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Original Research Article

Isolation and Identification of Gram-Positive and Gram-Negative Bacteria from Infertility Patients in Tikrit City

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Abstract: The objectives of the current study were conducting a statistical study to find out the number of men with primary and secondary infertility, immune infertility, and unexplained infertility in the city of Tikrit. Isolation and identification of Gram-positive and Gram-negative aerobic bacteria, and the study of some patterns of sensitivity to antibiotics of the isolated bacteria. The results showed that the percentage of primary infertility was the highest among infertility diseases, as the percentage reached 57%, while the lowest percentage was for immunological infertility, as it amounted to 7%. While the percentage of secondary and unexplained infertility was 15% and 31%, respectively. About the bacteriological study, the results showed two types of isolates, single bacterial isolates (52%), mixed bacterial isolates (38%). Percentages of isolates from E. coli and Klebsiella spp. and Staphylococcus spp. and Streptococcus spp. were 100%, 12%, 18%, and 10%, respectively. E. coli showed total (100%) resistance to amoxicillin/clavulanic acid and ampicillin. 50% resistance to Nalidixic. Otherwise, Escherichia coli showed resistance of 40%, 20%, 60%, 20% and 50% to Imipenem, Amikacin, Gentamicin, Azithromycin and Cefepime respectively. *Klebsiella* showed total (100%) resistance to amoxicillin/clavulanic acid and ampicillin. 40% resistance to Nalidixic. Otherwise, Klebsiella spp. showed resistance of 20%, 10%, 10%, 40% and 20% to imipenem, amikacin, gentamicin, azithromycin and cefepime respectively. Staphylococcus aureus showed complete resistance to penicillin, oxacillin and cefoxitin. 10%, 0%, 30%, 30%, 0%, 90% and 50% are resistant to gentamicin, nitrofurantoin, erythromycin, azithromycin, rifampicin, trimethoprim and ciprofloxacin respectively. Streptococcus spp. completely resistant to penicillin, oxacillin and cefoxitin. 30%, 0%, 20%, 10%, 0%, 70% and 30% are resistant to gentamicin, nitrofurantoin, erythromycin, azithromycin, rifampicin, trimethoprim and ciprofloxacin respectively.

Keywords: Infertility, Gram-negative aerobic bacteria, antibiotics, immune infertility.

INTRODUCTION

Infertility is defined as the inability to conceive after at least one year of marriage and sexual intercourse between a man and a woman, and affects about 8-12% of couples worldwide. 20-25% of the inability to conceive can be due to the male alone (WHO 2018). Between different countries and states, rates of infertility vary widely, consistent with the incidence of preventable conditions, which can lead to infertility. In some regions, particularly in sub-Saharan Africa, up to a third of couples are infertile and about 52% suffer from acquired infertility. On the contrary, the rate of secondary infertility is lowest in Asia and developed countries; 23% and 29%, respectively (Larsen 2000). Infertility is a problem because even the most comprehensive work-up that includes physical examinations, serological and hormonal tests, detailed semen analysis, and imaging techniques may fail to detect the etiology of reproductive disorders (Crosignani *et al.*, 1993). The term primary infertility is used to refer to couples who have never had children, and the term secondary infertility refers to couples who have had at least one pregnancy but are unable to achieve it again (Kazemijaliseh *et al.*, 2015). However, the most common diagnosis is unexplained idiopathic infertility, which accounts for about 60-70% of patients. People with unexplained infertility have all standard sperm counts intact, genital tract

exams are normal, and wife exams are also normal (Saleh *et al.,* 2003). One of the causes of infertility is immune reactions (autoimmune) against sperm cells, as studies have shown that there are anti-sperm antibodies (ASA) in 8-21% of infertile men, while there are 19-2.1% of men. In men who are fertile, this data confirms that not all anti- sperm antibodies are responsible for infertility (Krause 2009). Recently, there has been a lot of discussion about the role of the immune system in fertility. The immune system in general works to protect the body from cells and bacteria. (Rumke 2008), but sometimes problems within the immune system prevent it from working properly. Many couples facing infertility problems may already have an immune system abnormality that prevents them from conceiving. Fortunately, new tests are now available to identify these immune factors in infertility (Flint and co. 2015). So, the objectives of the current study included the following: Conducting a statistical study to find out the number of men with primary and secondary infertility, immune infertility, and unexplained infertility in the city of Tikrit.

