JOURNAL OF TECHNIQUES

Journal homepage: http://journal.mtu.edu.iq

RESEARCH ARTICLE - MEDICAL TECHNIQUES

The Effect of Gram Negative Bacteria on Semen Parameters of a Sample of Infertile Men and Antibiotic Susceptibility Tests

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Article Info.

Article history:

Received
15 February 2022

Accepted
25 April 2022

Publishing
30 June 2022

Abstract

The most Gram statistically significant cause of male infertility is male urogenital tract infection, being related to 8%-35% of male infertility. The detrimental effect of bacterial infection on male infertility was in no whole agreement. The aim of this work showed the influence of Gram negative bacteria on semen quality among infertile males. A total of 50 semen specimens were collected from infertile men and 50 samples from the healthy individual as the control group were attending Kamal Al-Sammrai Hospital / Baghdad during the period 25 May 2020 to 15 October 2020. Bacteriospermia identification in semen specimens was first prepared by the classical culture methods and confirmed by Vitek 2 system. The present study revealed that 38% of isolates were Gram negative and 62% was no growth. Escherichia coli (22%) followed by Enterobacter cloacae (4%), Klebsiella pneumonia (4%), and each of Raoultella ornithinolytica, Pseudomonas aeruginosa, and Citrobacter freundii at a percentage (2%) and only one (1%) unidentified. This work indicated that some Gram negative bacteria lead to poor health of seminal fluid. Record of Gram negative bacilli was sensitive to Vancomycin, Rifampicin, and Trimmethoprim/sulfamethoxazole combination drug while Fusidicacid was resistant from most Gram negative bacilli. Semen investigation for bacterial detection in infertile males should be done regularly because bacteria may be hurting the semen quality.

Publisher : Middle Technical University

Keywords: Antibiotic Sensitivity; Infertility; Seminal Fluid; Vitek 2 Compact System; Gram Negative Bacteria.

1. Introduction

Sterility or subfertility is well-distinct by way of the disappointment to succeed in a clinical gestation after 1 year of consistent and undefended sexual intercourse. Subfertility male is a clinical condition that records for just about 30% of propagative-aged couples globally [1, 2]. There are various reasons for male infertility including spermatogenesis disorders, chronic diseases, sexually transmitted diseases (STDs), and Male urogenital tract infections [3]. The last cause was the most important condition as infection in the genital tract and inflammation have been concomitant with 8-35% of male infertility circumstances [4]. One of the primary and important issues in male infertility is bacteriospermia, which leads to nonstandard semen quality in addition to causing weakness of sperm roles and seminal tract obstacles [5]. Bacteriospermia and subsequently leukocytospermia can deleteriously impact male fertility through numerous mechanisms, including participation in spermatogenesis, weakening of sperm function, and dysfunction of the genital tract [6]. The standards for infection-associated infertility have been laid down in the World Health Organization (WHO) manuals [7]. To detect pathogenic bacteria found in seminal fluid, methods that require culturing of bacteria have routinely been used [8-10]. This work aimed to detect the influence of Gram negative bacteria on semen parameters amongst infertile men and their antibiotic susceptibility.

2. Materials and Methods

2.1. Seminal fluid collection

Fifty semen specimens were collected from each infertile and fertile man (control group), attending Kamal Al-Samarrai Hospital for Infertility & *In Vitro* Fertilization Infants in Baghdad in the period from 25 May 2020 to 15 October 2020. Collected samples were done by masturbation into a glass with wide-mouth or plastic containers, provided by the laboratory, after 3–7 days of sexual abstinence. The sample was transmitted to the research laboratory directly and located in an incubator at 37 ^{o}C until whole liquefaction.

Nomenclature			
WHO	World Health Organization	N	Not tested
AST	Antimicrobial Susceptibility Testing	S	Sensitivity
TAT	Turnaround Time	R	Resistance
GNB	Gram Negative Bacilli	I	Intermediate
SPSS	Statistical Package for the Social Sciences		

2.2. General seminal analysis

Semen analysis was achieved according to WHO recommendations to assess pH, volume, the existence of pus/immature cells, sperm motility, sperm concentration, and normal/abnormal morphology [7].

