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Diagnosis of *Trichomonas vaginalis* and Bacterial Co-infections in Male Urogenital Tract and their Effects on Fertility among Attending to Private Clinics in Maysan Governorate.

A thesis

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بسم الله الرحمن الرحيم

﴿لِلَّهِ مُلْكُ ٱلسَّمَٰوَٰتِ وَٱلْأَرْضِ يَخْلُقُ مَا يَشَآءُ يَهَبُ لِمَن يَشَآءُ إِنَٰثًا وَيَهَبُ لِمَن يَشَآءُ ٱلذُّكُورَ ﴿٤٩﴾ أَوْ يُزَوِّجُهُمْ ذُكْرَانًا وَإِنَٰثًا ۖ وَيَجْعَلُ مَن يَشَآءُ عَقِيمًا ۚ إِنَّهُۥ عَلِيمٌ قَدِيرٌ ﴿٥٠﴾﴾

سورة الشورى

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Supervisor's Certificate

We certify that this thesis entitled " Diagnosis of *Trichomonas vaginalis* and Bacterial co-infections in male urogenital tract and their effects on fertility among attending to private clinics in Maysan Governorate "

"has been prepared under our supervision at the College of Science, University of Misan; as a partial fulfillment of the requirements for the degree of Master of Biology"

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Recommendation of the Head of Department Biology

In view of the available recommendations; I forward this thesis to debate by the examining committee

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DEDICATION

To my Father, may God have mercy on him, who strived, sacrificed, and spent for the sake of our education and the completion of our scientific career

To my Mother, may God extend her life, which may God grant me success with the blessings of her supplication and her satisfaction

To my Wife and children, who have been patient and patient with me and helped me in this effort

To everyone who taught me a letter throughout my scientific career

I dedicate this work as a candle that lights the path of knowledge

And from God is success

Uday

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Summary

Trichomonas vaginalis is flagellated protozoan extracellular obligate parasite. It is a common widespread parasite around the world. It is a sexually transmitted pathogen in both male and female and causes a disease known as trichomoniasis. The current study was conducted to diagnosis of *T. vaginalis* and bacterial co-infections in male urogenital tract and their effects on fertility among attending to private clinics in Maysan Governorate. Ninety-seven urine and semen samples had been collected from 97 male who agreed to participate in this study whose ages ranged between 18 to 50 years, from the private clinics in Al-Amara City, Maysan governorate, Southern Iraq, from January 23 to November 20, 2022.

The information of each participant male was recorded in a questionnaire sheet, including the patient's name, age, marital status, educational level, residence. Wet mount, Rapid antigen detection and molecular techniques were used to identify *T. vaginalis* in the collected urine and semen samples

In semen and urine, this study found the infection rate (IR) of *T. vaginalis* among males was 31.8% and the IR of bacteria among males was 17.53%. This study showed that *T. vaginalis* has a statistically significant effect on sperm total count, mean of sperm per milliliter (ml), the viscosity of semen, semen pus cells, and semen volume, while it was no statistically significant effect on semen liquefaction time, sperm velocity, sperms death, sperm sluggish, sperm slowing, RBC in semen, pus cells in the urine, count of RBC in the urine, and the bacteria was a statistically significant effect on sperm total count, the mean number of sperm per ml, semen viscosity, while did not statistically significant effect on the liquefaction time of semen, sperm velocity, sperms death, sperm sluggish, sperm slowing, semen pus cells, semen RBC, semen volume, pus cells in the urine, urine RBC, urine pH.

Nevertheless, the interaction between *T. vaginalis* and bacteria was a statistically significant effect on the semen pus cells. Alternatively, not statistically significant effect on sperm total count, the density of sperm/ml semen, semen viscosity, sperm velocity, death of sperms, sperm sluggish, sperm slowing, RBC in the semen, pus cells in the urine, RBC in urine. The results of diagnosis of bacteria by the Vitek 2 system and PCR in urine was distributed following species, *Staphylococcus aureus* (4), *Pantoea spp* (2), *Sphingomonas paucimobilis* (1), *Methylobacterium spp* (1), *Halomonas* spp (1), while in semen was distributed following species, *Staphylococcus aureus* (4), *Enterobacter aerogenes* (1), *Serratia marcescens* (1), *Pseudomonas putida* (1), *Sphingomonas paucimobili*s (1), *Aeromonas Sobria* (1), *Burkholderia cepacia* (1), *Aerococcus viridans* (1), *Pseudomonas aeruginosa* (1), *Staphylococcus epidermidis* (1), *Lactobacillus jensenii* (1), *Halomonas* spp (1).

List of Figures

List of Appendixes	
NO.	**Tital**
Appendix (A)	The Questionnaire paper used in the current study in the English language
Appendix (B)	The kit of RADT (Laboquick) and the procedure of the RADT
Appendix (C)	*16S rRNA* Sequencing data

List of Abbreviations	
Meaning of	**Term**
STIs	Sexually transmitted infections
IR	Infection Rates
WHO	World Health Organization
ELISA	Enzyme-Linked Immunosorbent Assay
IFA	Indirect Fluorescent Technique
PCR	Polymerase Chain Reaction
NAAT	Nucleic Acid Amplification Tests
RT-qPCR	Real-time quantitative polymerase chain reaction
NHANES	National Health and Nutrition Examination Survey
CDC	Centers for Disease Control and Prevention
CPLM	Cysteine-Peptone-Liver-Maltose
EPS	Extracellular Polymeric Substances
CDF	Cell-Detaching Factor
ROS	Reactive Oxygen Species
C-ELISA	Conventional Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
DW	Distilled water
BPH	Benign prostatic hyperplasia
SPSS	Statistical Package for Social Science

RNA	Ribonucleic acid
NCBI	National Centre for Biotechnology Information
DNA	Deoxyribonucleic acid
T	Test
C	Control

Chapter One

INTRODUCTION

1. Introduction

Sexually transmitted infections (STIs) are world most common diseases (Kim *et al*., 2017). In 2016 WHO reported that 370 million new infections of only three treatable STIs, *T. vaginalis, C. trachomatis*, and *N. gonorrhoeae* which were distributed as 156,127,87 million respectively (Rowley *et al*., 2019).

T. vaginalis is a human flagellated protozoan-obligated parasite, it transmitted through sexual intercourse (Riestra *et al*., 2019). Trichomonas vaginitis infection depends on several factors including, strain, virulence, host immunity, age, sexual behavior, and other socio-demographic parameters (Harp *et al*., 2011; AL-Majidii and AL-Saady, 2020).

T. vaginalis has a simple life cycle with no cyst, only the trophozoite stage. It infected the urethra and prostate in male as well as female vagina, and feed on epithelial cells, bacteria, leukocytes, erythrocytes, yeast, and sperms (Burch *et al*., 1959).

There are many techniques for the diagnosis of *T. vaginalis* like, Immunological methods antigen detection tests (ADT), Direct microscopy (wet mount), Enzyme-linked immunosorbent assay (ELISA), Immune fluorescent assay (IFA), Latex agglutination test (LAT), Molecular methods: Polymerase Chain Reaction (PCR), Nucleic acid amplification tests (NAAT), and DNA sequencing (Paxton *et al*., 2019).

The infection was more frequently in female by some symptoms than in male (Brookings *et al*., 2013), while in some time female harbor the infection asymptomatically for 2-3 years, this may be

related to variations in the genitourinary tract (Swygard *et al*., 2004). The chronic infection by *T. vaginalis* in infertility of males by the inflammatory process is not fully understood (Mielczarek and Blaszkowska, 2016).

On the other hand, the genitourinary tract of male and female are infected with some sexually transmitted bacteria such as *C. trachomatis, N. gonorrhea, U. urealyticum,* and *M. genitalium* are known to cause complications during pregnancy and are associated with tubal infertility in female, and associated with male fertility such as prostatitis, urethritis, and epididymitis (Brookings *et al*., 2013).

1.2:The Aims of the Study

The current study aims to investigate for following objectives:

1- Identification of *T. vaginalis* and concomitant bacteria in the genitourinary tract of males and investigation of the IR of their spread by using the molecular technique, Conventional polymerase chain reaction (PCR) and Real time-quantitative PCR (RT-qPCR) to study the molecular genetics of *T. vaginalis* and the concomitant bacteria.

2- Determination if there relationship between *T. vaginalis* and bacterial co-infection on male fertility.

Chapter Two

LITERATURES REVIEW

2. Literatures Review

2.1: General Concepts

T. vaginalis is a flagellated protozoan parasitized in both male and female urogenital systems, it was discovered by Donne in 1836 in vaginal secretion (AL-Ethafa, 2021). Trichomoniasis is one of the most common non-viral-sexually transmitted diseases (STDs), it infected about 156 million cases of the global population each year (Rowley *et al*., 2019). Vaginal irritation, yellowish, green moderate discharge, redness, itching, inflammation, scorching urine, infertility, and may lead to cervical cancer are the common manifestation of this infection in female (AL-Majidii and AL-Saady, 2020). This parasite can cause premature labor, dysuria, foul-smelling, and vaginal discharge (Dalimi and Payameni, 2021). Sexual contact is the most prevalent route of transmission for this parasite, the parasite also can be transmitted through the use of public baths and sharing of underwear-infected female (AL-Majidii and AL-Saady, 2020). In males, trichomoniasis is frequently asymptomatic or includes urethritis, which is often linked with cystitis, prostatitis, and epididymitis (Dalimi and Payameni, 2021).

2.1.1: Morphology of *T. vaginalis*

T. vaginalis is a flagellated extracellular obligated and anaerobic protozoan parasite (Hinderfeld and Simoes-Barbosa, 2020). It has 4 free anterior flagella, the 5th flagellum that returns along the edge of the undulating membrane and ends in the middle of the body (Owino, 2020). The size of *T. vaginalis* is ranging from 7 – 32 x 5 -12 μm (Roberts and Janovy, 2009). *T. vaginalis* axostyle is very clear and measures 3-14μm in length, with no free whip, the undulating

membrane extends about 2/3 of the parasite's body length (Figure 2-1) (Owino, 2020). When *T. vaginalis* is attached to the epithelial cells, it takes an oval or pear shape, and sometimes, it takes an amoeba shape (Mahmud *et al*., 2018).

This parasite lacks mitochondria and is replaced by hydrogenosomes which had double membrane organelles and are responsible for energy production (Schneider *et al*., 2011). It is reproduced by longitudinal binary fission (Harp *et al*., 2011).

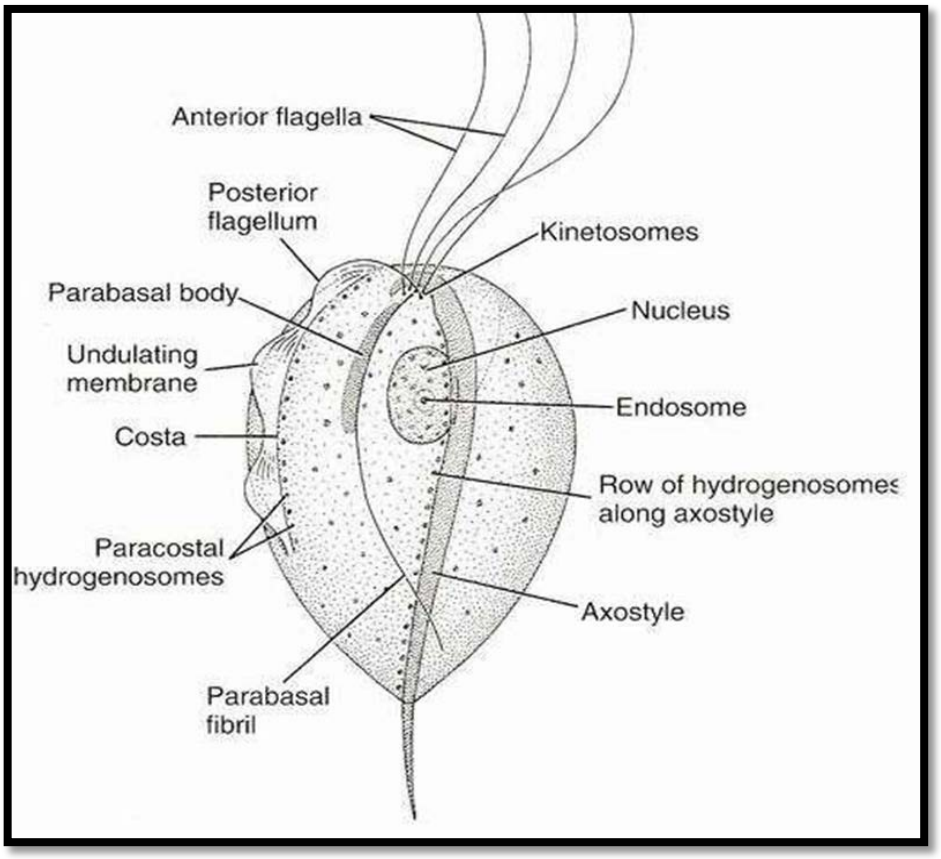

Figure 2-1: The Structure of *T. vaginalis* adapted from (Roberts and Janovy, 2000).

2.2: Classification of *T. vaginalis*

T. vaginalis is classified according to Margulis (1990) as follows:

Domain: Eukarya

Kingdom: Protista

Phylum: Zoomastigina

Class: Parabasaila

Order: Trichomondida

Family: Trichomonadidae

Genus: *Trichomonas*

Species: *T. vaginalis* (Donne, 1836)

2.3: Life cycle of *T. vaginalis*

T. vaginalis is known to be had only the trophozoite stage, no cyst was found in its life cycle (Figure 2-2), in some time, it takes an amoeboid form when adhesive to cells of epithelial tissue (Petrin *et al*., 1998), and forms a pseudocyst, under light microscopy, the pseudocyst is a round shape, without motility, and lacks a true cyst wall (Singh, 2018)**.** There are described as structures with internalized flagella found in endocytic vesicles, where they continue to beat motion (Pereira *et al*., 2003). Human is the only host of *T. vaginalis* no has reservoir or vector hosts in their life cycle (Kusdian and Gould, 2014).

This species lives in the lower genital tract in females and the prostate and urinary tract in males (Squire *et al*., 2019). It is transmitted from infected females to males or from males to females through sexual intercourse (D'Ancona *et al*., 2019).

The rapid transformation of the *T. vaginalis* from a free-swimming pyriform into an adherent amoeboid cell is an essential component for the rapid and efficient colonization of the urogenital tract (Rein, 2020). Occasionally, this parasite can be created multinucleated forms which can migrate actively on the host cells and can be budded off (Kusdian and Gould, 2014).

In chronic infection, *T. vaginalis* can be cytoadherence and established in the human urogenital tract by complex mechanisms involving some cytoskeleton and surface proteins called "adhesins" and glycoconjugates (Sethowa, 2017).

The microscopic studies showed that *T. vaginalis* is capable of phagocytic, quickly ingesting and degrading various cells including lactobacilli, cervical and vaginal epithelial cells, leukocytes, erythrocytes, yeast, sperms, and prostatic cells (Pereira-Neves and Benchimol, 2007).

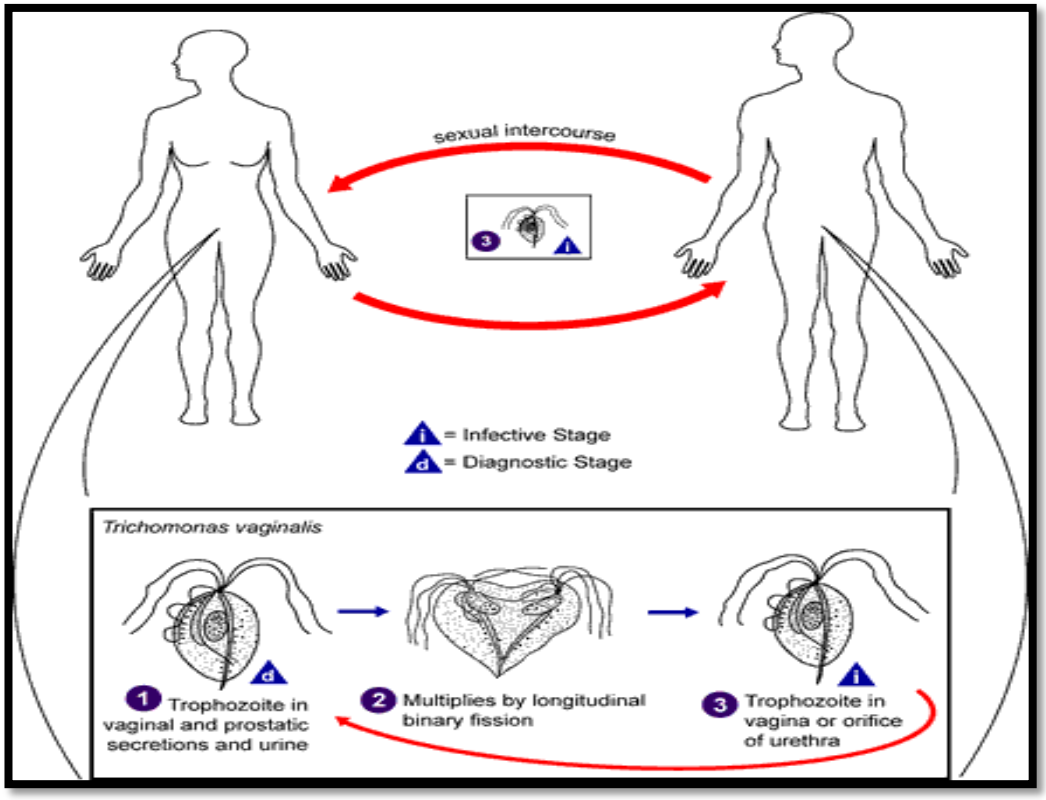

Figure 2-2: Life cycle of *T. vaginalis* (http://www.dpd.cdc.gov/dpdx).

