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Determination of Semen Quality and Antibacterial Susceptibility Pattern of Bacteria Isolated from Semen of Iraqi Subjects

Anwer Jaber Faisal[1] and Hamzah Abdulrahman Salman[2]*

[1]*Department of Medical Laboratory Techniques, Al-Farabi University College, Baghdad 10023, Iraq*
[2]*Department of Medical Laboratory Techniques, College of Medical Sciences Techniques, The University of Mashreq, Baghdad 10022, Iraq*

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Infertility is a key issue affecting mood and behavior in men. Microorganisms are one of the primary etiological agents that may be associated with infertility. The objective of the present study was to identify bacterial causative agents from the semen of infertile subjects and determine the effect of bacterial infection on sperm quality, as well as determine the susceptibility of these bacteria to drugs. Forty semen samples from 30 infertile patients and 10 fertile individuals were collected. The pH, volume, motility, and concentration of semen were analyzed. The samples were processed and identified by biochemical testing using API identification kits. The antibiotic susceptibility pattern was determined using the disc diffusion method. Abnormal sperm quality was observed. The mean age of the individual and their sperm morphology, concentration, progressive motility, pH level, and pus cell content were 31.9 years, 2.7%, 10.4 million/ml, 27.3%, 8.3, and 5.7, respectively. Among the tested samples, oligoasthenozoospermia was found to show the highest occurrence, at 27/30 samples, followed by teratozoospermia, at 25/30 samples, and asthenozoospermia, at 22/30 samples. Of the tested infertile patients' sperm, 19, 6, and 5 isolates were identified as *Escherichia coli*, *Klebsiella pneumonia*, and *Staphylococcus epidermidis*, respectively. The results also revealed multi-drug resistance in the bacteria. Compared to that shown by the other tested antibiotics, amikacin showed higher activity against all isolated bacteria. However, the bacteria exhibited maximum resistance against gentamicin, cefotaxime, levofloxacin, and ampicillin. In conclusion, leukocytospermia and bacterial infections are possibly responsible for sperm abnormalities. Multi-drug resistant bacteria were detected. Gentamicin, cefotaxime, levofloxacin and ampicillin were shown the highest resistance, while amikacin was the most effective antimicrobial agent against the isolated bacteria.

Keywords: **Infertility, multi-drug resistant, antibiotics, Amikacin, Leukocytospermia, *E. coli***

Introduction

Semen is a mixture of spermatozoa and fluids produced by the epididymis, bulbourethral, urethral, and prostate glands [1]. Multiple factors contribute to infertility in humans, including defects in reproductive function. Urogenital tract infection is one of the most common causes of infertility in men [2]. Approximately 15% of male infertility is thought to be caused by genital tract infections [3]. Moreover, 65–80% of cases of chronic bacterial prostatitis are caused by *Escherichia coli* causes various male genital tract infections such as epididymitis, urethritis and orchitis, and it is the furthermost recurrently isolated bacterium [4]. In addition to *E. coli*, other bacteria are also isolated from sperm, such as *Staphylococcus epidermidis*, *Streptococcus viridans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* [5, 6].

***Corresponding author**
Tel.: +9647732914480, Fax: +9647732914480
E-mail: hamza.alayash@gmail.com

Leucocytospermia refers to high levels of white blood cells in semen which affect sperm motility and reach up to 30% of infertile cases [7, 8]. By one of these methods, bacteriosperm alters the normal process of fertility: degradation of spermatogenesis, decreasing sperm motility, modified acrosomal response, changed shape, creation of the DNA fragmentation index reactive oxygen species, production of blood test barrier antibody anti-sperm and the genital tract impairment owing to inflammation and fibrosis [9, 10].