2.3. Semen culture

The collected seminal fluid specimens were left for 30 minutes for dissolving before processing. Four solid media were used to inoculate 0.1ml of the specimen for routinely laboratory cultures, the media used were Chocolate agar, Blood agar, MacConkey agar, and Mannitol agar. The routine media were incubated in aerobic atmosphere at 37^0C for 24hours while chocolate agar was incubated in an anaerobic jar at 37^0C [11].

2.4. Vitek 2 compact system

Fig. 1 Vitek 2 system is a compact, programmed device addressing microbial proof of identity and antibiotic susceptibility test (AST) through decreasing performance time to improved workflow and fast recording. The TAT is 2 to 18 h, although primary organism isolation is necessary. Vitek 2 system is considered a cost-effective, space-good system. The principle of the technology used by this system depends on a fluorogenic methodology for organism documentation and a turbidimetric system for antibiotic susceptibility tests.

Fig 1. Vitek 2 compact system

3. Results

3.1. Distribution of Gram negative bacteria in Semen according to their species

The distribution of organism's species is diagnosed by Vitek 2 Compact method shown in Table 1. Of a total of 50 semen specimens, 19 (38%) were culture positive and 31 (62 %) showed no growth by classical culture method. From 19 (38%) positive cultures, 18 (36%) showed significant Gram negative bacilli (GNB) and only one (2%) was an unidentified organism by vitek 2 compact. Escherichia coli was the most frequent with an occurrence of 11(22%) followed by Enterobacter cloacae and Klebsiella pneumonia as 2 (4%) for each one and 1 (2%) for each of Raoultella ornithinolytica, Pseudomonas aeruginosa, and Citrobacter freundii.

Table 1 Distribution of Gram negative bacteria in semen according to their species

Organisms by classical culture method	Number (50)	Percentage% 100.00%
Organisms by vitek 2 compact	19	38%
Gram negative bacilli	18	36%
Escherichia coli	11	22%
Enterobacter cloacae	2	4%
Klebsiella pneumonia	2	4%
Raoultella ornithinolytica	1	2%
Pseudomonas aeruginosa	1	2%
Citrobacter freundii	1	2%
Unidentify	1	2%
No growth	31	62%

3.2. Comparison of the effect of Gram negative bacteria on seminal fluid physical parameters

The results in Table 2 show a comparison of semen volume and semen pH according to the results of the culture results. It has been reported that the semen volume decreases in patients infected with E. cloacae (1.0±00) showing a significant difference p value ≤ 0.01 compared with control however, it shows low significance according to WHO. While E. coli and K. pneumoniae have no significant differences in comparison to the control group. Regarding semen pH, it was no significant difference between Gram negative bacteria and semen pH compared to the control group. The results of R. ornithinolytica, P. aeruginosa, and C. freundii were not worthy when showed a significant effect on semen volume and pH.

Table 2 Comparison of the effect of Gram negative bacteria on seminal fluid physical parameters

Parameter / Type of bacteria	Volume/mL N (1.5ml-6ml) Mean±SD	pH N (7.2-8.0) Mean±SD
Control	2.41±0.82	7.48±0.85
E. coli	1.94±1.11*****	7.57±0.09 *****
E. cloacae	1.0±00 **b**	7.65±0.07 *****
K. pneumoniae	1.10±1.27 *****	7.65±0.21 *****
R.ornithinolytica	1.0±0	7.80±0
P. aeruginosa	1.0±0	7.80±0
C.freundii	4.0±0	7.70±0

*(non-significant), b (P≤0.01)

3.3. Comparison of the effect of Gram negative bacteria on sperm count and motility

The effect of isolated Gram negative bacteria on semen count and the type of motility as shown in Table 3 reveals a highly significant difference in sperm count in infected patients with K. pneumoniae were a p-value ≤0.01. While E. Coli and E. cloacae show no significant were p ≥ 0.05 in comparison with the control group, a highly significant difference was seen in comparison with those fixed by WHO.