2.4: Symptoms and Pathogenicity in Males

In males, *T. vaginalis* is recognized to be bound to host tissue and as a mucosal pathogen (Tompkins *et al*., 2020). *T. vaginalis* causes some complications but is usually asymptomatic, these complications including urethritis, chronic prostatitis, epididymitis, infertility, and in some time prostate cancer might occur if the patient does not take therapy (Schwebke *et al*., 2018). Symptoms of the disease may exhibit in the infected individual during the period of incubation, which is between 5-28 days, but some individuals do not exhibit any symptoms (CDC, 2017).

The urogenital tract, including the vagina, urethra, and endocervix in females and the prostate tissues, seminal vesicles, and urethra in males is the infection site of *T. vaginalis* (Pekmezovic *et al*., 2019).

The parasite caused damage to the host cells through the axostyle acute trichomoniasis (Sethowa, 2017).

The presence of *T. vaginalis* in the seminal fluid affects the quality of spermatozoa and their capability to fertilize the ova. The fertilization induced by trichomoniasis is usually related to physical damage in spermatocytes and this is linked to the intensity of *T. vaginalis* in seminal fluid (Martinez *et al*., 1996). Some semen features are affected by *T. vaginalis* infection (Sena *et al*., 2007), such as motility, viability, morphology, and seminal fluid viscosity (Gopalkrishnan *et al*., 1990).

T. vaginalis is adhering to the epithelial tissue of the urogenital tract and sometimes it degrades the epithelial cells and RBC, leading to break down of the innate barriers, which allows other pathogens such as HIV to pass from one partner to another (Stewart *et al*., 2020).

Trichomoniasis may be caused many complications through inflammatory damage such as affecting sperm function (Mali *et al*., 2006; LaVignera *et al*., 2011), and then decreasing the capability of fertilization (Lloyd *et al*., 2003)**,** prostate cancer (Stark *et al*., 2009), that increase about 40% compared to uninfected (Stark *et al*., 2009).

2.5: Epidemiology of *T .vaginalis*

T .vaginalis is widespread in all regions (Figure 2-3) of the world (Masha *et al*., 2019), all environments, and in seasonal conditions (Rayan *et al*., 2019). The gestational age group (14-49 years) had the highest infection rates (Sherrard, 2020), and it showed in the age group 18-24 years 2.3% and 4% 25 years or older (Miller, 2005). In 2008, the global of infection cases with *T. vaginalis* was estimated at

276.4 million cases (WHO, 2008). Trichomoniasis is related to some sociodemographic factors like education level, age group, marital state, pregnancy state, residency, and pH levels (AL-Majidii and AL-Saady, 2020).

In the USA, the number of trichomoniasis cases is estimated to be 3.7 million individuals (Shahraki *et al*., 2020). In Denver, Colorado USA, 0.8% of males under ≤30 years old and 5.1% >30 years(Joyner *et al*., 2000), and according to National Health and Nutrition Examination Survey (NHANES) from 2013 to 2016, 0.5% of American males are infected with *T. vaginalis* (Kreisel *et al*., 2021).

Previous studies reported that the *T. vaginalis* IR in males, in the European region was 0.6% (WHO, 2016). In Australia was 17% (Upcroft and Upcroft, 2001). Asia 0.1%, the Middle East 3%, and South America 5.2% (WHO, 2012). 2-17% of newborns get the infection from an infected mother through delivery birth (Shehabi *et al*., 2009).

In Iraq, some previous studies found the IR of *T. vaginalis* among males in some provinces such as Basra, 12% (Khalaf *et al*.**,** 2010), Babylon, 9.29% (Al-Quraishi, 2014), Al-Najaf, 17.64% (Al-Khafagy and Al-Hadraawy**,** 2014), Erbil, 4.84% (Al-Jadoa and Mawlood, 2010).

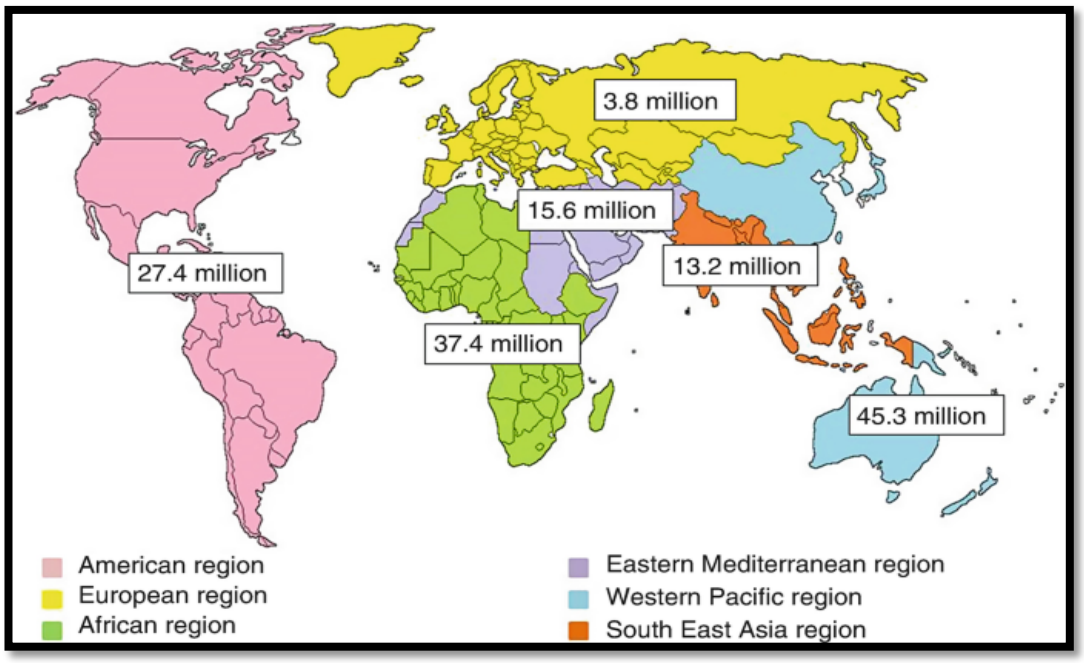

Figure 2-3: The distribution of trichomoniasis infection rate in the WHO regions (WHO, 2012).

2.5.1: Transmission of *T .vaginalis* between genders

The main route of *T .vaginalis* transmission between human partners is sexual intercourse, sometimes it can be transmitted through some routes such as infected fomites, contaminated water cycle, and contaminated baths (Squire *et al*., 2019; AL-Majidii and AL-Saady, 2020). The trichomoniasis infection rate of female is approximately ten times more than male (Van Der Pol, 2007). Some factors influenced the *T .vaginalis* infection and exhibited the symptoms like age, immunity, nutritive, and the status of the body's health (Sena *et al*., 2007).

2.5.2: The relationship of *T. vaginalis* infection with sociodemographic factors

2.5.2.1: The age

The *T. vaginalis* infection rate was related to age (Secor *et al*., 2014). In the USA, and Colorado, the IR of trichomoniasis among males less than 30 years old was 0.8%, and more than 30 years old was 5.1% (Khan *et al*., 2016).

In İzmir, Turkey was 6.5% among male 18-50 years age group, (Mutlu Yar *et al*., 2017), and In Tehran, Iran the male IR among the 20-40 years age group of *T. vaginalis* was 65.95% (Dalimi and Payameni, 2021).

The trichomoniasis IR in Iraq's males in some regions including Basra, 12% among the 20-40 years age group (Khalaf *et al*., 2010). In Tikrit and Baijim, 13.6% among the 16-49 years age group (Mohammed, 2012). In Babylon, 9.29% of the 20-50 years age group (Al–Quraishi, 2014).

2.5.2.2: The gender

Many studies found that trichomoniasis in females was higher than in males (Kissinger, 2015). In Africa, South-East Asia, and the Western Pacific, the IR of *T. vaginalis* among female was 8.08%, and in males, in South-East Asia was 1.0% (WHO, 2011).

In the USA, the overall IR among males between 2013-2016 was 0.49% (Daugherty *et al*., 2019). In the USA, the IR of *T. vaginalis* in 2018 among female and males are 1.8% and 0.5% respectively (Patel *et al*., 2018). Schwebke *et al*. (2019) showed that the IR in the West Coast, Midwest, Southern states, and East Coast of the USA the IR of

T. vaginalis was 2.7% in males. The IR of trichomoniasis in African males was 1.2% (WHO, 2016). In South Africa, the IR was in male13.4%, and in female 33.8% (Lewis *et al*., 2013). In European, the *T. vaginalis* IR was 0.2% among males and 1.6% among female (Jane *et al*., 2019).

In Iraq, it showed the infection rate of *T. vaginalis* among males in Al-Najaf province was 17.64% (Al-Kafagy and Al-Hadraawy, 2014), and 27.9% among femlae (Al-Abbas and Radhi, 2019). In Babylon province, showed the IR of *T. vaginalis* among female was 7.38% and among males 4.2% in urine in urban, but the infection rate in urine samples for female and males in rural was 12.16% and 5.09% respectively (Al–Quraishi, 2014).

In Maysan province, a study by AL-Majidii and AL-Saady (2020) showed that the infection rate among the female who invested in the gynecological clinic was 75.22% but there is no study had been conducted in Maysan on males.

One previous study involving some cities and villages of different provinces of Iraq in the period 2013 to 2017 showed that Baghdad had the highest IR of 85.5% whereas Erbil had a lower IR of 3.1% (Al-Marjan and Sadeq, 2022).

2.5.2.3: Marital status

In the USA, the IR for males who were married or living with partners was 0.6%, 2.4% for non-married, and 2.0% for widows, divorced individuals, and single individuals. (Patel *et al*., 2018). In USA Carolina, the IR of single males is 69.7%, while in separated, divorced, or widowed is 13% (Sena *et al*., 2007). In South Korea,

single males 3.6%, married 4%, and divorced 25% (Seo *et al*., 2014). In Turkey, single males are 6.55%, married 12.3%, and widows 8.5% (Erbil *et al*., 2019). In Iran Tehran, the IR in males had multiple sexes with females at 65.95%, (Dalimi and Payameni, 2021).

In Iraq, Basra province, the IR among males married was 12%, compared to 0% of unmarried males (Khalaf *et al*., 2010). In Al-Najaf province, 17.64% of married males (Al-Kafagy and Al-Hadraawy, 2014). In Tikrit, and Baiji City, 13.6% of married (Mohammed, 2012). In Babylon, 9.29 % of single and married (Al–Quraishi, 2014).

2.6: Diagnosis of *T. vaginalis*

The identification of *T. vaginalis* infections in infected males depends on the diagnosis technique such as microscopic, cultural media, immunological, serological, and molecular methods (Bruni *et al*., 2019), and sample types such as urine and seminal fluid used in diagnosed *T. vaginalis* (Menezes *et al*., 2016). The main features used to diagnose *T. vaginalis* are flagella and axostyle (Owino, 2020).

2.6.1: Microscopic method

2.6.1.1: wet mount test

A wet mount is a widely used method because it is simple, easy, and cheap. It is found that the sensitivity of wet mount ranged between 38%-82%. The sensitivity depended on the laboratory worker's experience, the period from sample collection and examination, and the parasite's intensity ($>10^4$ organisms/ml) (Hobbs *et al*., 2013). The disadvantage of this technique is not the most accurate and reliable method for diagnosing this parasite in males and has lower sensitivity and specificity (Menezes *et al*., 2016).

At some time when the intensity of the parasite is low, it used the culture media to proliferate this parasite, including Diamond's TYI liquid broth media, cysteine-peptone-liver-maltose (CPLM), Feinberg and Whittington's medium, InPouch TV medium (Divakaruni *et al*., 2018). InPouch TV medium is the golden method for propagating and diagnosing the *T. vaginalis* parasite, which is sensitive to a few parasites (10^2 organisms /ml) in the sample (Garber, 2005). When compared to microscopy, the culture media had specificity reached 100% and high sensitivity of 75–85% (Pattullo *et al*., 2009).

2.6.2: Immunological Methods

There are several immunological methods for detecting *T. vaginalis* in the genital fluids of males and female including rapid antigen detection tests (Laboquick *Trichomonas* rapid test, OSOM *Trichomonas* rapid test), Enzyme-linked immunosorbent assay (ELISA), Immunofluorescent Assay (IFA), Latex agglutination test and others, these methods differ in their sensitivity and specificity for detecting this parasite (Engbaek *et al*., 2003).

2.6.2.1: Rapid Antigen Detection Test

Most of the rapid antigen detection tests are based on immunochromatographic capillary flow and the presence of antibodies in blood samples (Meites *et al*., 2015). The advantage of this test is easy and rapid (takes about 30 min), safe (as a compact strip), and has high sensitivity of 82–95% and specificity 97–100% (Postenrieder *et al*., 2016).

2.6.3: Molecular Techniques

2.6.3.1: Conventional polymerase Chain Reaction (PCR) Technique

Conventional Polymerase chain reaction (PCR) was invented by Mullis in 1983, and this invention led him to obtain a patent in 1985 (Ahady *et al*., 2016). The basis of this technique is based on the polymerization of the specific fragment of the target nucleic acid in the lab. In this, it can be produced million or more copies of the target-specific fragment of the extracted DNA of the sample depending on the DNA template (primer) (Tipple *et al*., 2018). This technique involves many enzymatic reactions with different temperature degrees for simple detection of DNA (Noh *et al*., 2019).

2.6.3.2: Real-time quantitative PCR (RT-qPCR)

This method combines RT-PCR and qPCR to enable the measurement of levels of RNA using cDNA in a qPCR reaction, allowing for the quick detection of changes in gene expression (Deprez *et al*., 2002). Real-time quantitative PCR (RT-qPCR) uses RNA as a template to produce complementary DNA (cDNA), a single-stranded copy of the cDNA is produced by using the enzyme reverse transcriptase and then amplified using a DNA polymerase to create double-stranded cDNA, this is done through a standard PCR-based amplification process although it can be used to clone molecularly important genes, this step is considered the first step in RT-qPCR (Adams, 2020). RT-qPCR can be performed as a single reaction using a more specific commercial enzyme, or as two separate reactions (Ali *et al*., 2023). The RT-qPCR technique can be carried out either with a single enzyme that serves as both reverse

transcriptase and a thermophilic DNA polymerase or with separate reverse transcriptase and DNA polymerase enzymes (Deprez *et al*., 2002). This technique is used to identify pathogens and estimate the copies of specific DNA sequences, and has high sensitivity and specificity (Adams, 2020).

2.7: Relationship between *T. vaginalis* Infection and Fertility

T. vaginalis affect the reproductive system and the fertility of the male (Škerk *et al*., 2002). The *T. vaginalis* infection impacts sperm motility and viability (Ifeanyi *et al*., 2018). The interaction between *T. vaginalis* and bacteria caused inflammation lead to damage male genital tract infected the urethra, prostate, seminal vesicles, and epididymis that effect on quantity and quality of sperms (Mielczarek and Blaszkowska, 2016).

T. vaginalis affect sperm motility, it causes rapidly immobilized and killed sperms (Tuttle *et al*., 1977; Benchimol *et al*., 2008), and may lead to agglutination and phagocytic sperms (Mali *et al*., 2006). Sperm motility, viability, and functional integrity were exhibited significantly reduced when the sperms were exposed to extracellular polymeric substances (EPS) (Lucena *et al*., 2014). However, rare cases of infertile male with trichomoniasis point to the important role these protozoans play in disrupting fertility. One of the difficult diagnoses for *T. vaginalis* orchitis was diagnosis in male with severe oligoasthenoteratospermia (Lloyd *et al*., 2003).

2.8: Relationship between Trichomoniasis and Prostate enlargement

The infection with *T. vaginalis* related to some prostate complications such as benign prostatic hyperplasia (BPH), and prostate cancer (Mitteregger *et al*., 2012; Kim *et al*., 2016). Mitteregger *et al*, (2012) found that 34% of male with BPH and 21.2% of chronic prostatitis and urethritis are infected with *T. vaginalis*. Moreover, BPH in addition to *T. vaginalis* is linked to other microorganisms like gram-positive, and gram-negative bacteria, and fungi and their interaction (Langston *et al*., 2019).

2.9: Impact of some Urogenital Bacteria and their Effect on Male Fertility

Different species of gram-negative and gram-positive bacteria impact the urogenital tract in males and affect fertility which is described as follows:

2.9.1: *Staphylococcus aureus*

S. aureus is a Gram-positive bacteria that is aggressive and opportunistic. *S. aureus* has been observed as the causative organism responsible for (68.2%) of infections in seminal fluid. The most dominant microbe implicated in primary infertility in both males and females is likely *S. aureus* and it is the predominant flora in infertile male, and sperm motility has significantly decreased. Immobilization of human spermatozoa was caused by *S. aureus* when spermatozoa were coincubated with this bacteria. Similar studies on the impact of certain uropathogenic microorganisms on human sperm motility parameters were done by Liu *et al*, (2002), they found a significant decrease in sperm motility when spermatozoa were coincubated with

S. aureus. While other researchers have identified evidence for the production and secretion of soluble spermicidal factors by bacteria in the extracellular medium (Gupta and Prabha, 2012).

2.9.2: *Pantoea* spp and *Burkholderia cepacia*

In a research study, there are no reports published on *Pantoea* spp. These species were isolated from grasses and identified as a diazotrophic endophyte. Burkholder first identified *B. cepacia*, a non-fermenting Gram-negative rod, from rooted onions in 1950. It is occasionally isolated from the soil and roots of bee trunks, and it is less frequently associated with male infertility. Because molecular biological techniques can characterize an organism even if it is unculturable based on its genotype rather than its phenotype (Anuradha *et al*., 2004).

2.9.3: *Pseudomonas aeruginosa*

P. aeruginosa frequently causes urinary tract infections in humans (Wu *et al*., 2015). This microorganism produces 3-oxododecanoyl-L-homoserine lactone, a quorum-sensing signaling molecule that is harmful to spermatozoa (Tateda *et al*., 2003; Rennemeier *et al*., 2009). Exotoxin A from *P. aeruginosa* has also been shown to have cytotoxic effects on cells at the chromatin level. Many different spermatozoa problems have been caused by this effect. The detrimental effects on the spermatozoa tail were more severe than in the other parts of the spermatozoa because toxin target proteins concentrated mostly in the tail (Altaee *et al*., 2013). The epithelial cell line derived from seminal vesicles is susceptible to apoptosis by the porin from *P. aeruginosa*. Porins can directly affect sperm parameters

because they contain receptors for the sperm plasma membrane (Farsimadan *et al*., 2020).