Isolation and identification of Gram-positive and Gram-negative aerobic bacteria, and the study of some patterns of sensitivity to antimicrobials of the isolated bacteria.

MATERIALS & METHODS SUBJECTS

The study included 100 married men who attended the Urology Department at Salah Al-Din Hospital and private laboratories in the city of Tikrit and its environs for the period from April 2021 to February 2022, their ages ranged from (20-50) years, and they were divided into two main groups as follows:

Patients group (non-fertile):

- It included (80) men and was divided into four sub-groups, namely:
- The group of patients with primary infertility included 20 individuals.
- The group of patients with secondary infertility included 20 individuals.
- Immune infertile disease group included 20 individuals.
- The group of patients with unexplained infertility included 20 individuals.

The group of normal individuals (fertile): It is the fourth group, which represents the control group, which consisted of 20 individuals.

Data Collection

Data was collected through direct interview using a questionnaire designed for this study. A medical history was taken and a questionnaire was conducted, which included several questions for each patient studied. These patients were admitted to the Urology Department at Salah El-Din Hospital and private laboratories for the period from April 2021 to February 2022, and the inclusion criteria were as follows:

- Infertile males who have never conceived after 12 months or more of regular, unprotected sexual intercourse.
- Infertile males who have had at least one pregnancy but are unable to achieve it again.
- Sterile males who have all the standard sperm parameters intact and normal, as well as the examination of the genital tract is sound, and the wife's examinations are also sound.
- Males who are sterile for immunological reasons, which in turn led to the non-occurrence of pregnancy.

The control group consisted of (20) men who had at least one child under the age of one year and had similar criteria to patients in terms of age and nature of marital status.

Semen Collection

The samples were collected using the masturbation method, after an abstinence period of not less than three days, in a sterile wine container with a wide mouth, after giving the patient the necessary instructions on how to take the sample without causing external contamination as much as possible.

Culture of Seminal Fluid

Bacterial culture of semen samples that had previously been diagnosed to contain large numbers of white blood cells was carried out according to the (Lewis, *et al*., 1981) method, as it began by mixing the semen samples well, then transferring part of it using planning on three media: blood agar and McConkey agar. The chocolate was agar and all the dishes were incubated in the incubator for 24-48 hours at 37 °C, after which the growing colonies were examined and the required isolation and diagnostic tests were performed.

Isolation and Identification of Bacteria

The following tests were performed on all isolated samples:

A- Microscopic examination.
B- Gram stain for colonies for microscopic examination and identification.
C- Biochemical tests (traditional methods), which included:

Indole test, catalase test, oxidase test, methyl red test, fermentation test, starch hydrolysis, citrate consumption test, coagulase test, urease test and test

Antibiotic Sensitivity Test

The sensitivity of ten types of antibiotics was tested based on what was stated in (CLSI. 2021).

Statistical Analysis

Data were expressed as the mean (mean ± SD) standard deviation (SD) from at least three replicates. Statistical analysis was carried out using GraphPad Prism version 12. A t-test was also carried out for the purpose of multiple comparisons, and the P value was less than 0.05 for the purpose of showing significant differences between the arithmetic means.

RESULTS & DISCUSSION

Infertility Rate

Table (1) shows percentages and numbers of the types of infertility that were dealt with in the current study. Where the results showed that the percentage of primary infertility was the highest among infertility diseases, as the percentage reached 57%, while the lowest percentage was for immunological infertility, as it amounted to 7%. While the percentage of secondary and unexplained infertility was 15% and 31%, respectively.

