus faecalis*, and *Streptococcus faecalis*. Furthermore, the most common potentially pathogenic bacteria isolated from semen were *P. aeruginosa*, *Streptococcus spp.*, *Staphylococcus spp.*, *Proteus*

spp., and *Bacillus spp.* (Skau and Folstad, 2018). Another study was in agreement with our suggestion, they reported that infectious bacteria may adversely affect the reproduction and may consequently influence ejaculate production (Skau and Folstad, 2018). Spermatozoa are perceived as “non-self” by the immune system and are exposed to immunological attacks in the male reproductive tract. In another study, pathogenic microorganisms in seminal fluid have decreased sperm density, motility and morphology. For this reason, male infertility could increase (Sasikumar *et al.*, 2013). There are several medical cases of irregular seminal fluid are correlated to hormonal changes, inflammations, and Prostate volumes (Meriggiola *et al.*, 2005). However, the personal bad habits such as sexual promiscuity and poor hygiene could increase the microbial isolates and change the basic characteristics of semen.

The relationship between pathogenic bacterial growth and male infertility, as well as some quantitative and qualitative features of the sperm has been studied. Researchers noted the most common bacterial isolate was *S. aureus*. Therefore, the presence of isolated bacteria in seminal fluid may strongly affects the survival of sperm (Ekhaise and Richard, 2011) these findings have confirmed in the present study. On the other hand, a previous study reported that, the presence of pathogenic bacteria in seminal fluid failed to show cause-and-effect relationship between bacterial infections in seminal fluid and male infertility (Ombelet *et al.*, 1997). To further complicate the problem, the presence of bacteria, in sperm samples of infertile men, has a prevalence very similar to that seen in fertile men. The clinical significance of bacteria still unclear (Ombelet *et al.*, 1997). Normal sperm makes easy and successful fertility, whereas abnormal sperm infected by bacteria or virus

obstruct sperm motility (Robertson and Sharkey, 2016).

All bacterial isolates were identified using the advanced molecular technique based on amplifying the 16S rRNA segment using sequencing analysis method and PCR products. Several researchers identified bacterial species based on this technique to confirm their species (Alnefaieet *al.*, 2019). The molecular identification for six bacterial strains revealed that the identification of microbial isolates using the morphological features, the Gram stain, and the biochemical tests were correct, thus yielding the correct identification for all tested bacterial isolates. For instance, consistent with our data, which reported a molecular size of 16S rRNA region nearly 500 bp for all bacterial isolates were mentioned by (Chen, *et al* 2014).

The conventional embryo culture media for IVF procedure mixed with antibiotics such as gentamycin, penicillin, or streptomycin has an effort to prevent the growth of pathogenic microorganisms while some types of bacteria can be resistant to these particular antibiotics (Lemeireet *al.*, 2007). In our study, we have identified the effects of two different common antibiotics including (Clindamycin, and Metronidazole) on all bacterial isolates using disk diffusion method. We found that, the most only susceptible bacterial isolate to Clindamycin was *S. warneri* according to Clinical and Laboratory Standards Institute (CLSI, 2016).

Conclusion

This study investigated the bacteria within seminal fluid from males undergoing IVF procedure. All the collected semens was not sterile and contained some bacteria that may affect the IVF procedure outcomes. Furthermore, the microbiological analysis of semen from the male partners of couples whose undergoing IVF procedure may

provide an opportunity to initiate antimicrobial treatment prior to the next conception.

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