Because of their limited medical resources, developing nations suffer predominantly from resistant microorganisms [11–13]. Iraq has a broad and unregulated prescription of antibiotics. However, insufficient data are available on the antibiotic necessities and patient outcomes [13]. Millions of antibiotics were provided in industrialized nations for patients, most of which were unneeded and inappropriate [14]. Many cases of multi-antibiotic resistance bacteria (MAR) have been reported in different countries [11, 13, 15, 16]. However, many studies focused on plant extracts as an alternative to antibiotics for treating bacterial infection [17–19]. Microorganisms may impact male reproductive function by altering cell shape, limiting the ability to perform the acrosome response, and producing motile sperm agglutination [20]. This study aimed to examine the bacterial infection from semen samples of infertile men and determine the influence of bacterial infection on sperm quality and the antibacterial susceptibility testing.

Materials and Methods

Sample collection and processing

Forty semen samples (30 infertile patients and 10 fertile) aged 19–47 years were taken. All patients did not have antibiotic intake for at least 2 weeks. The purpose of the study was fully explained to all the subjects, and a signed consent form was obtained from each individual. Masturbation into a sterile wide-mouth beaker was used to collect sperm samples after a 24 h period of continence. According to World Health Organization [8], all semen samples were liquefied for 30 min at room temperature. The pH, volume, motility, and concentration were tested using an automated sperm quality analyzer machine (CN Meditech, China) following the manufacturer's protocol. The fertile males (control group) had at least one child and normal seminal fluid examination.

Microbiological examination and identification

The semi-quantitative culture approach was used to culture the semen samples, 0.001 ml of un-centrifuged semen was inoculated on the blood agar and MacConkey agar (HiMedia, India). The plates were then aerobically incubated at 37℃ for 24 to 48 h. Any growth of bacteria greater than 10,000 colony forming units (CFU/ml) on the blood agar was considered to be significant [21]. Suspected isolates were identified further by inoculation on different selective culture media, including Eosin Methylene Blue (EMB) agar and Mannitol Salt agar (MSA) (HiMedia). A fixed smear from each suspected colony was prepared on a microscopic slide, and the smear was stained with Gram stain to observe the reaction, cell shape and arrangement. The isolates were subjected to identification based on biochemical tests by using API identification kits (API Strept test, API Staph test, API 20 NE test and API 20 E test) (Bio-merieux, France). Furthermore, a coagulase test was performed for staphylococci species.

Antibiotic susceptibility testing

Antibiotic susceptibility testing of bacterial isolates was performed by the Kirby-Bauer disc diffusion method according to CLSI recommendations [22, 23]. Briefly, one colony from a pure culture of each bacterial isolates was separately inoculated into Mueller Hinton broth (HiMedia) and incubated for 18 h at 37℃. The turbidity of the inoculum was then adjusted to match the 0.5 McFarland standards. The inoculum of each bacteria was then swabbed on the Mueller Hinton agar (HiMedia) using a sterile cotton swab and the antibiotics discs were aseptically applied. All the plates were incubated for 18–24 h at 37℃, and the zone of inhibition was measured by milliliter using a ruler. The tests were repeated thrice to confirm the reproducibility and reliability of the results. The antibiotic discs used in this study were amikacin (10 μg), nitrofurantoin (100 μg), azithromycin (15 μg), cefixim (5 μg), chloramphenicol (10 μg), amoxicillin (10 μg), ciprofloxacin (10 μg), cefotaxime (30 μg), ceftriaxone (10 μg), levofloxacin (5 μg), tobramycin (10 μg), ampicillin (10 μg), tetracycline (10 μg), rifampin

(5 µg), streptomycin (10 µg), and gentamicin (10 µg) (HiMedia).

Statistical analysis

The mean average of the study and other statistical analyses were determined using Microsoft Excel 2013.

Results

All thirty semen samples (patient group) showed abnormal sperm quality. The mean average age of the patients was 31.9 years. The mean average of sperm morphology (2.7%), concentration (10.4 million/ml), sperm progressive motility (≤ 27.3%), pH level (8.3) and pus cell (5.7). The high value of pH and pus cells indicated bacterial infection in semen. While in the control group, the sperm quality was as follows sperm morphology (≥ 4%), concentration (≥ 38 million/ml), progressive motility (42%), and the pH level were between (7.1–7.5). The samples were categorized based on sperm concentration, total motility, progressive motility and morphology, as shown in Table 1.

