Journal of Pharmaceutical Research International

32(23): 85-92, 2020; Article no.JPRI.61405
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Bacterial Analysis for Seminal Fluid befor *In-vitro* Fertilization Procedure

S. O. Noor[1*], A. Albalawi[2], H. Abduljabbar[3], Ebtesam H. Alosaimi[1], Sabah M. Hassan[1,4] and A. Najjar[1]

[1]Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.
[2]Microbiology Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia.
[3]Department of Obstetrics and Gynecology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.
[4]Department of Genetics, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Authors' contributions

This work was carried out in collaboration among all authors. Author SON designed the study, wrote the protocol and helped draft of the manuscript. Author AA collected the samples, managed the analyses of the study, performed the statistical analysis. Author EHA wrote the ethical approval. Author HA provided all clinical samples and clinical data and managed the analyses. Author AN managed the literature searches and supervised practical work. Author SMH helped to finalizing the manuscript. All authors read and approved the final manuscript.

Article Information

DOI:10.9734/JPRI/2020/v32i2330793
Editor(s):
(1) Dr. Paola Angelini, University of Perugia, Italy.
Reviewers:
(1) B. Eswari, Sree Vidyanikethan Degree College, India.
(2) Muhjah Falah Hassan, University of Kerbala, Iraq.
Complete Peer review History: http://www.sdiarticle4.com/review-history/61405

Original Research Article

Received 10 July 2020
Accepted 17 September 2020
Published 29 September 2020

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

**Corresponding author: E-mail: sonoor@kau.edu.sa;*

bacterial growth. The prevalent bacterial isolates were *Staphylococcus* spp., *Enterococcus* spp., *Catellicoccus* 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.

Keywords: Seminal fluid; In vitro fertilization; semen; Infertility.

1. 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 [1]. 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 [2]. 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 [3,4]. The most frequent bacterial isolates in seminal fluid are *Staphylococcus* spp., *Escherichia coli*, *Enterococcus* spp., *Neisseria gonorrhea* and *Streptococcus* spp. [5]. 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 [6].

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.

2. MATERIALS AND METHODS

Study population: 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 semin samples from males and foleculafluied from femailes 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 previousone month prior to sampling and men with chronic diseases such ascancer 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 tasted with antibiotics and submitted to DNA amplification and gene sequencing [7].

2.1 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) [8]. 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) 5 µg, and Clindamycin (DA) 10 µg. 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 37ºC for 18-24 hours. After incubation, the antibiotic inhibition zone diameters (IZD) were measured in millimeters (mm) [9]. 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 [10].

2.2 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.

3. RESULTS

3.1 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 bacteriawere Gram-positive bacteria; *Enterococcus* spp. (56%) followed by, *Staphylococcus* spp. (31%), and *Catellicoccus* 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).

3.2 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 *Catellicoccus* spp., SF5B was 82% similar to *Staphylococcus warneri,* and SF6B was similar to *Enterococcus hirae* by 88% as shown in Table (2).

3.3 Antimicrobial Test

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

4. 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 [11]. 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 [12]. Bacteria can affect the male reproductive ability, causing the agglutination of motile sperm, decreasing the causing alterations in cell morphologyand indirectly, ability of acrosome reaction and through the production of reactive oxygen species generated by the inflammatory response to the infection [13].