2.9.4: *Serratia marcescens*

S. marcescens is an opportunistic pathogen of the Enterobacteriaceae family which often colonizes the genitourinary tract, was found to impair sperm motility in vitro by causing sperm agglutination. *S. marcescens* causes negative effects on seminal parameters, induced spermatozoa to decapitate, and caused histological changes. These effects were demonstrated by the decrease in sperm concentration, motility, and viability which was very well correlated with an increase in *S. marcescens* populations (Rana *et al*., 2017).

2.9.5: *Enterobacter aerogenes*

One of the most frequently common species in human semen and genitourinary infections, particularly epididymitis, is *Enterobacter aerogenes*. in vitro study, Sufficient bacterial concentration of these bacteria causes an environment change or high energy consumption that may lead to sperm motility loss and its impact on fertility (Berktas *et al*., 2008). Human spermatozoa are affected by *Enterobacter aerogenes* in many different ways, including sperm motility, viability, mitochondrial oxidative state, DNA fragmentation, and caspase activity (Marchiani *et al*., 2021).

2.9.6: *Lactobacillus spp*

lactobacillus bacteria affect sperm during the colonization by adhesion, which determines the ability of probiotics and the pathogens' ability for invasion. Bacterial adherence may increase the load of spermatozoa, which would then reduce sperm motility.

Spermatozoa with less motility are more likely to adhere together, and bacterial binding increases cell loads, which in turn lowers spermatozoa's motility (Shokryazdan *et al*., 2014).

There have been rare reports of studies of *Lactobacillus* adherence to spermatozoa. In recent years of in vitro investigation, the *Lactobacillus* vaginalis strains have confirmed their ability to adhere to spermatozoa. This consistency significantly reduced sperm motility in a co-incubation test with a single bacterial species. (Wang *et al*., 2019). *Lactobacillus* adherence is a complicated mechanism. Pili, a fimbrial adhesin, and interfacial free energy are generally thought to play a role in this process. Pili may have a specific effect on sperm motility without having any negative effects on the morphology or viability of these cells (Mashaly *et al*., 2016).

2.9.7: *Halomonas spp*

Halomonas spp are a gram-negative aerobic bacteria that are halophilic and/or halotolerant and are typically found in saline environments, which has been recognized as a distinct genus since 1980 (Garrity *et al*., 2005). The first case was detected in the renal care center in Santa Clara Valley Medical Center when culture dialysis fluids (Arnow *et al*., 1998). Standard medical microbiology texts which absent from *Halomonas species*, which suggests that their potential for pathogenesis is not fully understood. Recently, the only case of human pathology that has been described as a wound infection brought on by a fish bite (von Graevenitz *et al*., 2000).

2.10: Bacterial diagnosis methods

2.10.1: wet mount

Microscopical examination of microorganisms reveals significant details about their morphology but little about their biological characteristic. We must observe microorganisms in cultivation to obtain this information (Vandepitte *et al*., 2003). Microscopically examine one drop of the sediment for leukocytes, erythrocytes, bacteria, and yeasts between a slide and a coverslip called wet mount (Varghese *et al*., 2014). Wet microscopy is a secondary test used in the diagnosis of vaginitis and urethritis. However, there is little data on the effect of the sampling site. Wet mount microscopy is a useful tool used for the diagnosis of mixed infections (Donders *et al*., 2000 ; Bornstein, 2019). The sensitivity and specificity were 82.6% and 92.45 respectively (Vieira-Baptista *et al*., 2022).

2.10.2: Automation Method by Vitek 2 Compact

Bacteria that were grown on different culture media and with different growth conditions were diagnosed after 24 hours of incubation using the Vitek 2 compact device from the French company biomérieux. The Vitek 2 is an automated system for examining microorganisms that use growth-based technology. The system allows for colorimetric reagent cards that are automatically incubated and interpreted. There is a different type of colorimetric reagent card used to identify gram-positive bacteria and gram-negative bacteria and other microorganisms (Pincus, 2006).

The 64 wells on the reagent cards can each contain a unique test substrate. Various metabolic activities, including acidification, alkalization, enzyme hydrolysis, and growth when inhibitory

substances are present, are measured by substrates, while maintaining a sealed vessel that prevents contact with the organism-substrate admixtures, an optically clear film present on both sides of the card allows for the appropriate level of oxygen transmission. A transfer tube for inoculation is already inserted into each card (David and Pincus, 2009).

The gram-positive card is used for the most significant 115 taxa of non-spore-forming Gram-positive bacteria that are identified automatically, the Gram-positive identification card is used based on existing biochemical techniques and recently created substrates. 43 biochemical tests measure resistance, enzymatic activity, and carbon source utilization. Final identification results are given in eight hours or less (Poyart *et al*., 2002).

The gram-negative card is used to automatically identify 135 taxa of the most important fermenting and non-fermenting Gram-negative bacteria bacilli. The Gram-negative card identified card is used based on existing biochemical techniques and recently created substrates measuring carbon source utilization, enzymatic activities, and resistance. There is one negative control well and 47 biochemical tests. Final identification results are available in 10 hours or less (Chang *et al*., 2002).

2.10.3:Polymerase Chain Reaction

Nucleic acid amplification tests (NAAT) use polymerase chain reaction (PCR). The sensitivity of the test is much higher than that of unamplified tests. Modern assays can discover infections linked to certain clinical syndromes by targeting a single pathogen or

multiplexed panels of targets. *C. trachomatis* and *N. gonorrhea* in urine samples for sexually transmitted illnesses are examples of NAAT tests that the Food and drug administration (FDA) has approved (Levinson *et al*., 2022). These techniques show great promise for rapidly detecting viable microorganisms, but non-culturable ones in urine and sperm, and this method is now being used to detect bacteria associated with idiopathic inflammatory disorders (Lacroix *et al*., 1996). The nucleic acid amplification techniques used to assess and determine bacterial viability status could have more significant advantages for the food, environment, and health sectors, by improving detection speed and sensitivity, and use for pathogenic bacteria detection in droplet microfluidics (Birch *et al*., 2001; Azizi *et al*., 2019).

2.11: The Association between *T. vaginalis* and some Urogenital Bacteria in Male

The male genital tract and semen may contain different species of bacteria whose abundance and relevance are affected by the geographic variation and the etiology of the males in the presence of bacteria in semen effect on quality and quantity of sperms (Gimenes *et al*., 2014). The bacteria *C. trachomatis*, *N. gonorrhoeae*, *Mycoplasma spp*, *Ureaplasma spp*, and *T. pallidum* are the most sexually transmitted diseases (STD) pathogens that affect male semen (Mackern-Oberti *et al*., 2013).

The risk influence of interaction between *T. vaginalis* and some bacteria may be more affect the upper genital tract than each *T. vaginalis* or bacteria alone (Tsevat *et al*., 2017). Interleukins such as (IL-8) which are associated with *T. vaginalis*-*C. trachomatis*

infections can affect sperm function by reducing intensity, velocity, morphology, viability and altering the pH of the semen, and reducing ejaculate volume (Gimenes *et al*., 2014).

Male with *N. gonorrhoeae* infection are typically symptomatic, but asymptomatic urethral infections may occur in at least 10% of cases. The most prevalent gonococcal infections in males affect the urethral mucosa. Dysuria and a white purulent penile discharge are typical signs and symptoms. The absence of secretions may also indicate unilateral epididymitis (Cristaudo and Giuliani, 2020). *T. vaginalis* with *N. gonorrhoeae* infection increase of penile discharge and pelvic inflammatory disease (Lemly and Gupta, 2020).

The relationship of *T. vaginalis* with *M. hominis* is one of the more symbiotic relationships, it enters and lives and proliferation in *T. vaginalis* cells. It showed the interaction between *T. vaginalis* and *M. hominis* increases the production of inflammatory cytokines in macrophages which increases the local inflammation in the infection site (Edwards *et al*., 2016).

For this, *T. vaginalis* plays as a Trojan horse with harbors the bacteria inside it, this *T. vaginalis*-bacterial symbiont was leading to stable maintenance of this microbial relationship, and this is capable of module the host immune response through the arginine dihydrolase pathway which used the arginine as an energy source (Morada *et al*., 2010). *M. hominis* can be regulated the production of some proinflammatory cytokines like IL-8, IL-1β, and tumor necrosis factor-alpha TNF- α THP-1 in response to *T. vaginalis* stimulation (Fiori *et al*., 2013). This inflammation increased the risk of prostate

cancer, tissue damage, and secretion of toxic molecules, the interaction between *T. vaginalis* and *M. hominis* increased hemolytic activity, production of ATP, and parasite replication (Dessì *et al*., 2019).

Chapter Three

MATERIALS AND METHODS

3: Materials and Methods

3.1: Materials

3.1.1: The apparatus, materials, and tools

The apparatus, materials, and tools that had been used in this study was summarized in (Table 3-1):

Table 3-1: The apparatus, materials, and tools that had been used in this study.

No.	The apparatus, materials, and tools	The company
1	Agarose	Condalab
2	Amies Transport Medium Swab	CE
3	Aura Tm PCR Cabinet	Euroclone
4	Bio TDB-100, Dry block, Thermostat built	Bio San
5	Blood agar	Himedia
6	Burner	H-e Hemc
7	Centrifuge	Hettich
8	Collection Tubes (2 ml)	Sterile Eo
9	Combi-spin	Bio San
10	Conical flask (250ml)	Memmert
11	Cover slide	Hirshman
12	Cylinder(250 ml)	Supertek
13	Disposable syringe (5ml)	Kilani medical
14	Distillation apparatus	Lab teach
15	Document system	Labnet
16	Electrophoresis cell	CBS, Scientific
17	Eppendorf tube	Bioneer

18	Ethanol Absolute (70%), (95%)	M.P.C
19	Giemsa stain	Solar bio
20	Glass slide	Supertek
21	Gram Stain	Afco
22	Hood	Lab teach
23	Hot Plate	Harry Gestigkeit
24	Incubator	Memmert
25	KAPA SYBR® FAST qPCR Master Mix (2X) Kit	KAPA
26	Laboquick kit	Koroglu Tibbi
27	Ladder 100 bp, 1000 bp	Intron
28	Light microscope	Olympus
29	Litmus paper	Machery –Nage
30	Loading dye, 6X	Intron
31	Loop	Himedia
32	MacConkey agar	Himedia
33	Mannitol salt agar	Biomark
34	Maxime PCR PreMix kit (i-Taq)	Intron
35	Medical Gloves	Han-Care
36	Micropipettes (50 µL)	Transferpette
37	Micropipettes Tips (100µL)	Sterellin Ltd
38	Microspin	Bio San
39	Microspin 12, High-speed Mini-centrifuge	Bio San
40	Microwave	Gosonic

41	Mini-Power Supply 300 V, 2200V	Supplier
42	Modified Thayer martin agar	Himedia
43	MultiGene OptiMax Gradient Thermal Cycler	Labnet
44	Nanodrop	Nabi
45	Normal saline 0.9% NaCl	Pioneer
46	Petri Dish	Biozak
47	Pipettes different sizes	Labnet
48	Premix PCR	Intron
49	PrimeScriptTM RT reagent Kit	TaKaRa
50	Quick-DNA Miniprep kit	Zymo Research
51	Rack	Afco
52	Real time-qPCR(RT-qPCR)	Sacace
53	RedSafe™ Nucleic Acid staining solution (20,000x)	Intron
54	Refrigerator	Concord
55	RNA extraction (Direct-zol™ RNA MiniPrep)	Zymo
56	Sensitive Balance	Kernpfb
57	Specific primer of *β-tubulin* gene	Alpha DNA
58	TBE buffer, 10 X	Intron
59	Universal primer *16SrRNA* of gene	Alpha DNA
60	UV transilluminator	Vilber Lormat
61	Vitek 2 Compact system	Biomerieux
62	Vortex	Digsystem

63	Water bath	Thermo Scientific
64	ZR Fungal/Yeast/Bacterial DNA MiniPrep	Zymo

3.2:Ethics approved and informed consent

The Ethical approval of this study was obtained from the Maysan Health Directorate, Ethical Review Board with reference No. 2178 on 29/11/2021.

Before urine and semen samples were collected. The author described to participant the native of the study, the objectives of the study, and the risk of *T. vaginalis* and its effect on fertility, then the subjects were given the choice to accept or refuse to participate in this study. The consent of the participants in this study was taken by filling out the questionnaire and signing acceptance at the end of it on their consent study engagement.

3.3: Methods

3.3.1: Population study

Urine and semen samples were collected from 97 human males whose ages ranged between 18-50 years, from Private clinics, in Al-Amara City, Maysan Governorate. Southern Iraq, from January 23 to November 20, 2022. Some sociodemographic factors were recorded among each participant such as the name, age, marital status, period of married, number of children, fertility, educational level, and residence.

3.3.2: pH measurement of semen and urine

The pH of semen and urine was done according to (Sgibnev and Kremleva, 2020).

1**.** One drop of semen or urine was taken by doper and placed it on a piece of litmus paper (Figure 3-1)**.**

2**.** Wait one minute and then examine the color.

3**.** Compared the color of the result with the label of color gradations attached to the manufacturer's package.

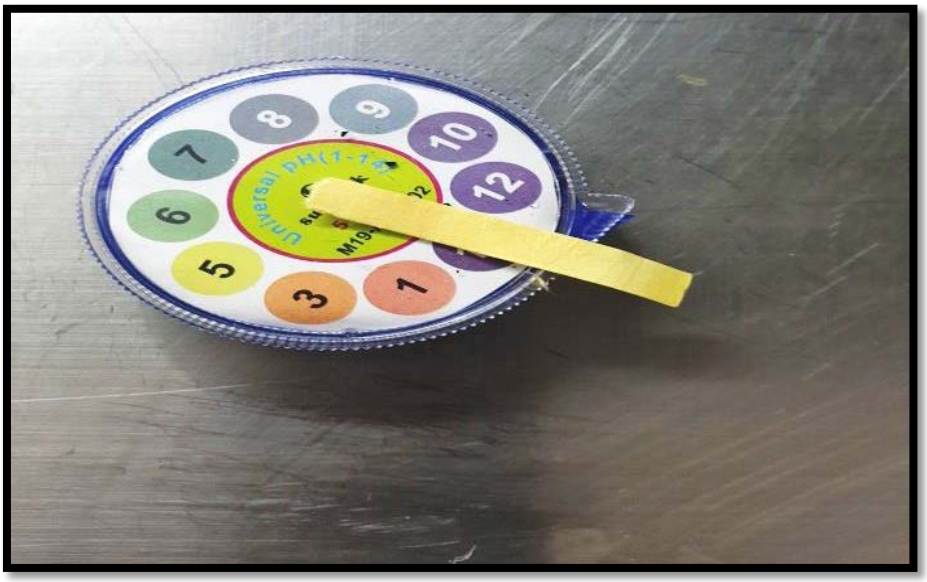

Figure 3-1: Litmus paper and the label of the colors.

3.3.3: Diagnosis methods of *T. vaginalis*

In the current study, there are three methods have been used to detect *T. vaginalis* in the semen and urine of males: wet mount (WM), Rapid antigen detection (RADT), and molecular techniques, such as Conventional Polymerase Chain reaction (PCR), and Real-time quantitative Polymerase Chain reaction (RT-qPCR). RADT uses the Laboquick technique and PCR uses the *β-tubulin* gene for detecting *T. vaginalis*, and RT-qPCR uses the *β-tubulin* gene for the identification of *T. vaginalis*.

3.3.3.1:Direct microscopic examination (Wet mount)

1**.** A 0.5 ml of semen or urine were centrifuged at 3,000 rpm for 5 minutes.

2**.** The supernatant was discarded and maked three slides from the precipitate of each sample

3. The slides were examined under magnification 40x.

4**.** The results were recorded in the specific field in the questionnaire.

3.3.3.2: The Rapid Antigens Detection Test (RADT)

3.3.3.2.1: The Idea of the Test

In *T. vaginalis* antigen detection test, the colored immunochromatographic capillary flow technique is used. The procedure requires that trichomonas proteins from the genitourinary secretion be dissolved by mixing into the sample solution. If trichomonas is found in the sample, it will form a complex with anti-trichomonas primary antibodies which conjugated with dyed latex particles have a red color. The complex will subsequently be attached to a second trichomonas antibody covered through a membrane made of nitrocellulose. A positive result will be determined by the presence of both a test line and a control line.

3.3.3.2.2: The Procedure of the Test

1. One cassette and one dilution bottle containing buffer was taken from the Laboquick kit for each sample.

2. Added 0.5 ml of each semen or urine inside the dilution bottle.

3. The bottle well were mixed for 60 seconds.

4. Added about 3-4 drops of the mixture to the sample well of the cassette.

5. The result were read after 15-30 minutes by appearing the reaction line between the anti-trichomonas primary antibodies and the antigen of *T. vaginalis*.

6. The display field consists of two lines (T and C), the first line (C) is coated with goat anti-*T. vaginalis* antibody. The second line (T) is coated with *T. vaginalis* antibodies which are used to detection of *T. vaginalis* antigens.

3.3.3.3: Conventional PCR of *T. vaginalis* DNA

3.3.3.3.1: Samples and DNA extraction

The DNA was extracted as following protocol:

1. Ten microliters of semen or urine samples were mixed with 400 µl of genomic lysis buffer in a Zymo-Spin IIC™ Column[1].
2. Mixed by vortex for 4-6 sec.
3. Let the mixture stand at room temperature (RT) for 5-10 min.
4. The mixture was transferred to a Zymo-Spin IIC™ Column [2] in a collection tube.
5. Centrifuged at 12000 rpm for one min.
6. The collection tube with flow through it was discarded.
7. The Zymo-Spin IIC™ Column was transferred into a new collection tube.
8. Two hundred µl of DNA pre-wash buffer were added to the spin column.
9. The new mixture was centrifuged for one minute at 12000 rpm.
10. Five hundred µl of g-DNA wash buffer was added to the spin column, and the centrifugation was repeated at 12000 rpm for one min.
11. The spin column was transferred to a clean microcentrifuge tube.
12. Fifty µl of DDW or DNA elution buffer was added to spin column [3].
13. The mixture was incubated for 2-5 min at (RT).