Table 3 Comparison of the effect of Gram negative bacteria on sperm count and motility

Parameter / Type of bacteria	Count million sperm/ml N (15Million sperm/ml) Mean ± SD	progressive motility N (32% or more) Mean ± SD	Total motility N (at least 40%) Mean ± SD	Dead cells N (Not more than 60%) Mean ± SD
Control	46.54±19.38	20.37±9.60	56.70±13.82	43.30±13.82
E. coli	35.86±31.0 *****	0.45±1.50 **c**	28.63±20.74 **a**	53.18±30.35 *****
E. cloacae	32.5±3.53 *****	2.50±3.53 *****	47.5±10.60 *****	52.5±10.60 *****
K. pneumoniae	35.50±48.79 **b**	0.0±0 **a**	32.5±38.89 **b**	67.50±38.89 **b**
R.ornithinolytica	15.0±0	0.00±0	25.0±	75.0±0
P. aeruginosa	18.0±0	0.0±0	45.0±0	55.0±0
C.freundii	14.0±0	0.0±0	45.0±	55.0±0

*(non-significant), a (P≤0.05), b (P≤0.01), c (P≤0.001), Diverse litters=significant difference

Regarding sperm motility, the present study showed that there was statistically significant progressive motility P≤0.001 in E. coli and P≤0.05 in K. pneumoniae, while in the case of E. cloacae, no significant difference was seen in comparison to the control group. There was a statistical difference in the total motility of infected patients' sperm P≤0.05 and P≤0.001 in patients infected with E. coli and K. pneumoniae respectively, while in the case of E. cloacae, there was no such significance in comparison to the control group. K. Pneumonia revealed a statistical difference of P≤ 0.01 between dead cells and the control group among infertile men. In contrast to the control group, P 0.05, there was no significant difference between E. coli, E. cloacae, and dead cells. The results of R. ornithinolytica, P. aeruginosa, and C. freundii were not worthy when they showed a significant effect on semen count and sperm motility.

3.4. Comparison of the effect of Gram negative bacteria on sperm morphology

Table 4 shows the mean proportion of defective and normal sperm morphology based on Gram negative bacterial isolates as follows:
Concerning abnormal sperm, there are statistically significant differences between E. coli and K. pneumoniae (P≤ 0.05). However, no significant difference between E. cloacae and the control group (P ≥0.05).
Regarding normal sperm, there are no statistically significant differences between E. coli and E. cloacae (P ≥0.05). However, a significant difference between K. pneumonia and the control group (P≤ 0.05).
The results of R. ornithinolytica, P. aeruginosa, and C. freundii were not worthy when they showed a significant effect on semen abnormality and normal sperm.

Table 4 Comparison of the effect of Gram negative bacteria on sperm morphology

Parameters / Types of bacteria	Abnormal Mean ± SD	Normal N (30% or more) Mean ± SD
Control	38.60±16.15	61.40±2.28
E.coli	55.0±29.32 **a**	26.81±17.21 *****
E. cloacae	67.5±3.53 *****	32.5±3.53 *****
K. pneumoniae	62.5±38.89 **a**	37.50±38.89 **a**
R.ornithinolytica	70.0±0	30.0±0
P. aeruginosa	75.0±0	25.0±0
C.freundii	70.0±0	30.0±0

a (P≤0.05), *(non-significant)

3.5. Comparison of the effect of Gram negative bacteria on semen immature cells and pus cell

The results in Table 5 show a comparison between immature cells, pus cells, and Gram negative bacteria. The patients infected with *E. cloacae* and *K. pneumoniae* had a significant difference of P≤0.01 in immature cells in comparison with the control group. While the results of patients infected with *E. coli* were a highly significant difference of P≤0.001of immature cells in comparison with the control group. However, the patients infected with *E. cloacae, K. pneumoniae*, and *E. coli* showed significant differences according to WHO.

Table 5 Comparison of the effect of Gram negative bacteria on semen immature cells and pus cell

Types of bacteria \ **Parameters**	**Immature cells N (<5 HPF)**	**Pus cells N (≥1 × 10^6/mL**
	Mean ± SD	Mean ± SD
Control	0.76±0.79	0.52±0.76
E. coli	2.72±3.52 **c**	9.18±5.60 **c**
E. cloacae	0.0±0 **b**	7.0±4.24 **c**
K. pneumoniae	0.0±0 **b**	6.0±2.82 **c**
R.ornithinolytica	6.0±0	12.0±0
P. aeruginosa	8.0±0	20.0±0
C.freundii	0.0±0	6.0±0

b (P≤0.01), c(P≤0.001), Diverse litters=significant difference

In terms of pus cells, there were statistical differences (P≤0.001) between groups infected with E. coli, E. cloacae, and K. pneumoniae and the control group. All isolated bacteria have been highly significant in contrast with WHO. The results of R. ornithinolytica, P. aeruginosa, and C. freundii were not worthy when they showed a significant effect on semen pus cells and immature cells.