Table 1: Shows the rates of infertility in the study groups

Groups	Number	Percent
primary infertility	57	%57
secondary infertility	15	%15
immune infertility	31	%31
unexplained infertility	7	%7
Total	**100**	**%100**

Infertility remained a public health challenge and has evolved into a psychiatric disorder with most of the burden in developing countries (Oladokun *et al.,* 2009). The current study showed that primary infertility was more common than other types of infertility, which was similar to other studies. Razzak *et al.,* (2002) reported that among the 250 infertile couples studied in Dohuk, Iraq, 77.2% had primary infertility and 22.8% had secondary infertility26. In Iran, approximately 81% were Couples experienced primary infertility and 19% had secondary infertility (Tayebi and co.2007).

Primary infertility appears to have been more common in Iraq, Iran, and some neighboring countries. Also, in Sudan, about 80% of infertile couples experience primary infertility (Abdalla and co. 2011).

Samples Distribution

The current study included 55 samples from infertile patients. The results found that 100 (100%) of the total samples showed positive results for bacterial growth that was grown on optimal media such as blood agar, mantol agar, and MacConkey agar, as shown in Table (2).

Table 2: Distribution of study samples according to growth

Groups	Number	Percent
Negative growth -ve	0	%0
Positive growth +ve	100	%100
Total	**100**	**%100**

Identification

The shape, diameter, and morphologies of the bacterial isolates were determined on blood agar, Manthol agar, and MacConkey agar. Microscopic and biochemical examinations, which included qualitative tests for each type, were used to confirm the results of the biochemical determination. The Vitek-2 results matched the biochemical tests. The results showed two types of isolates, single bacterial isolates (52%), mixed bacterial isolates (38%). Percentages of isolates from E. coli and Klebsiella spp. and Staphylococcus spp. and Streptococcus spp. were 100%, 12%, 18%, and 10%, respectively.

Table 3: Total bacterial isolates in the current study

Groups	Number	Percent
Single isolates	52	52
Mixed isolates	38	38
Total	**100**	**100%**

Table 4: Total Gram-negative bacterial isolates in the current study

Gram-negative	Number	Percent
Escherichia coli	100	100
Klebsiella spp.	12	12

Table 5: Total Gram-positive bacterial isolates in the current study

Gram-positive	Number	Percent
Staphylococcus *spp*	18	18
Streptococcus spp.	10	10

The two most common bacteria isolated from semen, E. coli and S. aureus were the main organisms with the greatest negative impact on sperm quality (Sanocka-maciejewska *et al.,* 2005, Ibadin and Ibeh 2008), and S. epidermidis plays an important role in the impairment of spermatogenesis due to infertility (Golshani *et al.,* 2006). Therefore, these pathogenic bacteria must be eliminated by antibiotics or other antimicrobial agents from semen especially before assisted reproductive technology is used.

Antibiotic Susceptibility Test Escherichia E. coli

E. coli showed total (100%) resistance to amoxicillin/clavulanic acid and ampicillin. 50% resistance to Nalidixic. Otherwise, Escherichia coli showed resistance of 40%, 20%, 60%, 20% and 50% to Imipenem, Amikacin, Gentamicin, Azithromycin and Cefepime respectively as shown in Table (6).

Table 6: Antibiotic sensitivity test for E. coli

Antibiotics	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	%
AMP(10mg)	R	R	R	R	R	R	R	R	R	R	100
AMC(10/20mg)	R	R	R	R	R	R	R	R	R	R	100
CN (10mg)	R	R	R	S	R	R	S	S	R	S	60
CPM(30mg)	R	R	R	R	S	S	S	R	R	S	50
IMI(10mg)	S	R	S	S	R	R	R	S	S	S	40
AK (30mg)	S	S	R	S	S	S	S	S	S	R	20
AZM(15mg)	S	S	S	R	S	S	R	S	S	S	20
NA (30mg)	S	R	R	S	R	S	R	S	S	R	50
TMP(5mg)	S	R	S	S	S	S	R	R	S	S	30
CIP (5mg)	R	R	R	S	S	S	S	S	S	R	40

R=Resistant, S=Sensitive, AMP= Ampicillin, AMC= (Amoxicillin and Clavulanic acid (Augmentin)), CPM=Cefepime, IMI=Imipenem, CN=Gentamicin, AK=Amikacin, AZM=Azithromycin, NA=Nalidixic acid, TMP=Trimethoprim.