14. The mixture was centrifuged at a high speed of 16000 rpm for 30 sec to elute the DNA.
15. The eluted DNA was used immediately for a molecular technique or stored ≤-20ºC until used.

3.3.3.3.2: Estimated the Purity and Concentration of DNA by Nanodrop

The purity and the concentration of DNA were estimated by a Nanodrop spectrophotometer **(**Nabi/ Korea**)** using wavelengths 260 and 280 nm and then determine the concentration by the ratio 1.8-2 according to (Ilbeigi *et al*., 2021).

3.3.3.3.3: Preparation of primers

A specific primer (Table 3-2) targeting the conserved *T. vaginalis β-tubulin* gene **(**dos Santos *et al*., 2015**)** (Alpha DNA/ Canada USA) was used to identify the *T. vaginalis* in urine or semen. Primer was prepared according to manufacturing company instructions by following steps:

1. The lyophilized primer was dissolved in DDW to obtain a final concentration of 100 pmol/µl as a stock solution.
2. Stock solution was kept in the freezer at -20 Cº until used.
3. Ten pmol/µl was prepared as a working primer by mixing 10 µl of the stock solution with 90 µl of the free ions DDW water (final volume of 100 µl).
4. A mixture of 25µl in each PCR reaction tube contained 5µl from Taq PCR PreMix (Table 3-3),1 µl of Forward primer **(**10 picomols/µl), 1 µl of Reverse primer (10 picomols/µl), and 3 µl of sample DNA and 15 µl of DDW and added in Eppendorf tube and mixes well then use in PCR.

Table 3-2: A specific primer of the *β-tubulin* gene.

Primer	Sequence	Tm (Cº)	GC (%)	Product size
Forward	5′-TCCGTGGCCGTATGTCATCT-3′	56.83	50%	**169 base pair**
Reverse	3′-GCTGTTGTGTTGCCGATGAA-5′	54.78	50%	

dos Santos *et al*. (2015).

3.3.3.3.4: Master Mix Component

The components of the maxime PCR premix kit (i-Taq) was shown as follows in (Table 3-3):

Table 3-3: Maxime PCR PreMix kit (i-Taq).

Material	Volume
i-Taq DNA Polymerase	5U/µl
DNTPs	2.5Mm
Reaction buffer (10X)	1X
Gel loading buffer	1X

3.3.3.3.5: Molecular Diagnosis of *T. vaginalis*

The Polymerase chain reaction (PCR) was done as described by dos Santos *et al*. (2015) (Table 3-4).

Table 3-4: The material used in the PCR Amplification protocol.

Component	Concentration
Taq PCR PreMix	5µl
Forward primer	10 picomols/µl (1 µl)

Reverse primer	10 picomols/µl (1 µl)
Sample DNA	3 µl
Distill water	15 µl
Final volume	25µl

3.3.3.3.6:The Thermal Cycler Conditions of Gene Amplified

The conditions used to amplify the *β-tubulin* gene are shown in (Table 3-5):

Table 3-5: The Steps and the Conditions of PCR Amplification.

The step	Phase	Tm (°C)	Time	No. of cycles
1-	Initial Denaturation	94	5 min	1
2-	Denaturation -2	94	45 sec.	40
3-	Annealing	63	45 sec.	
4-	Extension-1	72	45 sec.	
5-	Extension -2	72	10 min	1

dos Santos *et al*. (2015)

3.3.3.3.7: DNA Agarose Gel Electrophoresis

Each PCR product was electrophoresed through 1.5% agarose gel to determine the DNA fragments according to Sambrook *et al*. (1989) protocol.

3.3.3.3.8: Preparation of Agarose Gel

1. 1.5% agarose gel was prepared by dissolving 1.5 g of agarose in 100 ml TBE solution and then heated to a boil until become clear and then cooling at 50 °C in a water bath.

2. The gel was poured gently onto to pour plate carefully to avoid the forming of air bubbles
3. The comb was fixed in agarose gel to make wells for loading samples.
4. The gel was left to polymerization for 30 min and the comb was gently removed from the solid agarose.
5. The plate was fixed in its place in the horizontal electrophoresis unit.
6. The tank was filled with TBE buffer which covered the surface of the gel.

3.3.3.3.9: Preparation of Sample

1. During the polymerization period, the PCR product of each DNA sample was prepared for running in agarose gel electrophoresis by mixing 5 µl of PCR product DNA with 3 µl of loading buffer (Intron/ Korea).
2. After the mixing process, the samples were loaded into the gel wells.
3. The samples were electrophoresed through the agarose gel at 7 volts/cm^2 for 1.5 hours until the dye reached about 2 cm before the end.
4. Stained the DNA band with a staining solution containing 3µl of RedSafe^{TM} Nucleic Acid stain in 500 ml DDW in the staining tray.
5. For showing the bands put the gel under the UV light source with a wavelength of 336 nanometers.

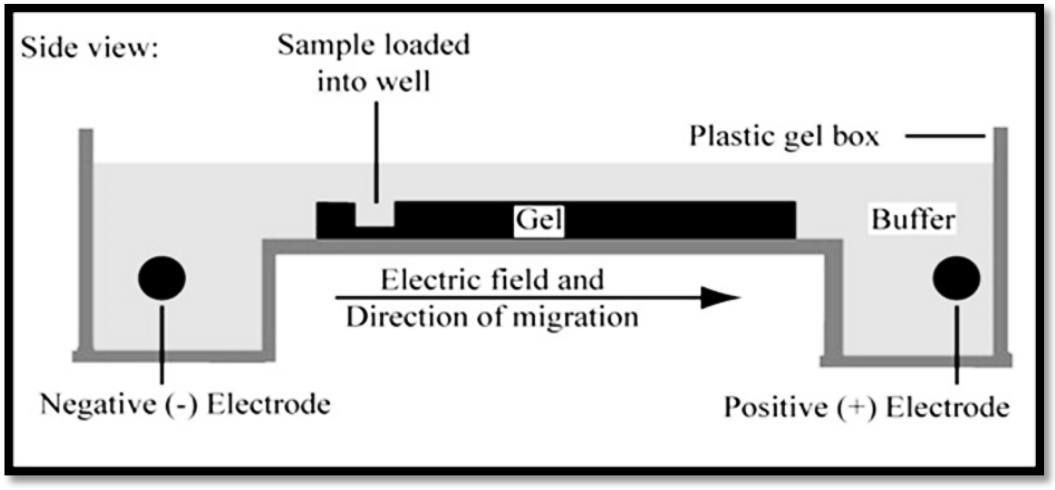

Figure 3-2: The side view sketch of the electrophoresis unit (https://ocw.mit.edu/courses).

3.3.3.4: Real-time-quantitative PCR (RT- qPCR)

3.3.3.4.1: The Washing Buffer Preparation

1. Approximately 10 ml of ethanol 95% was added to 40 ml of Direct-zol™ RNA PreWash [1] concentrate (Table 3-6).
2. Approximately 52 ml of 95% ethanol was added to 12 ml RNA wash buffer[2].

Table 3-6: Direct-zol™ RNA MiniPrep.

Direct-zol™ RNA Miniprep Kit Size (Preps)	R2051 (50)
TRI Reagent®	50 ml
Direct-zol™ RNA PreWash [1] (concentrate)	40 ml
RNA Wash Buffer [2] (concentrate)	12 ml
DNase I[3] (lyophilized)	1500 U
DNA Digestion Buffer	4 ml
DNase/RNase-Free Water	6 ml
Zymo-Spin™ IIC Columns	50 pcs
Collection Tubes	100 pcs

3.3.3.4.2: Sample Preparation

1. One hundred µl of the sample was taken and homogenized with 300 µl of TRI Reagent (USA, Zymo) and mixed well for 5 minutes.
2. The mixture was centrifuged at 12000rpm for 30 sec to remove the particulate debris.
3. The supernatant was transferred into an RNase-free tube.

3.3.3.4.3: RNA Purification

1. An equal volume of ethanol 95% was taken to a sample lysed in TRI Reagent and well mixed.
2. The mixture was transferred into a Zymo-Spin™ IIC Column[2] in a collection tube
3. The mixture was centrifugated at 12000 rpm for 30 sec.
4. The column was transferred into a new collection tube and discarded the flow through.
5. DNase I treatment in column [3]:

D1: Four hundred µl of RNA wash buffer was added to the column and centrifuged.

D2: Five µl of DNase I of concentration (6 U/µl) were added in an RNase-free tube then 75µl of DNA digestion buffer and well mixed and then transferred the mixture directly to the column matrix.

D3: The mixture was incubated at RT for 15 min.

6. Four hundred µl of Direct-zol™ RNA PreWash were added to the column and centrifuged after then, discard the flow-through and repeated this step one time.

7. Seven hundred µl of RNA wash buffer was added to the column and then centrifuge at 12000 rpm for 2 min to ensure complete removal of the wash buffer and the supernatant was discarded.
8. The column should be transferred carefully into an RNase-free tube.
9. Fifty µl of DNase/RNase-Free water was directly added to the column matrix to elute RNA and then centrifuged at 12000 rpm for 2 min to remove the wash buffer and discard the supernatant and the pellet was kept in deep freeze -20 until use.

3.3.3.4.4: Reverse transcription of RNA to cDNA

The following reaction mixture was prepared on ice according to the following steps.

1. Two µl from 5× PrimeScriptTM mix was added to the micro tube volume 1.5 ml, 8µl from total RNA reagent, and completed with RNase Free DDW up to 10µl.
2. The reaction mixture was incubated at 37℃ for 15 min (Reverse transcription), then at 85℃ for 5 sec (reverse transcriptase inactivation with heat treatment), then keep at 4℃.

3.3.3.4.5: Preparation of qPCR master Mix

The amplification of cDNA in the RT-qPCR system each micro tube (1.5 ml) contain 10µl of KAPA SYBR FAST qPCR Master Mix (2X) Universal, 1µ from forward primer, 1µ of reverse primer, 3µl of Nuclease-free water, and 5µl of Template DNA Sample.

3.3.3.4.6: Real-time quantitative PCR Cycling Program

The amplification of the cDNA in RT-qPCR had been done under the conditions described by dos Santos *et al.*(2015) with some modifications shown in (Table 3-7):

Table 3-7: RT-qPCR Cycling.

Step	Temp. (°C)	Time in min	Cycle
Pre-denaturation	95	5	Hold
Denaturation	95	2	40
Annealing	62	2	
Extension	72	2	

3.3.4: Diagnosis of Concomitant Bacteria

3.3.4.1: Primary Culture of Bacteria

The urine and semen samples were cultured to detect the bacterial species that were present in the samples. A part of each sample was placed in a tube containing a transport medium that kept and nourished the bacteria present in the samples, which is called Amies transport media as shown in Figure (3-3), then kept in the incubator until it was transferred to the laboratory for the culture process. The samples were cultured on different media, which are Blood agar, MacConkey agar, chocolate agar, Thayer martin agar, and Mannitol salt agar, and the media were incubated aerobically at 37ºC. As for the chocolate agar, Thayer martin agar media were grown in anaerobic conditions to detect the growth of fastidious bacteria using a candle jar in the presence of CO_2 gas. The bacterial growth was examined and it was confirmed that growth was found after 24 hours. To identify the bacteria, the samples that gave bacterial growth were tested by the Vitek 2 system, while the non-cultivable samples that did not give bacterial growth were tested with the conventional polymerase chain technique by using a primer *16S rRNA* gene.

Figure 3-3: Amies transport medium.

3.3.4.2: Preparation of the Cultured Media

All culture media were prepared according to the manufacturer's instructions. It is initially boiled to fully dissolve and sterilized in autoclaves at 121°C (15 Ibs pressure) for 15 minutes. To check for contamination, the sterile Petri dishes were then poured, incubated for 24 hours, and stored at 4°C until use. The cultural media are as follows:

1- Blood Agar

According to the manufacturer's instructions, the blood agar medium was prepared by suspending 40 g of blood agar in a flask with 1000 ml of DW, then the medium was heated to boiling and sterilized in an autoclaved at 15 par, 121 °C for 15 minutes, after Cooling to 45-50°C 10% of fresh blood were added. This medium is used to show colonial morphology and hemolysin production (Collee *et al*., 1996).

2- MacConkey Agar

The MacConkey agar medium was prepared by suspending 51gm from MacConkey agar base powder in 1000 ml DW, thoroughly

mixed heating gently until boiling. The medium was autoclaved for 15 minutes at a pressure of 15 par at 121°C. Either distributed into sterile tubes or pour into sterile Petri dishes. This medium was used to cultivate and differentiate coliforms and enteric pathogens based on their ability to ferment lactose (Parks, 2004).

3- Chocolate Agar

Chocolate agar medium was prepared by suspending 40 gm of blood agar base powder in 1000 ml of DW and thoroughly mixing and heating gently until boiling. The medium was autoclaved for 15 minutes at a pressure of 15 par at 121°C. Cooled to 45°–50°C. 100 ml of sterile sheep blood was added aseptically. Stirring regularly, gently heated to 85 °C for 5 - 10 minutes. cooled to 50 °C. This medium is used for the isolation and cultivation of different types of fastidious microorganisms (Ronald, 2004).

4- Modified Thayer-Martin agar

This Modified Thayer-Martin medium was prepared by suspending 68 gm from Modified Thayer-Martin agar base except for CNVT inhibitor (colistin sulfate, nystatin, vancomycin, trimethoprim lactate) and supplement solution to distilled water and bringing volume to 990.0ml, thoroughly mixed and heating gently until boiling. The medium is poured into tubes or flasks. Autoclaved for 15 minutes at a pressure of 15 par at 121°C. Cooled to 45°–50°C. 10.0 ml of sterile supplement solution was added and 10 ml of sterile CNVT inhibitor was added aseptically, then poured into sterile Petri dishes. This medium was used for the isolation of *Neisseria* species from samples containing mixed flora of bacteria and fungi (Parks, 2004).

5- Mannitol Salt Agar

This Mannitol salt medium was prepared by suspending 111 gm from Mannitol salt agar in 1000 ml of DW, thoroughly mixing, and heating gently until boiling. The medium was autoclaved for 15 minutes at 15 par at 121°C. Then poured the medium into sterile Petri dishes. This medium was used to selectively isolate, cultivate, and enumerate *staphylococci* from clinical and nonclinical samples. Medium-yellow color is produced by organisms that use mannitol with *staphylococcus aureus*, but if the medium is not fermented to a yellow color and remains on the same pink color, then it is diagnosed as *Staphylococcus epidermides* which is negative for the coagulase test (Ronald, 2004).

3.3.4.3: Confirmatory Diagnosis of Bacteria by Vitek 2 Compact System

The steps of the Vitek 2 system were performed as follows:

3.3.4.3.1: Preparation of Bacterial Suspension

A sterile wire loop is used to transfer a sufficient number of colonies of pure culture and to suspend the microorganism in 3 ml of sterile saline in a plain tube. The turbidity is measured using a turbidity meter called the DensiChek^{TM}. For gram-negative and gram-positive bacteria, the turbidity was 0.50-0.63.

3.3.4.3.2: Inoculation of Identification Card

Inoculated identification cards are with microorganism suspensions. The test tube containing suspension is placed into a special rack (cassette) and the transfer tube is inserted into the corresponding suspension tube while the identification card is placed in the adjacent slot. A maximum of 10 or 15 tests can accommodate in

the cassette. Manually inserting the filled cassette into a vacuum chamber station. The organism suspension is pushed into micro channels through the transfer tube, filling all tests well.

3.3.4.3.3: Card Sealing and Incubation

A mechanism was used to insert an inoculated card into the carousel incubator after cutting off the transfer tube and sealing the card. Carousel incubator that can hold about 30 to 60 cards. All types of cards are incubated online at around 35.5 + 1.0°C. Hence, each card is moved from the carousel incubator once every 15 minutes and returned to the incubator until the next reading time after being transported to the optical system for reaction readings. Throughout the whole incubation period, data were collected at intervals of 15 minutes.

3.3.4.4: Diagnosis of Bacteria by *16S rRNA* Gene

3.3.4.4.1: Extraction of Bacterial DNA

The following protocol for getting optimum performance, beta-mercaptoethanol (user-supplied) was added to the bacterial DNA-binding buffer until it reached a final dilution of (0.5%) (v/v) i.e., 500 µl per 100 ml.

1. Fifty-hundred mg (50 – 100 mg wet weight) of bacteria which equates to approximately 10^9 bacterial cells were added which have re-suspended in up to (200 µl) of isotonic buffer or water (e.g. Phosphate-buffered saline). The tube should contain (750 µl) of Lysis Solution.

2. In a bead beater fitted with a (2 ml) tube holder assembly, the mixture was secured and beat at maximum speed for approximately ≥ 5 minutes.

3. A microcentrifuge was used to centrifuge the ZR BashingBeadTM lysis tube at 12000 rpm for one minute.

4. Supernatant up to (400 µl) was transferred to a Zymo-Spin IV Spin Filter (Orange Top) in a collection tube, and it should be centrifuged at 5200 rpm for one minute.

5. Filtrate from Step 4 in the collection tube, was mixed with (1,200 µ l) of bacterial DNA binding buffer.

6. Eight hundred µl (800 µl) of the mixture from Step 5 was transferred to a Zymo-Spin™ IIC Column in a collection tube and then centrifuged at 12000 rpm for one minute.

7. Step 6 was repeated after discarding the flow through from the collection tube.

8. In a new collection tube, (200 µl) of DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column and centrifuged at 12000 rpm for one minute.

9. The Zymo-Spin™ IIC Column was centrifuged at 12000 rpm for 1 minute after (500 µl) of bacterial DNA Wash Buffer was added.