All the collected samples were found to have bacterial infections. Of the tested infertile patients' sperm, 19 isolates were identified as *E. coli*, 6 isolates were *K. pneumoniae* and 5 isolates were *S. epidermidis*. *K. pneumoniae* showed mucoid pink colonies on MaCconkey agar, while *E. coli* showed pink colonies and metallic greenish color colonies on MaCconkey agar and EMB agar, respectively. *S. epidermidis* showed coagulase-negative reaction and were light pick colonies on MSA agar. The antibiotics susceptibility testing against the isolated bacteria was listed in Table 2.

Table 1. Classification of sperm.

Classification	Control group (n = 10)	Patient group (n = 30)
Normozoospermia	10/10 (100%)	-
Oligoasthenozoospermia	-	27/30 (90%)
Asthenozoospermia	-	22/30 (73.3%)
Teratozoospermia	-	25/30 (83.3%)
Azoospermia	-	0/30
	Total = 10	Total = 30

Table 2. Antibiotics susceptibility testing of *E. coli, K. pneumoniae* and *S. epidermidis.*

Classes	Antibiotics	Bacteria					
		E. coli (19)		*Klebsiella pneumoniae* (6)		*Staphylococcus epidermidis* (5)	
		S	R	S	R	S	R
Aminoglycosides	Amikacin	17 (89.4%)	2 (10.5%)	5 (83.3%)	1 (16.6%)	4 (80%)	1 (20%)
	Gentamicin	4 (21%)	15 (78.9%)	2 (33.3%)	4 (66.6%)	1 (20%)	4 (80%)
	Tobramycin	8 (42.1%)	11 (57.8)	2 (33.3%)	4 (66.6%)	3 (60%)	2 (40%)
	Streptomycin	NA	NA	NA	NA	2 (40%)	3 (60%)
Nitrofurans	Nitrofurantoin	7 (36.8%)	12 (63.1%)	4 (66.6%)	2 (33.3%)	NA	NA
Cephalosporins 3rdG	Cefixime	8 (42.1%)	11 (57.8)	1 (16.6%)	5 (83.3%)	NA	NA
	Cefotaxime	4 (21%)	15 (78.9%)	2 (33.3%)	4 (66.6%)	3 (60%)	2 (40%)
Tetracyclines	Tetracycline	9 (47.3%)	10 (52.6%)	2 (33.3%)	4 (66.6%)	NA	NA
Quinolones	Levofloxacin	5 (26.3%)	14 (73.6%)	4 (66.6%)	2 (33.3%)	NA	NA
	Ciprofloxacin	8 (42.1%)	11 (57.8)	3 (50%)	3 (50%)	3 (60%)	2 (40%)
Chloramphenicol	Chloramphenicol	8 (42.1%)	11 (57.8)	4 (66.6%)	2 (33.3%)	2 (40%)	3 (60%)
Macrolides	Azithromycin	NA	NA	NA	NA	2 (40%)	3 (60%)
Penicillins	Amoxicillin	NA	NA	NA	NA	3 (60%)	2 (40%)
	Ampicillin	NA	NA	NA	NA	1 (20%)	4 (80%)
Rifamycin	Rifampin	NA	NA	NA	NA	2 (40%)	3 (60%)

R: resistant, S: Sensitive, NA: Not Applicable.

Discussion

The clinical issues of infertility are widespread. As both spouses age grows, infertility increases [24]. This study was in accordance with the previous report [25], which indicated that more men undergoing infertility evaluation are above 30 years old. Inflammation or infection was observed at 60% of patients treated with assisted reproductive technology. A strong correlation exists between male infertility and disease of the genital tract [26]. The microorganisms that infect the genital tract come through the urinary tract or unprotected sex [26]. Infertility therapy, including the use of In vitro fertilization and intra-uterine insemination, can be affected by the existence of microorganisms [27].