3.6. Antibiotic Susceptibility patterns of Gram negative bacteria in semen

Table 6 shows the antibiotic susceptibility patterns of Gram negative bacteria. According to the current study, the sensitivity rate of E. coli to Gentamicin, Rifampicin, and Vancomycin was 45.5%, 36.4%, and 27.3%, respectively. Nitrofurantoin, Trimmethoprim/sulfamethoxazole, Moxifloxacin, Levofloxacin, and Fusidic acid had resistance rates to E. coli of 72.7%, 54.5 %, 54.5%, 45.5%, and 45.5 % respectively. Vancomycin, Nitrofurantoin, and Trimethoprim/sulfamethoxazole were all found to be 50% effective against E. cloacae. Whereas Teichoplanin and Fusidic acid both have a high rate of resistance to E. cloacae 100.0%. K. pneumonia was sensitive to Linezolid in 50.0 % of cases and Vancomycin in 50.0 % of cases. The resistance rate of K. pneumonia to Erythromycin, Teichoplanin, Tetracycline, and Nitrofurantoin was 100.0%. C. freundii has a sensitivity rate of 100.0 % to Gentamicin, Levofloxacin, and Trimethoprim/sulfamethoxazole, as well as a resistance rate of 100.0 % to Nitrofurantoin.
Tobramycin, Clindamycin, Linezolid, Vancomycin, and Rifampicin have a 100% sensitivity rate against P. aeruginosa and R. ornithinolytica.

Table 6 Antibiotic Susceptibility patterns of Gram negative bacteria in semen

Organisms	**Antibiotic**	**Gentamicin**	**Tobramycin**	**Levofloxacin**	**Moxifloxacin**	**Erythromycin**	**Clindamycin**	**Linezolid**	**Teichoplanin**	**Vancomycin**	**Tetracycline**	**Nitrofurantoin**	**Fusidic acid**	**Rifampicin**	**Trimethoprim/ sulfamethoxazole**
***E.coli* (N=11)**	N%	-	90.9	36.4	45.5	72.7	81.8	81.8	45.5	72.7	72.7	-	54.5	54.5	-
	R%	54.5	-	45.5	54.5	27.3	-	-	27.3	-	27.3	72.7	45.5	9.1	54.5
	S%	45.5	9.1	18.2	-	-	18.2	18.2	-	27.3	-	18.2	-	36.4	45.5
	I%	-	-	-	-	-	-	-	27.3	-	-	9.1	-	-	-
***E. cloacae* (N=2)**	N%	50.0	50.0	100	100	50.0	50.0	100	-	50.0	50.0	50.0	-	-	50.0
	R%	50.0	50.0	-	-	50.0	50.0	-	100	-	50.0	-	100	50.0	-
	S%	-	-	-	-	-	-	-	-	50.0	-	50.0	-	-	50.0
	I%	-	-	-	-	-	-	-	-	-	-	-	-	50.0	-
***K.pneumonia* (N=2)**	N%	100	50.0	50.0	50.0	-	50.0	50.0	-	-	-	-	50.0	50.0	-
	R%	-	50.0	50.0	50.0	100	50.0	-	100	50.0	100	100	50.0	-	100
	S%	-	-	-	-	-	-	50.0	-	50.0	-	-	-	-	-
	I%	-	-	-	-	-	-	-	-	-	-	-	-	50.0	-
***C.feundii* (N=1)**	N%	-	100	-	100	100	100	100	100	100	100	-	100	100	-
	R%	-	-	-	-	-	-	-	-	-	-	100	-	-	-
	S%	100	-	100	-	-	-	-	-	-	-	-	-	-	100
***P.aeruginosa* (N=1)**	N%	100	-	100	100	100	-	-	-	-	100	100	100	-	100
	S%	-	100	-	-	-	100	100	-	100	-	-	-	100	-
	I%	-	-	-	-	-	-	-	100	-	-	-	-	-	-
***R. ornithinolytica* (N=1)**	N%	100	-	100	100	100	-	-	-	-	100	100	100	-	100
	S%	-	100	-	-	-	100	100	-	100	-	-	-	100	-
	I%	-	-	-	-	-	-	-	100	-	-	-	-	-	-