10. one hundred µl (100µl) (35µl minimum) of DNA Elution Buffer was added directly to the column matrix after transferring the Zymo-Spin™ IIC Column to a clean (1.5 ml) micro centrifuge tube. To elute the DNA, the column was centrifuged at 12000 rpm for 30 seconds.

3.3.4.4.2: Nanodrop of DNA

The purity and concentration of the DNA were estimated by Nanodrop as described in item (3.3.3.3.2).

3.3.4.4.3: Primer used in this study

Primers and the oligonucleotide sequence were prepared and done according to Srinivasan *et al*. (2015). As shown in the (Table 3-8).

Table 3-8: The universal primer *16Sr RNA* of the gene (Srinivasan *et al*., 2015).

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- AGAGTTTGATCCTGGCTCAG-3'	54.3	50.0	1250 base pair
Reverse	5'- GGTTACCTTGTTACGACTT- 3'	49.4	42.1	

3.3.4.4.4: Master Mix Component

The master mix component consisted of some material as summarized in (Table 3-9).

Table 3-9: The components of the maxime PCR premix kit (i-Taq).

Rial	Volume
i-Taq DNA Polymerase	5U/µl
DNTPs	2.5Mm
Reaction buffer (10X)	1X
Gel loading buffer	1X

3.3.4.4.5: Molecular Diagnosis of Bacteria

Polymerase chain reaction (PCR) components and conditions as described by (Waters and Shapter, 2014) as shown in (Table 3-10).

Table 3-10: The material used in the PCR amplification protocol.

Components	Concentration
Taq PCR PreMix	5µl
Forward primer	10 picomols/µl (1 µl)

Reverse primer	10 picomols/µl (1 µl)
Sample DNA	3 µl
Distill water	15 µl
Final volume	25µl

3.3.4.4.6: The Optimal Conditions

The steps and the conditions used in PCR amplification were done according to Waters and Shapter (2014) as described in Table (3-11).

Table 3-11: The steps and the conditions of PCR amplification.

The Step	Phase	Tm (^{o}C)	Time	No. of cycle
1-	Initial Denaturation	94^{o}C	3 min	1 cycle
2-	Denaturation -2	94^{o}C	45sec	35 cycle
3-	Annealing	56^{o}C	1 min	
4-	Extension-1	72^{o}C	1 min	
5-	Extension -2	72^{o}C	7 min	1 cycle

3.3.4.4.7: The DNA Electrophoresis

Each PCR product was electrophoresed through 1.5% agarose gel as described in item 3.3.3.3.7.

3.3.4.4.8: Statistical Analysis

The data of this study were statistically analysed by SPSS software (version 28) with using Chi-square test ($\chi 2$), t-test, and Analysis of Variance/ANOVA-F-test. The probability value "$p \leq 0.05$" was used as a statistically significant criterion.

Chapter Four

RESULT AND DISCCUSSION

4. The Results and Discussion

4.1: The IR of male with *T. vaginalis* and bacteria

The results (Table 4-1) showed that the IR of males with *T. vaginalis* is 31.82% (14/44), while the overall IR of bacteria is 17.53% (17/97). Of 44 Participant male (PM), the *T. vaginalis* and bacteria were present together (*T. vaginalis* +, bacteria+) in 11.36% (5/44), while the IR of *T. vaginalis* alone without bacteria (*T. vaginalis* +, bacteria-) is 20.45% (9/44), and the percentage of uninfected male (*T. vaginalis* -, bacteria-) is 68%.

Table 4-1:The Percentage of Infection with *T. vaginalis* and Bacteria Among Male.

Pathogens	No. exam	No. infected (%)
T. vaginalis	44	14 (31.8)
Bacteria	97	17 (17.53)
T. vaginalis +, bacteria+	44	5 (11.36)
T. vaginalis -, bacteria-	44	30 (68.18)
T. vaginalis +, bacteria-	44	9 (20.45)
T. vaginalis -, bacteria+	44	0 (0.00)

4.2: Effect of *T. vaginalis* and or Bacteria and their interaction on the semen parameter

4.2.1: Effect of *T. vaginalis* on Total Count of Sperm

The results of the current study (Table 4-2) show that the mean±SD (95%C.I) of the total count of sperm for trichomoniasis-infected males is $(85.07\pm66.23)x10^6$ $((95\%C.I=46.82-123.31)x10^6)$, compared to uninfected males of $(110.30\pm54.99)x10^6$ ((95%C.I=89.76-

130.83)x10⁶). There are non-statistically significant differences between infected and uninfected males in the total count of sperm (F=1.763, *P*> 0.05).

Table 4-2: Effect of *T. vaginalis* on the Total Count of Sperm.

T. vaginalis	No. males	Mean X 10^6	SD X 10^6	95% CI. X10^6
Uninfected	30	110.30	54.99	(89.7-130.8)
Infected	14	85.07	66.23	(46.82-123.31)
Total	44	102.272	59.222	-

F=1.763 ***P*> 0.05**

This study shows (Table 4-2) a decrease in the total number of spermatozoa but not significant. This decrease may be attributed to the release of some cytotoxic molecules by *T. vaginalis* "like perforin" which is capable of making pores in cell membranes like RBC (Gomez *et al*., 2010). Additionally, other nucleated cells degrade phosphatidylcholine (Harp and Chowdhury, 2011) which is considered a major component of the cell membrane (Yamashita *et al*., 2014) and in other sites of mammalian cells (Hishikawa *et al*., 2014), this impact on cell energy metabolism (van der Veen *et al*., 2017). These results agree with the study of Akgul *et al*. (2018), who found that trichomoniasis has no statistically significant negative effect on sperm total count. but disagree with Gong *et al*. (2018), who found that trichomoniasis leads to a decrease in sperm total count and a decrease in fertility.

4.2.2: Effect of Bacterial infection on total count of sperm

The results of (Table 4-3), show that the mean±SD of the total count of sperm in males with bacterial infection was (58.11±53.70)x10⁶ ((95%C.I=30.50–85.73)x10⁶) it is less than of uninfected males (75.50±58.20)x10⁶ ((95%C.I=62.54-88.45)x10⁶). However, there is no statistically significant difference between infected and uninfected males with bacterial infection and its impact on sperm total count (F=1.283, *P*>0.05).

Table 4-3: Effect of Bacterial Infection on Total Count of Sperm.

Bacteria	No. males	Mean x10⁶	SD x10⁶	95% CI. x10⁶
Uninfected	80	75.50	58.20	(62.54-88.45)
Infected	17	58.11	53.70	(30.50-85.73)
Total	97	72.45	57.55	-

F=1.283 ***P*>0.05**

This study is somewhat in line with Nabi *et al*. (2013) who stated that the mean of the total count of sperm of an infected individual with bacterial infection was found to be close to 54.62x10⁶. This decrease in the total count of sperm may be caused by an inverse correlation between the presence of various bacteria in the semen with sperm counts such as induction of apoptosis of sperms and bacterial toxins (Villegas *et al*., 2005).

4.2.3: The Interaction effect between the Infection of *T. vaginalis* and Bacteria on the Total Count of Sperm

The current study (Table 4-4) shows that the mean±SD of total count of sperm in cases infected with the two pathogens, *T. vaginalis*

and bacteria is (54.60±38.15)x10⁶, this is less than that infected with *T. vaginalis* alone (102.0±74.15)x10⁶ or uninfected. There is a statistically significant effect in the interaction of *T. vaginalis* and bacteria on the total count of sperm (F=47.05, p<0.05).

Table 4-4: The Effect of Interaction between *T. vaginalis* and bacteria on the total Count of Sperm.

Interaction	No. males	Mean x10⁶	SD x10⁶	F-test		95% CI x10⁶
				F value	*P*	
T. vaginalis + Bacteria	5	54.60	38.15	47.05	<0.05	(2.29-106.90)
NO *T. vaginalis* NO Bacteria	30	110.30	54.99			(88.94-131.65)
Infected *T. vaginalis* – NO Bacteria	9	102.0	74.15			(63.01-140.98)
Total	44	102.27	59.22			-

These results are in line with the findings of Nabi *et al*. (2013) who show a negative relationship between the interaction of *T. vaginalis* and concomitant bacteria on the total count of sperm in human male semen. This decrease in the total count of sperm may be due to the effect of sperm function by the agglutination of motile spermatozoa, disruption of acrosome reaction, alteration of morphology, and induction of apoptosis (Fode *et al*., 2016).

4.2.4: Effect of *T. vaginalis* Infection on the Mean of Sperm per ml

The results of the present study (Table 4-5) show that the mean±SD (95%C.I) of the number of spermatozoa per ml for trichomoniasis-infected males is 30.39±23.75x10⁶/ml ((95%C.I=16.68-44.11)x10⁶). It

is less than that of uninfected males (37.75±18.38) x10⁶/ml ((95%C.I=30.89-44.62)x10⁶), but there are non-statistically significant differences between infected and uninfected males in the mean of spermatozoa per ml (F=1.267, *P*> 0.05).

Table 4-5: Effect of *T. vaginalis* on the number of sperm/ml .

T. vaginalis	No. males	Mean X 10⁶/ml	SD X 10⁶/ml	95% CI. X10⁶
Uninfected	30	37.75	18.38	(30.89-44.62)
Infected	14	30.39	23.75	(16.68-44.57)
Total	44	35.416	20.265	-

F=1.267 ***P*> 0.05**

This result agrees with the findings of Akgul *et al*. (2018), which reported that the decrease in the density of spermatozoa/ml may be due to the mechanism of ingesting there by *T. vaginalis*, this mechanism depends on parasite intensity, strain, virulence and the microenvironment in the site of infection, these in later effect on the male fertility (Mielczarek and Blaszkowska, 2016), and disagree with the finding of Gimenes *et al*. (2014) who reported no decrease in the number of spermatozoa/ml.

4.2.5: Effect of Bacterial Infection on the mean Number of Sperm per ml

In a current study (Table 4-6), the mean±SD of the number of sperm per ml in males with bacterial semen infection is 27.44±32.54 x10⁶/ml ((95%C.I=10.70–44.17)x10⁶), as compared with uninfected males is 27.17±22.68 x10⁶/ml ((95%C.I=22.12-32.21)x10⁶). There are non-statistically significant differences between infected and

uninfected males with bacterial infection and there was no impact on the mean number of sperm per ml (F=0.002, *P*>0.05).

Table 4-6:Effect of Bacterial Infection on a mean Number of sperm/ml.

Bacteria	No. males	Mean x10⁶/ml	SD x10⁶/ml	95% CI. x10⁶
Uninfected	80	27.17	22.68	(22.12-32.21)
Infected	17	27.44	32.54	(10.70-44.17)
Total	97	27.21	24.49	-

F=0.002 ***P*>0.05**

This result disagrees with Fraczek and Kurpisz (2015), who found that the mean of the number of spermatozoa per ml of infected males is more than an uninfected individual.

4.2.6: The effect of the Interaction between *T. vaginalis* and Concomitant Bacteria on the Density of sperm/ml semen

Table (4-7) shows that the mean±SD of the density of sperm per ml semen in males infected with the two pathogens, *T. vaginalis* and bacteria (18.07±12.89)x10⁶/ml is less than that of uninfected males with any pathogens (37.75±18.38)x10⁶/ml and less than of infected with *T. vaginalis* alone (37.24±26.20)x10⁶/ml. There is a statistically significant effect in the interaction of *T. vaginalis* and bacteria on the semen volume per ml (F=11.578, *p*<0.05).

Table 4-7: The Effect of the Interaction between *T. vaginalis* and Concomitant Bacteria on the density of Sperm/ ml Semen.

Interaction	No. males	Mean x10⁶/ ml	SD x10⁶/ ml	F-test		95% CI x10⁶
				F value	*P*	
T. vaginalis + Bacteria	5	18.07	12.89	11.573	<0.05	(2.30-3.89)
NO *T. vaginalis* NO Bacteria	30	37.75	18.38			(3.11-3.75)
Infected *T. vaginalis* - NO Bacteria	9	37.24	26.20			(2.18-3.36)
Total	44	35.41	0.89			-

This result agrees with the finding of Ho *et al*. (2022), who confirmed that there is an association between *T. vaginalis* and bacterial infection on seminal oxidative stress which effect sperm total count through the generation of oxidative stress during the inflammatory process of the seminal vesicles (Aydemir *et al*., 2008). There is a clear effect of the interaction between *T. vaginalis* and bacteria on the density of spermatozoa(sperm/ml of semen), this effect leads to a decline in the density of spermatozoa to more than half (see Table 4-7).

4.2.7: Relationship between the *T. vaginalis* Infection and the Viscosity of Semen

The current study (Table 4-8) shows that 21.43% of the *T. vaginalis* infected males had high semen viscosity compared with 16.67% among uninfected males. There is non-statistical relationship between *T. vaginalis* infection and semen viscosity (χ^2=0.146, *P*>0.05).

Table 4-8: Relationship between the *T. vaginalis* Infection and Viscosity of Semen .

Viscosity		*T. vaginalis* diagnosis		Total
		Non –Infected	Infected	
High	No.	5	3	8
	% Viscosity	62.5	37.5	100.0%
	% *T. vaginalis* diagnosis test	16.67	21.43	18.2%
Normal	No.	25	11	36
	% Viscosity	69.4	30.6	100.0%
	% *T. vaginalis* diagnosis test	83.3	78.6	81.8%
Total	No.	30	14	44
	% Viscosity	68.2	31.8	100.0%
	% *T. vaginalis* diagnosis test	100.0	100.0	100.0%
	% of Total	68.2	31.8	100.0%

χ^2=0.146 ***P*> 0.05**

These results disagree with the findings of Elia *et al*. (2009), and Mielczarek and Blaszkowsk (2016), who found an increase in semen viscosity of *T. vaginalis*-infected males.

4.2.8: Effect of Bacterial Infection on Semen Viscosity

The results of this study (Table 4-9) found that 88.2% of infected cases with bacteria had high viscosity compared with 80% for uninfected. The statistical data showed no significant association between semen viscosity and bacterial infection (χ^2=0.629, *P*> 0.05). and that the percentage of high semen viscosity in infected individuals was 11.8%.

Table 4-9: Effect of bacterial infection on semen viscosity.

Viscosity		male diagnosis. For bacteria		Total
		Non –Infected	Infected	
Normal	No.	64	15	79
	% Viscosity	81%	19%	100.0%
	% Infected bacteria	80%	88.2%	81.4%
High	No.	16	2	18
	% Viscosity	88,9%	11.1%	100.0%
	% Infected bacteria	20%	11.8%	18.6%
Total	No. Count	80	17	97
	% Viscosity	82.5%	17.5%	100.0%
	% Infected bacteria	100.0%	100.0%	100.0%

χ^2=0.629 ***P*> 0.05**

A similar finding was found by Munuce (1999), who did not find a relationship between the viscosity of the seminal fluid and the presence of bacteria in semen. Therefore, a genitourinary tract infection may not always be related to seminal fluid hyperviscosity. But this result disagrees with Barbagallo *et al*. (2021), who found that the semen viscosity increased in bacterial infection by generating oxidative stress (Harchegani *et al*., 2019).

4.2.9: The Interaction Effect between the Infection of *T. vaginalis* and Bacteria on the Semen Viscosity

The current study (Table 4-10) shows that the mean±SD of the semen viscosity in cases infected with the two pathogens, *T. vaginalis* and bacteria is 1.20±0.44, this is less than that infected with *T. vaginalis* alone 1.22±0.44 and higher than non-infected 1.17±0.37.

There is a statistically significant effect in the interaction of *T. vaginalis* and bacteria on the semen viscosity (F=128.82, p<0.05).

Table 4-10: The interaction effect between the infection of *T. vaginalis* and bacteria on the semen viscosity.

Interaction	No. males	Mean	SD	F-test		95% CI
				F value	*P*	
T. vaginalis + Bacteria	5	1.20	0.44	128.82	<0.05	(0.84-1.56)
NO *T. vaginalis* NO Bacteria	30	1.17	0.37			(1.02-1.31)
Infected *T. vaginalis* - NO Bacteria	9	1.22	0.44			(0.95-1.49)
Total	44	1.18	0.39			-

The increase in the semen viscosity in males with infection of *T. vaginalis* and bacteria may be caused by hyperviscosity due to stimulation of a local inflammatory reaction which leads to more production in reactive oxygen (ROS) which affect sperm density (Aydemir *et al*., 2008; Agarwal *et al*., 2018).

4.2.10: The Relationship between *T. vaginalis* Infection and semen liquefaction time

The current study (Table 4-11) shows that the mean±SD (95%C.I) of the liquefaction time of the semen among trichomoniasis-infected males is 30.71±2.67 minutes (95%C.I=29.17–32.26), and among uninfected males is 33.33±8.84 minutes (95%C.I=30.03-36.63). There are statistically significant differences between infected and non-infected males in the semen's liquefaction time (F=414.21, P< 0.05).

The results of this study are similar to the results of the study of Gopalkrishnan *et al*. (1990) who found that the *T. vaginalis* parasite caused a decrease in the time of liquefaction of the semen. These results may be contributed to infection with *T. vaginalis* caused a decreased levels of Zinc which leads to impaired semen quality (Langley *et al*., 1987).

Table 4-11: The Relationship between *T. vaginalis* infection and semen liquefaction time.

T. vaginalis	No. males	Mean Min	SD Min	95% CI. Min
Uninfected	30	33.33	8.84	(30.03-36.63)
Infected	14	30.71	2.67	(29.17-32.26)
Total	44	32.50	7.510	-

F=414.21 *P*< 0.05

4.2.11: The relationship between Bacterial Infection and the liquefaction Time of Semen

Table (4-12) shows that the mean±SD of the liquefaction time of the semen among bacterial infected males is 30.29±1.21 (95%C.I=26.94–33.64), is low than uninfected males is 32.77±7.61 (95%C.I=31.22-34.32). There are statistically significant differences between infected and non-infected males in the liquefaction time for semen (F=1046.32, *P*< 0.05).