Among the sperm of the patients, 27/30 (90%) were characterized as oligoasthenozoospermia followed by teratozoospermia 25/30 (83.3%) and asthenozoospermia 22/30 (73.3%) (Table 1). Sperm count less than 15 million/ml in the male ejaculate is known as oligoasthenozoospermia. However, oligoasthenozoospermia is generally linked to sperm motility and morphological abnormalities [28]. In contrast, teratozoospermia is defined as the morpho-defect of more than 85% of sperm cells in ejaculate [29]. While asthenozoospermia refers to low sperm motility [30]. In the present investigation, all the sperm isolated from infertile patients were demonstrated abnormal morphology (2.7%), concentration (10.4 million/ml), progressive motility (27.3%), pH (8.3) and pus cells (5.7). This is maybe attributed to the presence of bacterial infections. Microorganisms can either directly impact male fertility, inducing sperm aggregation, decreasing acrosome responses, altering cell shape, or indirectly by producing reactive oxygen species caused by inflammatory responses to infection and diseases [31]. Bai *et al*., revealed that leukocytospermia is implicated in the reduction of sperm quality but not associated with sexually transmitted infections [32]. However, the effect of microorganisms on men's fertility is still a controversial topic [33].

The present study examined the bacterial infection of humans' seminal fluid. Among the isolated bacteria, *E. coli* (63.3%), *K. pneumoniae* (20%) and *S. epidermidis* (16.6%) were identified. However, previous studies indicated the gram-positive bacteria (*Streptococcus* spp., and *Staphylococcus* spp.) as the most abundant isolated pathogens from seminal fluid [34, 35]. *E. coli* was found to be responsible for reducing the concentration and motility of semen [36, 37]. However, the influence of bacteria on sperm quality remains uncertain [2]. Vilvanathan *et al*. [25] isolated very few strains of *E. coli* (n = 3) and *K. pneumoniae* (n = 2) compared to this study. Nevertheless, the most frequently isolated seminal fluid bacteria were *S. aureus* and *S. epidermidis* [38, 39]. In another study, *E. coli*, *Ureaplasma urealyticum*, *Mycoplasma hominis* and *Chlamydia trachomatis* were observed to influence the sperm [40]. Furthermore, *Enterococcus faecalis*, micrococci, and alpha-haemolytic streptococci have been isolated from infertile males [41].

Numerous research across the world have studied antibacterial sensitivity tests, but relatively few about seminal fluid-isolated microorganisms. The current investigation has shown antibiotics' resistance and sensitivity rates against semen-isolated bacteria (Table 2).

Aminoglycosides are a class of antibiotics responsible for the treatment of gram-positive and gram-negative bacteria. In the present study, aminoglycosides (amikacin, gentamicin, tobramycin and streptomycin) were shown variable susceptibility against the tested bacteria (Table 2). *E. coli* (89.4%), *K. pneumoniae* (83.3%) and *S. epidermidis* (80%) demonstrated the highest sensitivity rate to amikacin (Table 2). Vilvanathan *et al*., showed 100% susceptibility *E. coli* isolated from semen to amikacin [26]. Moreover, amikacin was established as a drug of choice against *E. coli* isolated from UTIs [42]. Our result was in accordance with the previously reported study that showed amikacin as an effective drug against carbapenem-resistant *K. pneumoniae* [43]. Rizwan *et al*., revealed that *E. coli* and *K. pneumoniae* were the least resistant to amikacin, and nitrofurantion, while ampicillin and gentamicin were the highest resistant [44]. Fanos *et al*. reported that 17.5% of *S. epidermidis* strains were resistant to amikacin, similar to what we got in the present study [45].