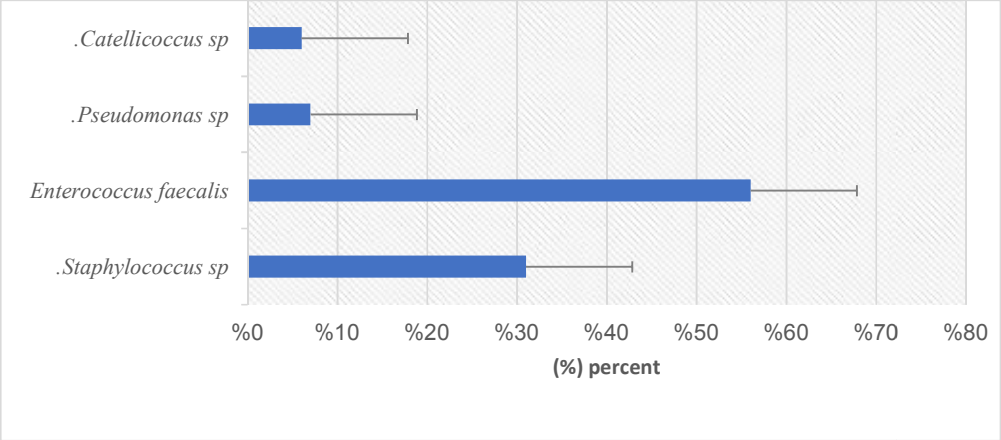

Fig. 1. The percentage of bacterial species detected within the seminal fluid samples
Graph showing that the predominant species was Enterococcus spp. (56%), Staphylococcus spp. (31%), Catellicoccus spp. (6%), and Pseudomonas spp. (7%)

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

	BA	CA
Isolate no.	**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 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)
S. spp.	-	-
	(-)	(-)
E. faecalis	-	-
	(-)	(-)
P. spp.	-	-
	(-)	(-)
*Catellicoccus*spp.	-	-
	(-)	(-)
S. warneri	11	-
	(s)	(-)
E. hirae	-	-
	(-)	(-)

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

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

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 [14] 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. [15] 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. Another study was in agreement with our suggestion, they reported that infectious bacteria may adversely affect the reproduction and may consequently influence ejaculate production [16]. 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 [17]. There are several medical cases of irregular seminal fluid are correlated to hormonal changes, inflammations, and Prostate volumes [18]. However, the personal bad habits such as sexual promiscuity and poor hygiene could increase the microbial isolates and change the basic characteristics of semen [19-21].

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 [22] 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. 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 [23-25]. The clinical significance of bacteria still unclear [26]. Normal sperm makes easy and successful fertility, whereas abnormal sperm infected by bacteria or virus obstruct sperm motility [27].

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 [28]. 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 [29].

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 [30,31,32]. In our study, we have identifiedthe 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 [10].

5. 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.

CONSENT

A cross-sectional study conducted for 60 couples undergoing IVF procedure gave their written informed consent to participate in the study

ETHICAL APPROVAL

Ethical approval was obtained from the Biomedical Ethics Unit at King Abdulaziz University Hospital, Jeddah, Saudi Arabia.

ACKNOWLEDGEMENT

This project was supported by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah. The authors, therefore, acknowledge with thanks DSR for technical and financial support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFRENCES

1. De Cuir JT, Dixson AD. So when it comes out, they aren't that surprised that it is there: Using critical race theory as a tool of analysis of race and racism in education. Educational Researcher. 2004; 33(5):26-31.
2. Lotti. Ultrasound of the male genital tract in relation to male reproductive health. Human Reproduction Update. 2014;21(1): 56–83.