This study agrees with Barbagallo *et al*. (2021), who confirmed that the liquefaction time of semen is negatively affected by bacterial infection through the generation of oxidative stress during the inflammatory process of the seminal vesicles, which generates an

increase in the viscosity of the semen and then prolongs the liquefaction time (Aydemir *et al*., 2008).

Table 4-12: Effect of Bacterial Infection on liquefaction time.

Bacteria	No. males	Mean Min	SD Min	95% CI Min
Uninfected	80	32.77	7.61	(31.22-34.32)
Infected	17	30.29	1.21	(26.94 -33.64)
Total	97	32.34	6.99	-

F=1.778 ***P*<0.05**

4.2.12: Effect of *T. vaginalis* infection on the Sperm velocity

Table (4-13) shows the mean of the sperms which had high velocity in the semen of the trichomoniasis-infected males was (12.97±9.51)x10^6 (95%CI) compared with uninfected of (18.44±14.76)x10^6, there are statistically significant differences between infected and uninfected males in the semen velocity sperm (F=11.599, *P*<0.05).

Table 4-13: Effect of *T. vaginalis* infection on sperm velocity.

T. vaginalis	No. males	Mean x10^6	SD x10^6	95% CI. X10^6
Uninfected	30	18.44	14.76	(12.92-23.95)
Infected	14	12.97	9.51	(7.47-18.46)
Total	44	16.700	13.452	-

F=11.599 ***P*<0.05**

These findings agree with the findings of Hernández *et al*. (2014) who found an effect of *T. vaginalis* infection on sperm velocity. This

effect may be due to some *T. vaginalis* secretes that induce hyperviscosity which prevents or capture the sperm to move or caused a decrease in the activity of the sperms (Mielczarek and Blaszkowska, 2016).

4.2.13: Effect of Bacterial infection on sperm velocity

The results of this study (Table 4-14) show the mean±SD of the sperm which had high velocity in the semen of infected males with bacteria was 13.55±11.66x10^6 ((95%C.I=6.52 –20.58)x10^6), is less than uninfected males which 18.68±15.13x10^6 ((95%C.I=15.43-21.92)x10^6). There are statistically significant differences between infected and uninfected males with bacteria on sperm velocity in semen (F=72.75 P<0.05).

Table 4-14: Effect of Bacterial Infection on Sperm Velocity.

Bacteria	No. males	Mean x10^6	SD x10^6	95% CI. x10^6
Uninfected	80	18.68	15.13	(15.43-21.92)
Infected	17	13.55	11.66	(6.52-20.58)
Total	97	17.78	14.66	-

F=72.75 **P<0.05**

A similar pattern of the result was obtained by Marchiani *et al*. (2021), who found that bacteria might release the soluble spermatotoxic molecules such as sperm immobilizing factor leading to a change in sperm morphology which causes a substantial decline in spermatozoa to move over time as a result of inflammatory reactions within the male urogenital tract which are strongly connected to oxidative stress (Fraczek and Kurpisz, 2015).

4.2.14: The Interaction Effect between the Infection of *T. vaginalis* and bacteria on the sperm velocity

The results of the current study (Table 4-15) show that the mean±SD of the count of the fast sperms in males infected with the two pathogens *T. vaginalis* and bacteria is 13.32±10.84x10⁶, this is less than that of uninfected males (18.44±14.76)x10⁶. There is a statistically significant effect in the interaction of *T. vaginalis* and bacteria on the count of the fast sperms (F=22.89, p<0.05).

Table 4-15: The Interaction Effect between the Infection of *T. vaginalis* and Bacteria on the sperm velocity.

Interaction	No. males	Mean x10⁶	SD x10⁶	F-test		95% CI x10⁶
				F value	*P*	
T. vaginalis + Bacteria	5	13.32	10.84	22.89	<0.05	(1.10-25.53)
NO *T. vaginalis* NO Bacteria	30	18.44	14.76			(13.45-23.42)
Infected *T. vaginalis* - NO Bacteria	9	12.77	9.39			(3.67-21.88)
Total	44	16.70	13.45			-

The decrease of the count of the fast sperms in the semen that is infected with two pathogens *T. vaginalis* and bacteria may be associated with the activity of the two pathogens like enzymatic secretions of *T. vaginalis* CDF and somebody's immune response such as interleukins, cytokines which may affect the vitality of sperms (Mielczarek and Blaszkowska, 2016). The ability of *T. vaginalis* to interact with pathogenic bacteria in semen constitutes a third pathogenic mechanism during endocytosis and can consume various

mammalian cells such as spermatozoids and cause sperm death to obtain essential sources of nutrients and helping to defend from the immune system (Fiori and Margarita, 2014).

4.2.15: Effect of *T. vaginalis* Infection on Sperms Death

The results of the current study (Table 4-16) showed that the mea±SD (95% CI) of the count of the dead sperms among trichomoniasis-infected males (35.69±12.42x10^6) ((95%C.I=28.52-42.86)x10^6) is higher than that of uninfected males (30.82±15.52)x10^6 ((95%C.I=25.02-36.61)x10^6). There are statistically significant differences between the means of the dead sperms of *T. vaginalis* infected and uninfected males (F=6.059, P<0.05).

Table 4-16: Effect of *T. vaginalis* Infection on Sperms Death.

T. vaginalis	No. males	Mean x10^6	SD x10^6	95% CI x10^6
Uninfected	30	30.82	15.52	(25.02-36.61)
Infected	14	35.69	12.42	(28.52-42.86)
Total	44			-

F=6.059 P<0.05

This is consistent with what Tuttle Jr *et al*. (1977) mentioned that infection with *T. vaginalis* leads to the death of sperms in infected males, and this is attributed to the protein secretions by this parasite which leads to restriction of sperms movement and death. And also showed *InVetro* that a high density of *T. vaginalis* from (10^4 to 10^7 *T. vaginalis* per ml) causes immotile of about 75% of the sperms and dead through one hour after the fresh sperms mixed with this parasite (Benchimol *et al*., 2008).

4.2.16: Effect of the Bacterial Infection on Sperms Death

The results (Table 4-17) show that the mean±SD of the count of sperm death in the semen of males infected with bacteria is 49.45±20.37x10^6 ((95%C.I=40.51–58.39)x10^6). It is higher than that of uninfected males (38.20±18.17)x10^6 ((95%C.I=34.08-42.32)x10^6). There are statistically significant differences between infected and uninfected males in the mean of the number of sperms death (F=229.65, *P*<0.05).

Table 4-17: Effect of the Bacterial Infection on Sperm Death.

Bacteria	No. males	Mean x10^6	SD x10^6	95% CI.x10^6
Uninfected	80	38.20	18.17	(34.08-42.32)
Infected	17	49.45	20.37	(40.51-58.39)
Total	97	40.17	18.96	-

F=229.65 ***P*<0.05**

The percentage of sperm that die in the semen of infected individuals was higher than that of non-infected, There are many suggestions that apoptosis is caused by different types of bacteria that cause sperm DNA damage during bacteriospermia. However, DNA fragmentation was observed in both apoptotic and necrotic sperm. In vitro by experimental semen infection, human spermatozoa showed reduced viability in hypoosmotic swelling (HOS) tests and an increased percentage of sperm death (Fraczek *et al*., 2014).

4.2.17: The Interaction Effect between the Infection of *T. vaginalis* and Bacteria on the mean of the Death of Sperms

The current study (Table 4-18) shows that the mean±SD of the death of sperm in cases infected with the two pathogens, *T. vaginalis* and bacteria is 43.94±8.45x10^6, this is higher than that of infected with *T. vaginalis* alone ((31.11±12.19) x10^6) or uninfected 30.82±15.51 x10^6. There is a statistically significant effect in the interaction of *T. vaginalis* and bacteria on the death of sperms (F=75.701, p<0.05).

Table 4-18: The Interaction Effect between the Infection of *T. vaginalis* and bacteria on the mean of the Death of sperms.

Interaction	No. males	Mean x10^6	SD x10^6	F-test		95% CI x10^6
				F value	*P*	
T. vaginalis + Bacteria	5	43.94	8.45	75.701	<.005	(30.96-56.91)
NO *T. vaginalis* NO Bacteria	30	30.82	15.51			(25.52-36.11)
Infected *T. vaginalis* - NO Bacteria	9	31.11	12.19			(21.44-40.78)
Total	44	32.37	14.63			-

The increase in the count of the death of sperms is related to more than one factor such as the pH of the semen fluid, and the enzymatic excretion from the two pathogens that affect the viscosity of semen (Aydemir *et al*., 2008; Agarwal *et al*., 2018) and some reported that interaction effect between the infection of *T. vaginalis* and bacteria on the death of sperms may cause DNA fragmentation during increased oxidative stress which leads to the death of sperm(Ho *et al*., 2022).

4.2.18: Effect of *T. vaginalis* Infection on Sperm Sluggish

The results of the present study (Table 4-19) show that the mean±SD (95% CI) of the sperms sluggish among *T. vaginalis* infected males is (26.04±13.52)x10^6 ((95%C.I=18.23–33.35)x10^6), this mean is higher than that of uninfected (19.38±11.92)x10^6 ((95%C.I=14.93-23.83)x10^6). There are statistically significant differences between *T. vaginalis* infected and uninfected males in the mean of sluggish sperms (F=5.342, $P< 0.05$).

Table 4-19: Effect of *T. vaginalis* Infection on Sperm Sluggish.

T. vaginalis	No. males	Mean x10^6	SD x10^6	95% CI x10^6
Uninfected	30	19.38	11.92	(14.93-23.83)
Infected	14	26.04	13.52	(18.23-33.85)
Total	44	21.50	12.68	-

F=5.342 **$P< 0.05$**

This effect of *T. vaginalis* on sperm sluggish was also shown by Elia *et al*. (2009), who attributed this phenomenon to the semen hyperviscosity due to the cytotoxic effect of *T. vaginalis* and its excreted material that caused a reduction in the motility of the sperm and then trapping the sperm and prevents the sperm from passing and progressive normally the female genital tract or this may be due to declining in fructose level in the semen hyperviscosity which caused a decrease in energy sources for sperm (Andrade-Rocha, 2005).

4.2.19: Effect of Bacterial Infection on Sperm Sluggish

The results of this study (Table 4-20) find the mean of the sluggish sperms in infected males with bacteria (15.15) x10^6 was less than uninfected. There are statistically significant differences between

infected and uninfected males concerning sperm sluggish (F=104.3, *P*<0.05).

These findings agree with Atere *et al*. (2017), who explained that bacterial infection affects some sperm parameters like increasing the sperm sluggish and this effected on male fertility during the production of soluble spermatotoxic material such as sperm immobilization factor (SIF) (Agarwal *et al*., 2012).

Table 4-20: Effect of Bacterial Infection on Sperm Sluggish.

Bacteria	No. males	Mean $x10^6$	SD $x10^6$	95% CI.$x10^6$
Uninfected	80	19.37	12.69	(16.54-22.21)
Infected	17	15.15	13.05	(9.01-21.29)
Total	97	18.63	12.79	-

F=104.31 ***P*<0.05**

4.2.20: The interaction Effect between the Infection of *T. vaginalis* and Bacteria on the Sperm Sluggish

The results of the present study (Table4-21) show that the mean±SD of sperm sluggish in cases infected with the two pathogens, *T. vaginalis* and bacteria is 23.92±17.21$x10^6$, this is less than that infected with *T. vaginalis* alone 27.22±12.01$x10^6$ and higher than uninfected with the tow pathogen (19.38 ±11.91)$x10^6$. There is a statistically significant effect in the interaction of *T. vaginalis* and bacteria on the sperm sluggish (F=44.007, *p*<0.05).

This study agrees with Elia *et al*. (2009), who show that the *T. vaginalis* are produced chemical materials that lead to reduced sperm motility which may be attributed to the trapping effect while the bacteria direct attachment to sperm cells by using some organelles

such as pili which causes agglutination of motile sperm (Zeyad *et al*., 2017).

Table 4-21: The Interaction Effect between the Infection of *T. vaginalis* and Bacteria on the Sperm Sluggish.

Interaction	No. males	Mean x10⁶	SD x10⁶	F-test		95% CI x10⁶
				F value	*P*	
T. vaginalis + Bacteria	5	23.92	17.21	44.007	<0.05	(12.58-35.25)
NO *T. vaginalis* NO Bacteria	30	19.38	11.91			(14.75-24.01)
Infected *T. vaginalis* - NO Bacteria	9	27.22	12.01			(18.77-35.67)
Total	44	21.50	12.68			-

4.2.21: Effect of Bacterial Infection on Sperm Slowing

This study (Table 4-22) shows that the mean±SD of the slow sperm in the semen of males infected with bacteria is 15.77±8.26x10⁶ ((95%C.I=11.52–20.02)x10⁶), whilst uninfected males is 22.04±11.96x10⁶ ((95%C.I=19.37-24.70)x10⁶). There are statistically significant differences between the mean of the slow sperm among infected and uninfected males with bacterial semen infection which impact slow sperm (F=165.00, *P*<0.05).

This study agrees with Sanocka *et al*. (2005), who explained that the increase in the mean of slow sperm as a result of the harmful effects of different microbial pathogens on sperm, not only result from interacting cells by tight adhesion but also from the expression of other virulent factors found on the bacterial surface such as lipopolysaccharides, cytotoxic necrotizing factor, a- hemolysins, b-hemolysins, and as a result from the release of soluble spermatotoxic

factors such as sperm immobilization factor (SIF). So SIF can inhibit the sperm and lead to sperm slowing and viability by decreasing mitochondrial ATPase activity (Kaur and Prabha, 2013).

Table 4-22: Effect of the bacterial infection on sperm slowing.

Bacteria	No. males	Mean $x10^6$	SD $x10^6$	95% CI.$x10^6$
Uninfected	80	22.04	11.96	(19.37-24.70)
Infected	17	15.77	8.26	(11.52-20.02)
Total	97	20.94	11.61	-

F=165.004 ***P*<0.05**

4.2.22: The interaction effect between the infection of *T. vaginalis* and bacteria on the sperm slowing

The results of this study (Table 4-23) show that the mean±SD of the slow sperm in cases infected with the two pathogens, *T. vaginalis* and bacteria is 18.76±4.14$x10^6$, this is less than that infected with *T. vaginalis* alone ((26.66±12.99)$x10^6$) and less than uninfected with these two pathogens (28.02±13.12)$x10^6$. There is a statistically significant effect in the interaction between *T. vaginalis* and bacteria on the slow of sperm (F=67.53, p<0.05).

The bacteria and *T. vaginalis* interact with the host's inflammatory and immunological response differently through their unique structures, specialized enzymes, and toxins, to evade the host's response. Regardless of the specific organ from which the inflammation results, there will be an increase in the level of polymorphonuclear leucocytes and granulocytes which can lead to an elevated in oxidative stress and defect spermatogenesis which affect sperm motility (Ahmad *et al*., 2017).

Table 4-23: The Interaction effect between the Infection of *T. vaginalis* and Bacteria on the Sperm Slowing.

Interaction	No. males	Mean x10⁶	SD x10⁶	F-test		95% CI x10⁶
				F value	*P*	
T. vaginalis + Bacteria	5	18.76	4.14	67.53	<0.05	(7.46-30.06)
NO *T. vaginalis* NO Bacteria	30	28.02	13.12			(23.40-32.63)
Infected *T. vaginalis* - NO Bacteria	9	26.66	12.99			(18.24-35.08)
Total	44	26.69	12.56			-

4.2.23: Effect of *T. vaginalis* on Semen Pus Cells

The result (Table 4-24) shows the mean of pus cells in the semen of infected males with *T. vaginalis* (1.35±2.46) is lower than that of uninfected 2.96±7.61. There are non-statistically significant differences between the *T. vaginalis* infected and uninfected males in the semen pus cells (F=0.589, *P*>0.05).

Table 4-24: Effect of *T. vaginalis* Semen Pus Cells.

T. vaginalis	No. males	Mean	SD	95% CI
Uninfected	30	2.96	7.61	(0.12-5.81)
Infected	14	1.35	2.46	(-0.07-4.41)
Total	44	2.45	6.44	-

F=**0.589** ***P*>0.05**

These results disagree with other studies like Henkel and Solomon (2019). But agree with the finding of Midlej and Benchimol (2010) where who found that *T. vaginalis* can efficiently digest and degrade

the lactobacilli, yeast cells, vaginal cells and cervical epithelial cells, leukocytes, erythrocytes, prostatic cells, spermatozoids (Benchimol *et al*., 2008).

4.2.24: Effect of Bacterial Infection on Semen Pus Cells

Table (4-25) shows the mean±SD of the pus in the semen of males infected with bacterial infection is 4.00±8.54 (95%C.I=-0.39–8.39), and among non-infected males is 3.82±7.05 (95%C.I= 2.25-5.39). There are statistically significant differences between infected and uninfected males in semen pus (F= 0.008, *P*<0.05).

Table 4-25: Effect of bacterial Infection on Semen Pus Cells.

Bacteria	No. males	Mean	SD	95% CI
Uninfected	80	3.82	7.05	(2.25-5.39)
Infected	17	4.00	8.54	(-0.39-8.39)
Total	97	3.85	7.29	-

F= 0.008 ***P*<0.05**

The result of this study is directly in line with the study of Henkel and Solomon (2019), who pointed out that the mean of the semen pus of an infected individual with bacterial infection was found to be more than non-infected. The increase in the semen pus due to the bacterial infection stimulates the leukocytes to secrete cytokines as an immune response which considers a proinflammatory factor that affects sperm vitality by producing large amounts of reactive oxygen species (ROS) via oxidative stress (Fraczek *et al*., 2016).

4.2.25: The Interaction Effect between the Infection of *T. vaginalis* and Bacteria on Semen Pus Cells

This study (Table 4-26) shows that the mean±SD of the semen pus in cases infected with the two pathogens, *T. vaginalis* and bacteria is 0.00±0.00, less than that infected with *T. vaginalis* alone 2.11±2.84 and uninfected. There is a non-statistically significant effect in the interaction of *T. vaginalis* and bacteria on the semen pus (F=2.37, *P*>0.05)**.**

Table 4-26:The Interaction Effect between the Infection of *T. vaginalis* and Bacteria on Semen Pus Cells.