E. coli (78.9%), *K. pneumoniae* (66.6%) and *S. epidermidis* (80%) were comparatively shown higher resistance to gentamicin (Table 2). Sorlózano-Puerto *et al*., indicated that gentamicin and other antibiotics are not a good treatment of choice for UTIs patients due to their resistance [46]. The present study is contrary to the previously reported studies, where *E. coli* (24.5%),

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K. pneumoniae (24%) and coagulase-negative staphylococci (7.7%) showed less resistance to gentamicin [47]. On the other hand, Salman *et al*., reported a higher resistance rate of *Salmonella enterica* serovar Typhi against gentamicin [13]. Furthermore, *S. epidermidis* (60%) showed higher susceptibility to tobramycin compared to *E. coli* (42.1%) and *K. pneumoniae* (33.3%). However, Sabir *et al*., demonstrated that *E. coli* strains were 100% sensitive to tobramycin [48].

The resistance rate of *E. coli* and *K. pneumoniae* to nitrofurantoin were 63.1% and 33.3%, respectively (Table 2). Thus, our study contradicts the previous report, where nitrofurantoin showed good activity against isolated bacteria and was considered the first choice of treatment for UTIs along with fosfomycin [49].

The 3rd generation of cephalosporin (cefixime and cefotaxime) demonstrated the highest resistance against the isolated bacteria (Table 2). The present results are comparable with the study of Iqbal *et al*., which exhibited a high rate of resistance *E. coli* and *K. pneumoniae* to cefixime and cefotaxime [50].

E. coli (52.6%) and *K. pneumoniae* (66.6%) exhibited resistance against tetracycline (Table 2). A systemic review demonstrated that tetracycline was the second-highest antibiotic-resistant against *E. coli* [51]. Aboderin *et al*., revealed that *E. coli* is totally resistant against tetracycline [52].

Levofloxacin and ciprofloxacin (quinolones) showed various resistant rates against isolated bacteria. Among quinolones, *E.coli* (73.6%) showed higher resistance to levofloxacin, while *K. pneumoniae* displayed less resistance (33.3%). However, the resistance rates of *E. coli* and *K. pneumoniae* were 57.8% and 50%, respectively, against ciprofloxacin (Table 2). Thus, the resistance of quinolones obtained in this report was higher than those reported earlier [53]. Therefore, the present study suggests that quinolones are inappropriate for empiric treatment of UTI, which is consistent with the previously reported study [53].

In the present investigation, the strains of *E. coli* (57.8%), *K. pneumoniae* (33.3%) and *S. epidermidis* (60%) were resistant to chloramphenicol (Table 2). However, the resistance rate of the present study was contrary to the reports published recently where chloramphenicol was a highly active treatment [54, 55].

Azithromycin is an anti-inflammation antibiotic that belongs to the class macrolide with excellent activity against gram-negative and gram-positive bacteria [56]. However, azithromycin has antiviral activity against several viruses, and it has been included in the treatment protocol of covid-19 [57]. *S. epidermidis* in the current study showed 60% resistance to azithromycin (Table 2). The resistance rate of *S. epidermidis* to amoxicillin and ampicillin were 40% and 80%, respectively. However, Božić *et al*., demonstrated that amoxicillin-clavulanic acid is an effective antibiofilm against *S. epidermidis* [58]. *S. epidermidis* was resistant to 60% rifampin (Table 2). Previously, rifampin was a very effective antibacterial agent against staphylococcal biofilm. Szczuka and Kaznowski, have demonstrated that the combination of rifampin and tigecycline gives higher antibiofilm activity [59].

The study concluded that the presence of microorganisms affects male fertility by influencing sperm quality. *E. coli* was the most prevalent bacteria in the semen, followed by *K. pneumoniae* and *S. epidermidis*. Multidrug resistance was observed in this study. Amikacin was the most effective antimicrobial agent against the isolated bacteria. While gentamicin, cefotaxime, levofloxacin and ampicillin were shown highest resistance. Therefore, not consuming these antibiotics is one of the priorities of publishing this project. Further investigations are warranted to find out natural antibacterial agents to reduce bacterial resistance.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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