Cephalosporins, especially the second and third generation, are generally used to treat Escherichia coli infection. Cefepime sensitivity was detected in this study only in 50% which is more than those reported in other studies (28.1%, 24%) (Aminzadeh and group, 2008; Sasirekha and group, 2010). With regard to aminoglycosides, they are generally prescribed against infections caused by gram-negative bacilli. Amikacin indeed showed a high sensitivity (80%), which is consistent with the findings of other researchers (Daoud *et al.,* 2003, Chlebicki *et al.,* 2004), while other studies (Shashikala, *et al.,* 2007, Husam *et al.,* 2009) revealed lower sensitivity which may be due to Extensive use of this drug in those areas. Sensitivity to gentamicin was 60%, which is similar to that reported in other studies (Daoud *et al.,* 2003, Abdulla *et al.,* 2005).

Klebsiella spp.

Klebsiella showed total (100%) resistance to amoxicillin/clavulanic acid and ampicillin. 40% resistance to Nalidixic. Otherwise, Klebsiella spp. showed resistance of 20%, 10%, 10%, 40% and 20% to imipenem, amikacin, gentamicin, azithromycin and cefepime respectively as shown in Table (7).

Table 7: Antibiotic susceptibility test for *Klebsiella spp.*

Antibiotics	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	%
AMP(10mg)	R	R	R	R	R	R	R	R	R	R	100
AMC(10/20mg)	R	R	R	R	R	R	R	R	R	R	100
CN (10mg)	R	S	S	S	S	S	S	S	R	S	20
CPM(30mg)	S	S	S	R	S	S	S	S	R	S	20
IMI(10mg)	S	S	S	S	R	S	R	S	S	S	20
AK (30mg)	S	S	S	S	R	S	S	S	S	S	10
AZM(15mg)	S	R	S	S	S	S	R	S	R	S	30
NA (30mg)	R	S	R	S	S	S	S	S	S	R	30
TMP(5mg)	R	R	R	S	R	S	R	R	R	R	80
CIP (5mg)	R	R	R	S	S	S	S	S	S	R	30

Cefepime, characterized, has very weak activity against Gram-negative bacteria such as E. coli, K. pnumoniae except S. paucimobilis was 90% sensitive to cefepime and superior action against Gram-causing bacteria. Cefotaxime, highly sensitive rate (100%) observed in Kl. pneumoniae, making it an appropriate choice for empirical therapy. Ceftriaxone, a higher rate of resistance was observed in E. coli, K. pnumoniae with the exception of S. paucimobilis and Str. agalactiae was highly sensitive to ceftriaxone. Our findings regarding the high rate of resistance to cephalosporins are in broad agreement with other studies in Iran (Ranjbar and co., 2009), and Turkey (Gökçe and co., 2017).

Staphylococcus spp.

Staphylococcus aureus showed complete resistance to penicillin, oxacillin and cefoxitin. 10%, 0%, 30%, 30%, 0%, 90% and 50% are resistant to gentamicin, nitrofurantoin, erythromycin, azithromycin, rifampicin, trimethoprim and ciprofloxacin respectively as shown in Table (4-9).

Table 8: Antibiotic sensitivity test for Staphylococcus spp.

Antibiotics	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	%
P (10 U)	R	R	R	R	R	R	R	R	R	R	100
OX (1mg)	R	R	R	R	R	R	R	R	R	R	100
CN (10mg)	S	S	S	S	S	S	R	S	R	S	10
F (300mg)	S	S	R	S	S	S	S	S	S	S	0
E (15mg)	R	S	R	S	S	S	R	S	S	S	30
CX (30mg)	R	R	R	R	R	R	R	R	R	R	100
AZM(15mg)	S	S	S	S	S	R	S	S	R	R	30
RP (5mg)	S	S	S	S	S	S	S	S	S	S	0
TMP(5mg)	R	R	R	R	R	R	R	R	S	R	90
CIP (5mg)	S	R	S	R	S	R	S	R	S	R	50

R=Resistant, S=Sensitive, I=Intermediate, P= Penicillin, OX=Oxacillin, CN=Gentamicin, F=Nitrofurantoin, E= Erythromycin, CX=Cefoxitin, AZM=Azithromycin, RP=Refampicin, TMP=Trimethoprim, CIP=Ciprofloxacin.