Interaction	No. males	Mean	SD	F-test		95% CI
				F value	*P*	
T. vaginalis + Bacteria	5	0.00	0.00	2.37	>0.05	(-5.89-5.89)
NO *T. vaginalis* NO Bacteria	30	2.97	7.61			(0.55-5.37)
Infected *T. vaginalis* - NO Bacteria	9	2.11	2.84			(-2.28- 6.50)
Total	44	2.45	6.44			-

This study with line Mielczarek and Blaszkowska (2016), found that *T. vaginalis* was phagocytosis for pus cells and RBC and other materials which contributes to some of the essential functions of this parasite, such as feeding, and its importance in vivo and in vitro genesis (Juliano *et al*., 1991).

4.2.26: Effect of *T. vaginalis* on the presence of RBC in semen

This results study showed that the mean of the RBC in the semen of infected males of 1.07±1.32 (95%C.I=0.52–1.62) is higher than that

of non-infected (Table4-27), There are statistically significant differences between the *T. vaginalis* infected and uninfected males in the RBC of the semen (F=4.536, *P*<0.05).

Table 4-27: Effect of *T. vaginalis* on the presence of RBC in semen.

T. vaginalis	No. males	Mean	SD	95% CI.
Uninfected	30	0.37	0.85	(-0.01-0.74)
Infected	14	1.07	1.32	(0.52-1.62)
Total	44	0.59	1.06	-

F=4.536 ***P*<0.05**

These findings agree with the findings of Khodamoradi *et al*. (2020), who noted that hematospermia most often accompanies cases of infertility which occur as a result of the generation of reactive oxygen or free radicals.

4.2.27: Effect of Bacterial Infection on semen RBC

Current results found statistically significant differences between infected and uninfected males in semen RBC (F= 20.122, *P*<0.05), and that the mean of semen RBC in infected individuals was 1.00 as shown in the table below (Table 4-28).

This finding agrees with Leocádio and Stein (2009), who stated that the mean of the semen RBC of an infected individual with bacterial infection was found about 1.00, and the semen RBCs can adversely affect semen quality and fertility by reactive oxygen species production is the most prominent issue as clarified Khodamoradi *et al*. (2020).

Table 4-28: Effect of Bacterial Infection on Semen RBC.

Bacteria	No. males	Mean	SD	95% CI
Uninfected	80	1.49	2.25	(1.00-1.97)
Infected	17	1.00	1.90	(-0.05-2.05)
Total	97	1.40	2.19	-

F= 20.122 ***P*<0.05**

4.2.28: The Interaction effect between the infection of *T. vaginalis* and bacteria on the count of RBC in the semen

Table (4-29) shows that the mean±SD of the count of RBC in the semen of males infected with the two pathogens, *T. vaginalis* and bacteria is 0.40±0.89, this is less than that infected with *T. vaginalis* alone 1.44±1.42 and higher than uninfected. There is a statistically significant effect in the interaction of *T. vaginalis* and bacteria on the count of RBC in the semen (F=7.98, *P*<0.05).

These findings are similar to that of Rijsselaere *et al*. (2004), who found that the defect generated by trichomoniasis and bacterial infection leads to hematospermia, and the toxic material of RBCs may affect the ability of sperm to fertilize. Sperm's vulnerability to oxidative stress is increased due to the high levels of polyunsaturated fatty acids in their plasma membrane due to hematospermia (Khodamoradi *et al*., 2020).

Table 4-29: The Effect of Interaction between Bacteria and *T. vaginalis* on the count of RBC in the semen.

Interaction	No. males	Mean	SD	F-test		95% CI
				F value	*P*	
T. vaginalis + Bacteria	5	0.40	0.89	7.98	<0.05	(-0.49-1.29)
NO *T. vaginalis* NO Bacteria	30	0.37	0.85			(0.00-0.73)
Infected *T. vaginalis* - NO Bacteria	9	1.44	1.42			(0.77-2.11)
Total	44	0.59	1.06			-

4.2.29: Effect of *T. vaginalis* Infection on Semen volume

The results of this study (Table 4-30) show that the mean±SD of the semen volume in trichomoniasis-infected males is 2.89±0.44 (95%C.I=2.42–3.36) is less when compared with uninfected males 3.43±1.00 (95%C.I=3.11-3.75). But there are non-statistically significant differences between infected and uninfected males in the semen volume (F=3.665, *P*>0.05).

The decline of the semen volume may due to infection of the prostate with this parasite (McConaughey, 2014), and the finding was agreed with Gopalkrishnan *et al*. (1990), who found no significant changes in the semen volume as a result of *T. vaginalis* infection.

Table 4-30: Effect of *T. vaginalis* Infection on Semen volume.

T. vaginalis	No. males	Mean	SD	95% CI
Uninfected	30	3.43	1.00	(3.11-3.75)
Infected	14	2.89	0.44	(2.42-3.36)

Total	44	3.26	0.899	-

F=3.665 ***P*>0.05**

4.2.30: Effect of Bacterial Infection on Semen volume

In a current study (Table 4-31), the mean±SD of semen volume in males with bacterial semen infection was 3.18±2.03 (95%C.I=2.50–3.84), less than in uninfected males 3.44±1.22 (95%C.I=3.13-3.75). There are statistically significant differences between infected and non-infected males with bacterial infection and its impact on semen volume (F=287.49, *P*<0.05).

Table 4-31: Effect of bacterial Infection on semen volume.

Bacteria	No. males	Mean	SD	95% CI
Uninfected	80	3.44	1.22	(3.13-3.75)
Infected	17	3.18	2.03	(2.50-3.84)
Total	97	3.40	1.39	-

F=287.49 ***P*<0.05**

Overall these findings are in accordance with that reported by Rivera *et al*. (2022), who clarified that the mean of the semen volume of an infected individual with bacteria was found to be less than non-infected. This decrease in the semen volume may be due to the effect of bacterial inflammation on the secretory functions of prostate glands which leads to reduce the volume of semen (Marconi *et al*., 2009).

4.2.31: Effect of *T. vaginalis* Infection on the Pus Cells in the Urine

Table (4-32) shows that the mean±SD of the pus cells in the trichomoniasis-infected males is 2.0±1.35 (95%C.I=1.21–2.78), less

than among uninfected males is 2.33±3.60 (95%C.I=0.98-3.48), There are statistically significant differences between infected and uninfected males in the urine pus (F=11.49, *P*<0,05).

Table 4-32: Effect of *T. vaginalis* on the pus cells in the urine.

T. vaginalis	No. males	Mean	SD	95% CI
Uninfected	30	2.33	3.60	(0.98-3.48)
Infected	14	2.0	1.35	(1.21-2.78)
Total	44	2.22	3.05	-

F=11.49 ***P*<0,05**

These results agree with the findings of Mielczarek and Blaszkowska (2016) who found that *T. vaginalis* phagocytosis different materials such as leukocytes, erythrocytes, and sperms to obtain nutrients. But disagree with Henkel and Solomon (2019) who found that *T. vaginalis* causes leukospermia.

4.2.32: Effect of Bacterial Infection on Pus Cells in the Urine

Table (4-33) shows that the mean±SD of the pus cells in urine in bacterial infection of males is 4.0±8.54 (95%C.I=-0.39–8.39), more than with uninfected males which is 3.82±7.05 (95%C.I=2.25-5.39), There are statistically significant differences between infected and uninfected males in the urine pus (F=37.90, *P*<0.05).

The results of this study agree with Menkveld *et al*. (2003) their findings show that male with inflammatory chronic prostatitis and chronic pelvic pain syndrome have sperm morphological changes which are associated with elongation and reduced acrosomal inducibility. These changes were attributed to leukocytes at the second

stage of an infection of the urogenital tract, leukocytes appear in addition to bacteriospermia (Fraczek and Kurpisz, 2015).

Table 4-33: Effect of Bacterial Infection on Urine pus.

Bacteria	No. males	Mean	SD	95% CI
Uninfected	80	3.82	7.05	(2.25-5.39)
Infected	17	4.0	8.54	(-0.39-3.78)
Total	97	3.85	7.29	-

F=37.90 ***P*<0.05**

4.2.33: The Interaction Effect between the Infection of *T. vaginalis* and Bacteria on the Pus Cells in the Urine

This study (Table 4-34) shows that the mean±SD of the pus cells in the urine of males infected with the two pathogens, *T. vaginalis* and bacteria is 2.00±1.17, this is less than that infected with *T. vaginalis* alone 2.00±1.52. There is a non-statistically significant effect in the interaction of *T. vaginalis* and bacteria on the pus cells in the urine (F=0.054, *P*<0.05).

These results agree with the finding of Pereira-Neves and Benchimol (2008) and Midlej and Benchimol (2010), who found that the *T. virginals* have mechanical stress and phagocytosis of the necrotic cells, leukocytes, erythrocytes for nutrition as the cytopathic effect. But disagree with Tambyah and Maki (2000), who found that the interaction effect between the infection of *T. vaginalis* and bacteria increases urinary tract infections which increase pyuria which is common and treatable.

Table 4-34: The Interaction Effect between the Infection of *T. vaginalis* and Bacteria on the Pus Cells in the Urine.

Interaction	No. males	Mean	SD	F-test		95% CI
				F value	*P*	
T. vaginalis + Bacteria	5	2.00	1.17	0.054	<0.05	(0.54-3.45)
NO *T. vaginalis* NO Bacteria	30	2.33	3.60			(0.98-3.67)
Infected *T. vaginalis* - NO Bacteria	9	2.00	1.52			(0.83- 3.16)
Total	44	2.22	3.05			-

4.2.34: Effect of *T. vaginalis* infection on the count of RBC in the Urine

This study (Table 4-35) shows that the mean±SD of the RBC in the urine among *T. vaginalis* infected males of 0.88±0.77 (95%C.I=0.46–1.31), is higher than that of uninfected males 0.41±0.74 (95%C.I=0.13-0.69), There are statistically significant differences between infected and uninfected males in the count of RBC in the urine (F=13.697, *P*<0.05).

Table 4-35: Effect of *T. vaginalis* infection on the count of RBC in the Urine.

T. vaginalis	No. males	Mean	SD	95% CI
Uninfected	30	0.41	0.74	(0.13-0.69)
Infected	14	0.88	0.77	(0.46-1.31)
Total	44	0.55	0.77	-

F=13.697 *P*<0.05

This result of the current study agrees with the result of the study of Chang *et al*. (2020), the increase in the count of RBC in the urine of *T. vaginalis* infected males may be due to the secretions activity of this parasite-like cysteine protease or CDF that leads to exfoliation of epithelial cell and the adhesive of this parasite to their damage of urogenital tract lead to pustular raspberry-shaped bleeding which gives greater opportunity to increase the exudative of RBC with urine (Núñez-Troconis, 2020).

4.2.35: Effect of Bacterial Infection on Urine RBC

This study as appeared in Table (4-36) pointed out that the mean±SD of the urine RBC for bacterial infection in males is 1.38±1.69 (95%C.I=0.85–1.91), while in uninfected males is 0.75±0.93 (95%C.I=0.51-1.00), There are statistically significant differences between infected and uninfected males in the urine RBC (F=37.90,*P*<0.05).

Table 4-36: Effect of Bacterial Infection on Urine RBC.

Bacteria	No. males	Mean	SD	95% CI
Uninfected	80	0.75	0.93	(0.51-1.00)
Infected	17	1.38	1.69	(0.85-1.91)
Total	97	0.86	1.11	-

F=32.38 ***p*<0.05**

This study found the mean of the urine RBC of an infected individual with bacterial infection was found to be more than with non-infected. This increase in urine RBC is due to the bacteria having special properties that enable them to adhere to the outer layer of the urinary membrane, penetrate cells, multiply, and eventually cause cell

lysis so which causes hematuria, this study agrees with Avellino *et al*. (2016).

4.2.36: The Interaction effect between the Infection of *T. vaginalis* and bacteria on the count of RBC in urine

The current study (Table 4-37) shows that the mean±SD of the urine RBC in males infected with the two pathogens, *T. vaginalis* and bacteria is 1.00±1.00, this is higher than that infected with *T. vaginalis* alone 0.77±0.66 and uninfected 0.41±0.74. There is a statistically significant effect in the interaction of *T. vaginalis* and bacteria on the urine RBC (F= 9.06, $P<0.05$).

Table 4-37: The Interaction effect between the Infection of *T. vaginalis* and bacteria on the count of RBC in urine.

Interaction	No. males	Mean	SD	F-test		95% CI
				F value	*P*	
T. vaginalis + Bacteria	5	1.00	1.00	9.06	<0.05	(0.31-1.68)
NO *T. vaginalis* NO Bacteria	30	0.41	0.74			(0.13-0.69)
Infected *T. vaginalis* - NO Bacteria	9	0.77	0.66			(0.26- 1.28)
Total	44	0.55	0.77			-

These results agree with the finding of Mathers *et al*. (2017), who found the presence of RBCs in the urogenital secretions of males can affect the fertilization potential of spermatozoa, and the secretion of cysteine proteinases and cell-detaching factors leading to the exfoliation of epithelial cells and damage to the structural integrity and defense barrier of the urogenital tract which increases the

susceptibility of the host to more urogenital infections and eventually causes cell lysis so which causes hematuria (Avellino *et al*., 2016).

4.2.37: Effect of Bacterial infection on urine pH

Table (4-38) shows that the mean±SD of the urine pH for bacterial infection in males is 4.58±0.90 (95%C.I=4.20–4.97), versus uninfected males is 4.55±0.77 (95%C.I=4.38-4.73), There are statistically significant differences between infected and uninfected males in the urine pH (F=1572.25, $P<0.05$).

Table 4-38: Effect of bacterial infection on urine pH.

Bacteria	No. males	Mean	SD	95% CI
Uninfected	80	4.55	0.77	(4.38-4.73)
Infected	17	4.58	0.90	(4.20-4.97)
Total	97	4.56	0.79	-

F=1572.25 **$P<0.05$**

This study agrees with Yang *et al*. (2014), who concluded that the mean of the urine pH of an infected individual with bacterial infection was found to be more than that of non-infected, and the pH plays an important role in the overall inhibitory activity. Although many agents behave similarly on most or all of the urinary tract pathogens tested, many of them only showed pH-dependent effects against specific microorganisms, and the acidification of urine is widely recommended for the treatment and prevention of urinary tract infections (Erdogan-Yildirim *et al*., 2011).

4.3: The Relationship between the infection of *T. vaginalis* and concomitant bacteria with sociodemographic factors

4.3.1: The Relationship between the infection of *T. vaginalis* and concomitant bacteria with education level

The results of the current study show that is a non-statistical significance of the relationship between the infection of *T. vaginalis* and concomitant bacteria with education level (χ^2=4.68, *P*> 0.05).

These results with line with the findings of Bai *et al*. (2022), who show no relationship between the infection of males with *T. vaginalis* and education level. But disagree with Daugherty *et al*. (2019), who found that males with lower levels of education have a higher rate of trichomoniasis. And agree with Bai *et al*. (2022), who show there are non-relationship between bacterial infection in semen and education level. But disagree Fasciana *et al*. (2021), who find that males with higher levels of education have a higher rate of bacterial infection in semen.

4.3.2: The Relationship between the infection of *T. vaginalis* and concomitant bacteria with age group

The current study showed (Table 4-39) that the highest incidence of *T. vaginalis* infection was in male in the age group (34-37) and the lowest infection in the age group (30-33) and (42-45) respectively.

This result is consistent with Dalimi and Payameni (2021), who show that the infection of trichomoniasis in the age group between (31- 40) is more than in other age groups due to who has the most sexual activity. But do not agree with Tompkins *et al*. (2020).

Table 4-39: The Relationship between the infection of *T. vaginalis* and concomitant bacteria with age group.

		Age group						
		22-25	26-29	30-33	34-37	38-41	42-45	Total count
		No.	No.	No.	No.	No.	No.	No.
Infective *T. vaginalis*	Un-infected	1	3	8	9	6	3	30
	Infected	2	3	1	4	3	1	14
	Total	3	6	9	13	9	4	44

4.3.3: The Relationship between the infection of *T. vaginalis* and concomitant bacteria with a residence

This study shows that is non-statistically significant the relationship between trichomoniasis-infected males with their residence (χ^2=3.07, P> 0.05).

This result is compatible with Al-Mayah *et al*. (2013), who confirmed that there are no statistically significant differences in the relationship between infection and residence (rural or urban). But disagree with Webb *et al*. (2021), who confirmed that there are statistically significant differences in the relationship between infection and residence.

4.4: The comparative between some diagnosis techniques for detecting *T. vaginalis* in males urine and semen.

Four diagnosis techniques were used to detect the *T. vaginalis* in males urine and semen including wet mount (WM), Rapid antigen detection test (RADT), Conventional PCR (PCR) and

Real time-quantitative-PCR (RT-qPCR). These methods differed statistically significant in their ability to detect this parasite (χ^2=50.355, p<0.001).

The results of this study (Table 4-40, Figures 4-1 and 4-2) show the RT-qPCR had the highest accuracy for detecting *T. vaginalis* in semen (100%) and urine (97.7%) and the highest sensitivity (100%) and specificity (100%) in semen and in urine (92.8 and 100% respectively). The WM had the lowest sensitivity (0.0% and the lowest accuracy (68.2%) (Figures 4-3 and 4-4).

The PCR techniques had a higher sensitivity, specificity, PPV, NPV and the accuracy for detecting the *T. vaginalis* in semen an urine (see Table 4-40, Figures 4-5). Similar study was conducted by Asmah *et al.* (2018) found that the PCR techniques were the golden test in diagnosis *T. vaginalis* and the WM and RADT had lowest sensitivity and specificity, and with Tchankoni *et al*. (2021), who reported that the WM had low sensitivity for detecting *T. vaginalis*.

Table 4-40: A comparative between different diagnostic methods for detecting *T. vaginalis* among males in Maysan province.