N: Not tested, S: Sensitivity, R: Resistance, I: Intermediate

3.7. Statistical Analysis

Data analysis was done by utilizing the Statistical Package for the Social Sciences (SPSS), version 24. The test used for comparison between means was the one-sample T-test, and the percentage (0.05 and 0.01) of probability can be found by using the Chi-square test.

4. Discussion

Many studies have found a link between bacterial infection in the genital tract and male fertility, but the evidence for a deleterious impact of bacteria on sperm density is still being contested [12]. Various bacterial species can directly affect male reproductive function, causing the clumping of sperm motility, decreasing the capability of acrosome reaction, and initiating variations in cell morphology—and indirectly, overproducing of reactive oxygen species created by the inflammatory reaction as a result of infection [13].

4.1. Distribution of organisms in semen according to their species by Vitek 2 compact method

The present study indicated that E. coli was the most frequent organism, followed by Enterobacter cloacae, K. pneumoniae, R. ornithinolytica, P. aeruginosa, and C. freundii as shown in the Table 1. The current study, similar to the study done by Hakim et al. [14], recorded Escherichia coli 9 (17.3%) as the most predominant isolate. The same finding was reported by Ekhaise and Richard [15] in Benin City, Nigeria. A study done by Sasikumar showed the most predominant bacteria is Staphylococcus aureus (43.33%), followed by Klebsiella spp. (10%), Proteus mirabilis (6.66%), Escherichia coli (3.33%) and Pseudonomas aeruginosa (3.35%) [16]. The reason for this diversity may be different techniques used for diagnosis. On the other hand, it is possible that patients do not follow all hygiene instructions when collecting samples, resulting in contamination of the specimen and the detection of a variety of bacteria species.

4.2. Comparison of the effect of Gram negative bacteria on seminal fluid physical parameters

The result of the present study about the effect of Gram negative on semen pH and volume was shown in Table 2. From a diagnostic point of view, semen pH cannot be mentioned as a tool to separate infected from non-infected patients due to its low sensitivity and specificity [17]. In terms of semen volume, the current study found no agreement with Al-Saadi [18], who found the highest semen volume (2.730.7) among Enterococcus-infected patients. This diversity may be because the patients don’t comply with the period of abstention before the semen analysis test.

4.3. Comparison of the effect of Gram negative bacteria on sperm count and motility

Another semen parameter is sperm concentration, which plays a vital role in male infertility [19]. The results of the present study were consistent with previous studies showing that sperm concentration was reduced in infected specimens in contrast with non-infected samples [20]. Another study done by Filipiak et al. [21] found the possible causative role of bacteriospermia. Consequently, leukocytospermia of infected samples has harmful effects on sperm concentration. Regarding sperm motility, E. coli and S. haemolyticus were in contact directly with sperm by their bacterial organelles. The attachment agents of the bacteria, such as pili or fimbriae and mannose receptor-dependent interactions with sperm, directly immobilize spermatozoa and affect their motility and morphology [22]. The result of a similar study found the motility of the sperm in the presence of E. coli showed a general reduction in grades A, B, and C, while grade D demonstrated higher motility [18]. Meanwhile, the sperm motility in the presence of Klebsiella sp. showed a decline in grade A motility with a p-value of 0.001. Furthermore, no significant differences ($p > 0.05$) were found in other grades [18], which disagrees with this result when K. Pneumoniae shows a decline in both grades A and B and higher in dead cells. On the other hand, a study done by Nasrallah et al. [23] disagrees with the current results, which find all the semen parameters were not significantly affected owing to bacteriospermia.