Bitew *et al.,* (2017) stated that they disagree with the current study showing that Staphylococcus species have sensitivity to nitrofuranction, rifampicin, gentamicin, and vancomycin with a sensitivity rate of (100.0%) in S. saprophyticus, (66.7%) in S. aureus and (33.3%) in S. aureus. lentus and (20.0%) in S. warneri, respectively.

Streptococcus spp.

Streptococcus spp. completely resistant to penicillin, oxacillin and cefoxitin. 30%, 0%, 20%, 10%, 0%, 70% and 30% are resistant to gentamicin, nitrofurantoin, erythromycin, azithromycin, rifampicin, trimethoprim and ciprofloxacin respectively as shown in Table (9).

Table 9: Antibiotic sensitivity test for Streptococcus spp.

Antibiotics	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	%
P (10 U)	R	R	R	R	R	R	R	R	R	R	100
OX (1mg)	R	R	R	R	R	R	R	R	R	R	100
CN (10mg)	S	R	S	R	S	R	S	S	S	S	30
F (300mg)	S	S	S	S	S	S	S	S	S	S	0
E (15mg)	S	S	S	S	S	S	R	S	S	R	20
CX (30mg)	R	R	R	R	R	R	R	R	R	R	100
AZM(15mg)	S	S	R	S	S	S	S	S	S	S	10
RP (5mg)	S	S	S	S	S	S	S	S	S	S	0
TMP(5mg)	S	S	R	R	R	R	R	R	S	R	70
CIP (5mg)	R	S	S	S	S	S	S	R	S	R	30

Mogram (1981) found that Streptococcus feacalis was sensitive to trimethoprim- sulfamethoxazole followed by nitrofurantoin and erythromycin. Bhatt *et al.,* (2015) did not agree with the present study which found that S. feacalis has a high sensitivity rate (100.0%) to nitrofurantoin and (71.4%) to gentamycin, levofloxacin and ampicillin- sulbactam, and (100.0%) resistance to cephalexin. Another study, Nasrallah *et al.,* (2018) discovered that Streptococcus spp. It had high sensitivity to linezolid, vancomycin, azithromycin, clindamycin, teicoplanin and erythromycin while the results of the current study showed high sensitivity of S. agalactia erythromycin towards rifampicin only.

A study by Uneke and Ugwuoru (2010) showed that half of S. aureus and Streptococcus isolates were sensitive to erythromycin and amoxicillin-clavulonic acid, but were completely resistant to penicillin and ampicillin. All strains of streptococcus were sensitive to clotrimoxazole and tetracycline, and half of the strains of Staphylococcus aureus were sensitive to clotrimoxazole and tetracycline.

CONCLUSION

- The percentage of white blood cells showed a rise in the different infertility groups, with an increase in the bacterial infection.
- Gram-negative bacteria had the largest percentage among the isolated sterile samples, especially E.coli 100%, followed by Klebsiella spp. by 27.2%.
- E. coli and Klebsiella spp. showed total (100%) resistance to amoxicillin/clavulanic acid and ampicillin.
- Gram-positive bacteria Streptococcus and Staphylococcus, isolated from the seminal plasma of infertile patients, were 100% sensitive to Rifampicin and Nitrofurantoin, and their resistance was 0% and 0%, respectively.
- Conducting several studies to discover the reasons for the increased resistance of bacteria isolated from semen to antibiotics, with the study of the genes encoding some of the virulence factors in them.
- The need to conduct genetic tests at the chromosomal level in the event of delayed childbearing in couples.

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