3. Merino G. Bacterial infection and semen characteristics in infertile men. Archives of Andrology. 1995;35(1):43-47.
4. Robertson, David Seminal fluid and fertility in women. Fertility and Sterility. 2016; 106(3):511-519.
5. Ruggeri, Cannas. Bacterial agents as a cause of infertility in humans. New Microbiol. 2016;39(3):206-209.
6. Rotimi, Al-Sweih, Feteih. The prevalence and antibiotic susceptibility pattern of gram-negative bacterial isolates in two ICUs in Saudi Arabia and Kuwait. Diagnostic Microbiology and Infectious Disease. 1998;30(1):53-59.
7. Chesbrough R, Brown J, Broyles M. U.S. Patent Application No. 10/715. 2004;587.
8. Pomeroy. Contamination of human IVF cultures by microorganisms: A review. J Clin Embryo. 2010;75(13):11-30.
9. Hudzicki J. Kirby-Bauer disk diffusion susceptibility test protocol; 2009.
10. CLSI, Performance standards for antimicrobial susceptibility testing. Clinical Lab Standards Institute; 2016.
11. La Vignera, Sandro. Microbiological investigation in male infertility: A practical overview. Journal of Medical Microbiology. 2014;63(1):1-14.
12. Haidl G. Macrophages in semen are indicative of chronic epididymal infection. Archives of Andrology. 1990; 25(1):5-11.
13. Tremellen K. Oxidative stress and male infertility—a clinical perspective. Human Reproduction Update. 2008;14(3):243-258.
14. Maciejewska, Ciupińska, Kurpisz. Bacterial infection and semen quality. Journal of Reproductive Immunology. 2005;67(1-2): 51-56.
15. Ruggeri, Cannas. Bacterial agents as a cause of infertility in humans. New Microbiol. 2016;39(3):206-209.
16. Skau PA, Folstad I. Do bacterial infections cause reduced ejaculate quality? A meta-analysis of antibiotic treatment of male infertility. Behavioral Ecology. 2003;14(1): 40-47.
17. Sasikumar S, Dakshayani D, Franklin A, Samuel R. An *In-vitro* study of effectiveness of uropathogenic yeast on male infertility. International Journal of Current Microbiology and Applied Sciences. 2013;2(5):233-246.
18. Meriggioli MN, Sanders DB. Advances in the diagnosis of neuromuscular junction disorders. American Journal of Physical Medicine & Rehabilitation. 2005;84(8):627-638.
19. Ghoneim IM. Evaluation of the microbial quality of fresh ejaculates of camel (*Camelus dromedarius*) semen. Animal Reproduction Science. 2014;149(3-4):218-223.
20. Hou Dongsheng. Microbiota of the seminal fluid from healthy and infertile men. Fertility and Sterility. 2013;100(5): 1261-1269.
21. Korrovits Paul. Seminal microflora in asymptomatic inflammatory (NIH IV category) prostatitis. European Urology. 2006;50(6):1338-1346.
22. Ekhais, Richard. Common bacterial isolates associated with semen of men attending the fertility clinic of the university of Benin teaching hospital (UBTH), Benin City, Nigeria. African Journal of Microbiology Research. 2011;5(22):3805-3809.
23. Ghasemzadeh Jal. Seminal Bacterial Contaminations: Probable factor in unexplained recurrent pregnancy loss. Iranian Journal of Reproductive Medicine. 2013;11(11):925–32.
24. Korrovits Paul. Seminal microflora in asymptomatic inflammatory (NIH IV category) prostatitis. European Urology. 2006;50(6):1338-1346.
25. Melania. Bacterial agents as a cause of infertility in humans. New Microbiol. 2016; 39(3):206-209.
26. Ombelet W, Bosmans E, Janssen M, Cox A, Vlasselaer J, Gyselaers W, Steeno O. Semen parameters in a fertile versus subfertile population: A need for change in the interpretation of semen testing. Human Reproduction (Oxford, England). 1997; 12(5):987-993.
27. Robertson, David. Seminal fluid and fertility in women. Fertility and Sterility. 2016; 106(3): 511-519.
28. Alnefaie Z, Mutawakil MH, Ahmed MMM, Molecular identification for camel udder microbiota. Advances in Environmental Biology. 2019;13(4):7-13.
29. Chen LC, Papandreou G, Kokkinos I, Murphy K, Yuille AL. Semantic image segmentation with deep convolutional nets and fully connected CRFs; 2014.
DOI: arXiv preprint arXiv:1412.7062
30. Ferlin, Gatta. Male infertility: Role of genetic background. Reproductive Biomedicine Online. 2007;14(6):734-745.

31. Merino G. Bacterial infection and semen characteristics in infertile men. Archives of Andrology. 1995;35(1):43-47.

32. Osoba. Candidemia and the susceptibility pattern of *Candida* isolates in blood. Saudi Medical Journal. 2003;24(10):1060-1063.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/61405