4.4. Comparison of the effect of Gram negative bacteria on sperm morphology

As shown in Table 4, Gram negative bacteria have a poor effect on normal sperm. Consistent with the current result, Khadim and Al-Bermani show there was a significant difference characterized by an extremely reduced percentage of normal sperm ($P \leq 0.01$) in E. coli, and there was no important alteration ($P \geq 0.05$) in the percentage of normal sperm between the control group and E. cloacae infection [24]. The harmful effects of numerous microbial pathogens on spermatozoa not only result from the tight adhesion of interacting cells, but also the expression of other surface virulent factors, such as lipopolysaccharides, cytotoxic necrotizing factor, a-haemolysins, b-haemolysins, and from the release of soluble spermatotoxic factors such as sperm immobilization factor [25]. For example, E. coli haemolysins might be involved in the molecular mechanism that ultimately alters the membrane integrity. No association was found between semen culture with bacterial growth and sperm parameters (concentration, motility, and morphology) were noticed by Esfandiari et al. [26]. The reason is that some bacteria show no harmful effect on sperm. This may be due to the patient being in the late stage of infection and the bacteria having no harmful effect on sperm.

4.5. Comparison of the effect of Gram negative bacteria on semen immature cells and pus cell

The presence of leukocyte in semen specimens, with or without bacteriospermia, had a detrimental influence on semen quality, including sperm concentration, motility, and morphology as indicated by Domes, et al. [27]. Leukocytospermia could be a predictor of bacterial infection in infertile men. This is because potential pathogens in the genital tract lead to an inflammatory process with an increase of leukocytes in the seminal fluid. In this regard, Feraczek et al. (2014) suggested that microbial detection should be recommended in semen samples with leukocytospermia, especially in infertile men. Several studies have found no statistically significant relationship between leukocytospermia and bacteriospermia in ejaculated sperm [28]. This controversy may be due to the elimination of bacteria in the urogenital tract by leukocytes in the final stage of the inflammatory process.

4.6. Antibiotic Susceptibility patterns of Gram negative bacteria in semen

To reduce the need for broad-spectrum antibiotics and treat infections with suitable drugs, the antimicrobial susceptibility of infecting bacteria needs to be determined in the early stage of infection to make a private treatment design. Therefore, rapid antimicrobial susceptibility testing (AST) is critical for treating infections with the correct antibiotics, which will reduce the death caused by antibiotic-resistant bacteria and slow down the occurrence of antibiotic resistance consequently [29]. In the study done by Bhatt et al. [30], there was no agreement with recent studies which showed E. coli was sensitive to Nitrofurantoin (76.9%), followed by Levofloxacin (69.2%), Ampicillin Sulbactam (57.6%), Gentamycin (61.5%), and Co-Trimoxazole (50%). Another study found E. coli, the more frequently isolated bacterium, showed a high level of resistance to 70.0% for Trimethoprim/Sulfamethoxazole and K. pneumoniae isolates demonstrated a high level of resistance of 66.7% for Trimethoprim/Sulfamethoxazole [31], which contradicts the current study, which revealed that the resistance rate of E. coli and K. pneumoniea to Trimethoprim/Sulfamethoxazole was 54.5% and 100%, respectively. The susceptibility pattern of gram-negative bacteria against antibiotic sensitivity discs was shown to be highly active (100%) toward Gentamycin [14]. This study is different from the present study because it found only C. freundii has 100% sensitivity to Gentamycin. A wide range of broad-spectrum antibiotics can be used as an empirical treatment for infertile patients, like Vancomycin, Rifampicin, and Trimmethoprim/sulfamethoxazole. Adjusting the seminal parameters and reducing the number of leukocytes in semen ejaculates could be beneficial.

5. Conclusion

Routine bacterial culture methods are still important in diagnosing bacteria. *E. coli* was the most common bacteria diagnosed by Vitek 2 compact method. Some isolated bacteria hurt semen parameters. Vancomycin, Rifampicin and Trimmethoprim/sulfamethoxazole were the most susceptible drug against Gram-negative bacteria.

Acknowledgement

I'd want to express my gratitude to my supervisors, Prof. Dr. Mohammed Sh. jebur and Lecture Dr. Jaleel Samanje, for their help and guidance**.** Many thanks to the staff of Kamal al-Samarrai Hospital-Fertility Center of Infertility Treatment Laboratory for helping me during my work.

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