Method	Sample	N0. Exam	TP	FP	∑P	TN	FN	∑N	(FP+ FN)/ 2	SE%	SP%	PPV %	NPV %	Acc %
WM	Urine	44	0	0	0	83	14	97	7	0.0	100	-----	86.2	68.2
	Semen	44	0	0	0	83	14	97	7	0.0	100	-----	68.2	68.2
RADT	Urine	44	5	1	6	29	9	38	5	35.7	96.7	83.3	76.3	77.3
	Semen	44	0	0	0	30	14	44	7	0.0	100	-----	68.2	68.2
PCR	Urine	44	8	0	8	30	6	36	3	57.1	100	100	83.3	86.4
	Semen	44	12	0	12	30	2	32	1	85.7	100	100	93.8	95.5
RT-qPCR	Urine	44	13	0	13	30	1	31	0.5	92.8	100	100	96.8	97.7
	Semen	44	14	0	14	30	0	30	0	100	100	100	100	100

TP: True positive, FP: False positive, ∑p: Summation positive, TN: True negative, FN: False negative, ∑N: Summation negative, SE: Sensitivity. Sp: specificity, PPV: Positive predictive value, NPV: Negative predictive value and Acc: Accuracy

This finding may be due to many reasons such as light infection, with low intensity of *T. vaginalis*, taking treatment, and experience of the microscopic examiner. Microscopically, this test depends on the finding of motile trophozoites in a wet mount (Nye *et al*., 2009). For this, some believe that this method is not useful in detecting *T. vaginalis* among males (Rogers *et al*., 2014).

With regard to the RADT method, this study showed it has a low sensitivity of 0.0, and 35.7% among semen and urine samples respectively, and it has a high specificity 96.7 and 100% respectively. These results agree with Sheele *et al*. (2019), who found that this test had 37.5% sensitivity and 82.9% specificity. But disagree with Asmah *et al*. (2018) who found that RADT had 25% sensitivity.

PCR had a high sensitivity (85.7%) and specificity (100%) for detecting *T. vaginalis* in semen samples and had moderate sensitivity (57.1%) and high specificity (100%) in urine samples. These results among semen are in line with what Van Gerwen *et al*. (2021) found to be of high sensitivity (97.2%) and the specificity (99.9%) and in urine, it agrees with Asmah *et al*. (2018) who found the sensitivity 40 % and specificity 100 % respectively.

RT-qPCR had the highest sensitivity (100%) and specificity (100%) in detect *T. vaginalis* in semen samples and high sensitivity of 92.4% and high specificity of 100 for detecting this parasite in urine. This findings agree with other studies which found that the RT-PCR technique had a high sensitivity (99.3%) and specificity (100%) (Elsherif and Youssef, 2013).

However, this result shows that the all RT-qPCR amplified products of cDNA of the *T. vaginalis β-tubulin* gene in urine and semen samples were detected at the cycle threshold value (Ct value) ranged from the 19^{th} to 21^{st} cycles. (Figure 4-1). This indicates that the sample had a high abundance of nucleic acid and indicates that the primers were specific for this technique and the sensitivity of the *β-tubulin* gene with RT-qPCR was high when using the optimum temperature (Figure 4-2) (Simpson *et al*., 2007; Elsherif and Youssef, 2013).

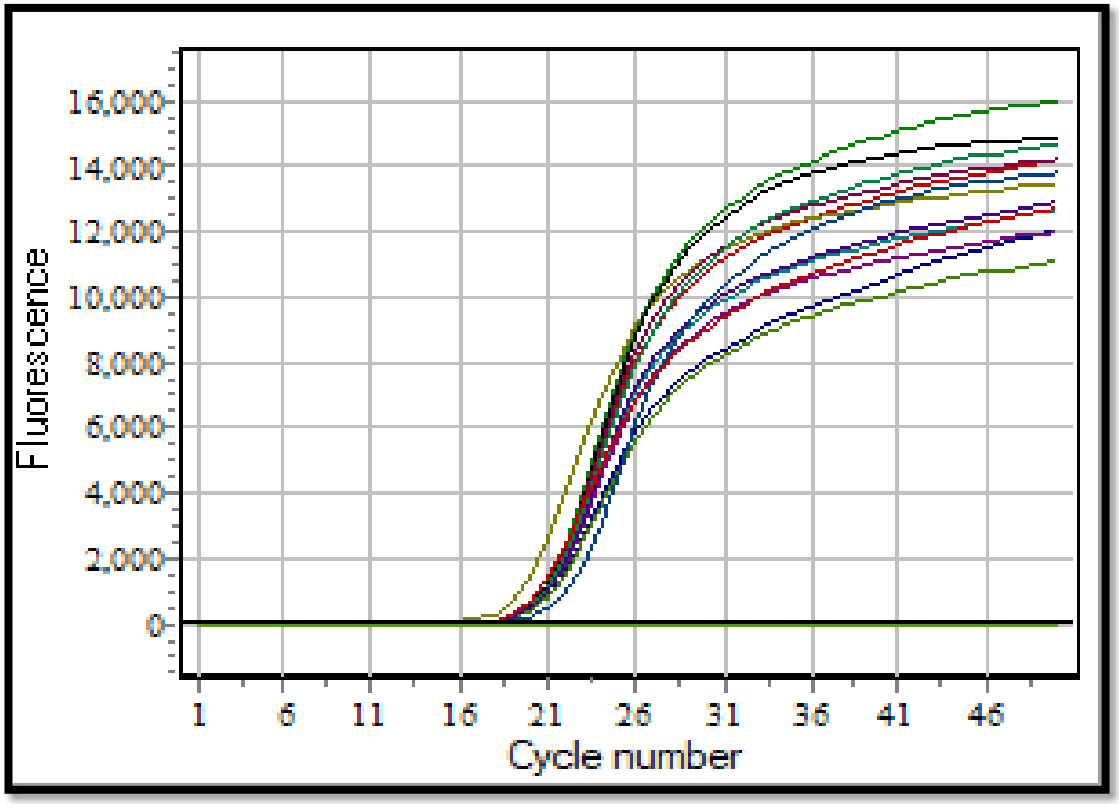

Figure 4-1: RT-qPCR cycle number.

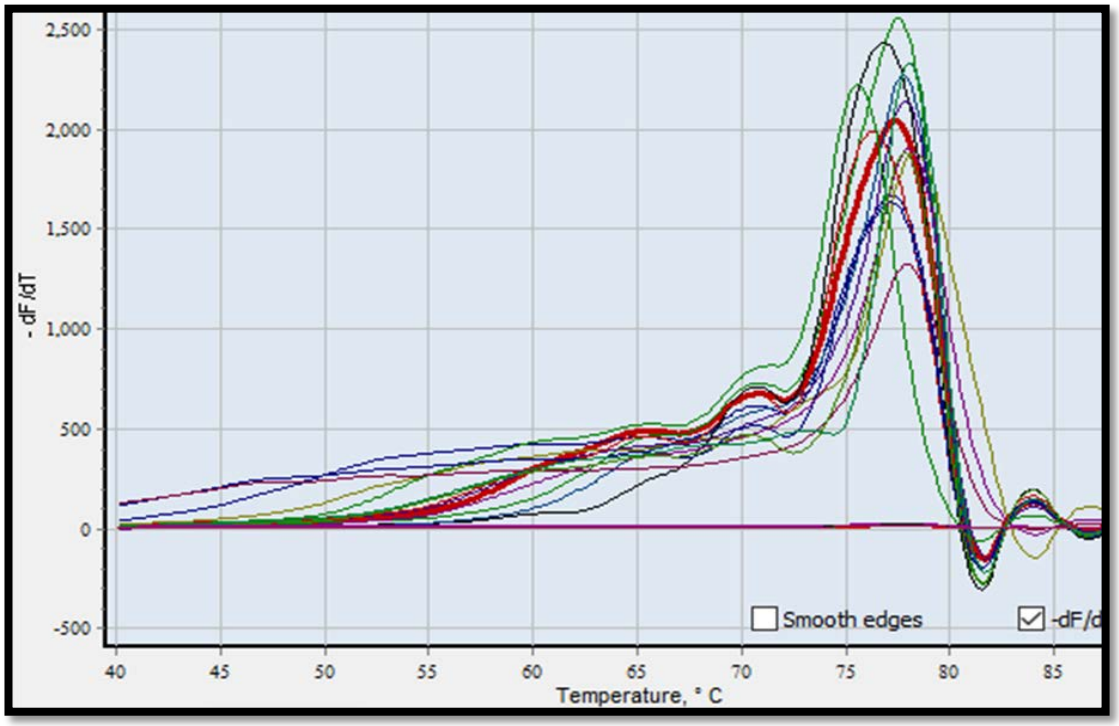

Figure 4-2: The melting curve analysis assessment of the dissociation characteristic of double stranded DNA during heating.

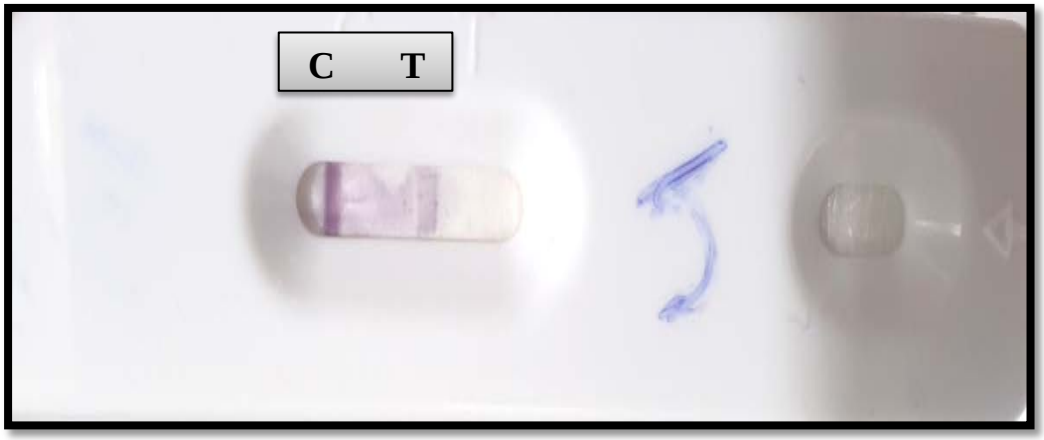

Figure 4-3: *T. vaginalis* in a Urine sample (Positive).

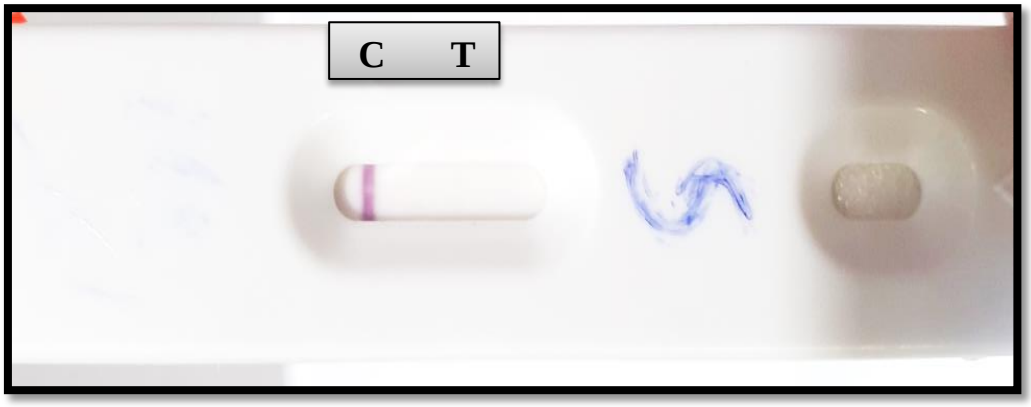

Figure 4-4: *T. vaginalis* in Semen sample (Negative).

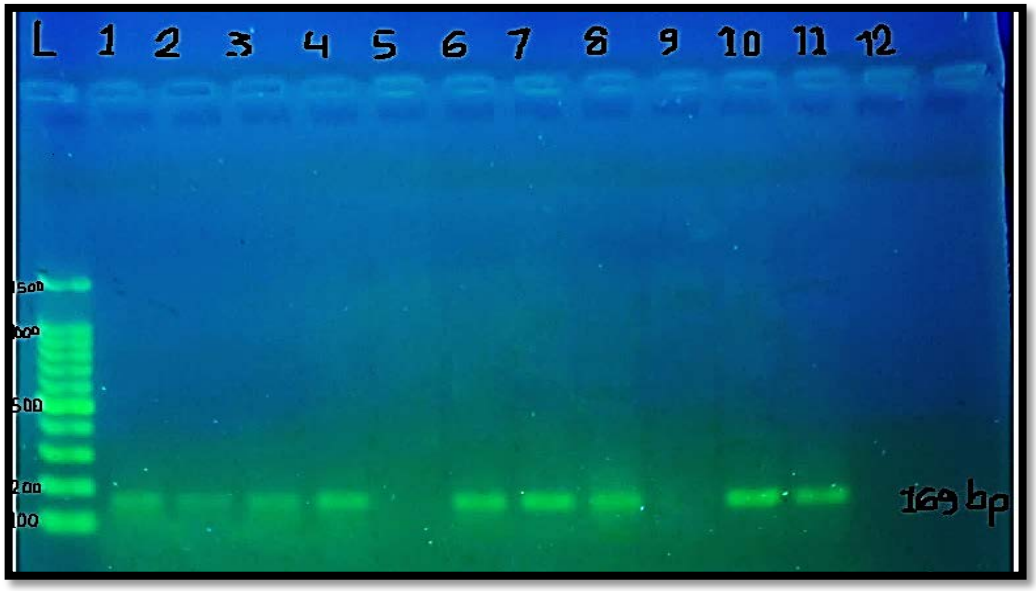

Figure 4-5: 1.5% agarose gel electrophoresis of the *T. vaginalis β-tubulin* PCR DNA product. L:100 bp DNA Ladder, 1, 2, 3, 4, 6, 7, 8, 10 *T. vaginalis* positive cases, 5,9: *T. vaginalis* negative case, 11: positive control, 12, negative control.

4.5: Diagnosis of bacteria

4.5.1: Results of Preliminary tests

The results of the preliminary tests were shown in (Table 4-44) where urine and semen samples were cultured on different culture media to ensure bacterial growth after incubation of the samples in the incubator for 24 hrs at a temperature of 37º, the result recovered different genera of gram-negative and gram-positive.

This study was in line with Nasrallah *et al*. (2018) and Ricci *et al*. (2018), who found that both Gram-positive and Gram-negative bacteria were detected in semen-infertile male which can alter sperm function such as *Enterococcus faecalis* which had have been associated with teratozoospermia and oligozoospermia (Moretti *et al*., 2009). While it has been proven that *Escherichia coli* leads to apoptosis within sperm cells and reduces their motility (Sanocka *et al*.,

2005), were *Ureaplasma urealyticum* and *Mycoplasma hominis* it was found that both types affect the motility and vitality of sperm (Lee *et al*., 2013), also *Pseudomonas* spp contributes to the deterioration of semen quality (Isaiah *et al*., 2011)**.**

In a study conducted by Stamatiou and Pierris, (2017) on semen and urine from male with chronic bacterial prostatitis where they found that the highest infection rate is for the bacteria *Escherichia coli* and *Staphylococcus* coagulase-negative and the lower infection rate for the bacteria *Klebsiella pneumonia*, but Iovene *et al*, (2018) who found that the *Enterococcus faecalis* high percentage in semen and urine and *Proteus mirabilis* is low percentage. The medium also used the mannitol salt agar and coagulase test for confirmatory *Staphylococcus aureus* Figure (4-7) Figure (4-8)**,** when the medium is fermented and turned yellow, the suspected bacteria were the *Staphylococcus aureus*, which was then confirmed by the coagulase test which is positive for this test. But if the medium is not fermented to a yellow color the suspected bacteria is the *Staphylococcus epidermidis* which is negative for the coagulase test (Bergey, 1994).

Table 4-41: Results of Preliminary tests for Urine and Semen Samples.

No. isolation	Type Samples	Gram stain	Blood agar	MacConkey agar	Chocolate agar	Thayer martin agar	Mannitol salt agar	Coagulase test
1	Urine	-ve	Pale yellow	Pink	Yellow	-	-	-
2	Urine	**-ve**	Pale yellow	Pink	Yellow	-	-	-
3	Urine	**-ve**	Creamy	Pink	White to creamy	-	-	-
4	Urine	**+ve**	Creamy	-	Yellow	-	Ferment Yellow	+ve
5	Urine	**+ve**	Creamy	-	Yellow	-	Yellow Ferment	+ve
6	Urine	**+ve**	Creamy	-	Yellow	-	Yellow Ferment	+ve
7	Urine	**+ve**	Creamy	-	Yellow	-	Yellow Ferment	+ve
8	Urine	**-ve**	Pink	Pink	Pink	-	-	-
9	Semen	**-ve**	White	Pink	White	-	-	-
10	Semen	**-ve**	Buff in color	Pink	Creamy	-	-	-
11	Semen	**-ve**	White	Creamy	Creamy	-	-	-
12	Semen	**-ve**	Creamy	White	Creamy	-	-	-
13	Semen	**-ve**	Creamy	Pink	Creamy	-	-	-
14	Semen	**-ve**	Yellow	Pink	Greenish	-	-	-
15	Semen	+**ve**	Creamy	-	Yellow	-	Yellow Ferment	+ve
16	Semen	+**ve**	Creamy	-	Yellow	-	Yellow Ferment	+ve
17	Semen	+**ve**	Creamy	-	Yellow	-	Yellow Ferment	+ve
18	Semen	+**ve**	Creamy	-	Yellow	-	Yellow Ferment	+ve
19	Semen	+**ve**	white or grey	-	White to greenish	-	-	-
20	Semen	**-ve**	Greyish white	Colorless	Brown greenish	-	-	-
21	Semen	+**ve**	White	-	White	-	Non-ferment	-ve

Figure 4-6: Bacteria grow on Mannitol Salt Agar where the Pink color Indicates the Mannitol non-fermented Bacteria and the yellow color Indicates the Mannitol Fermented Bacteria.

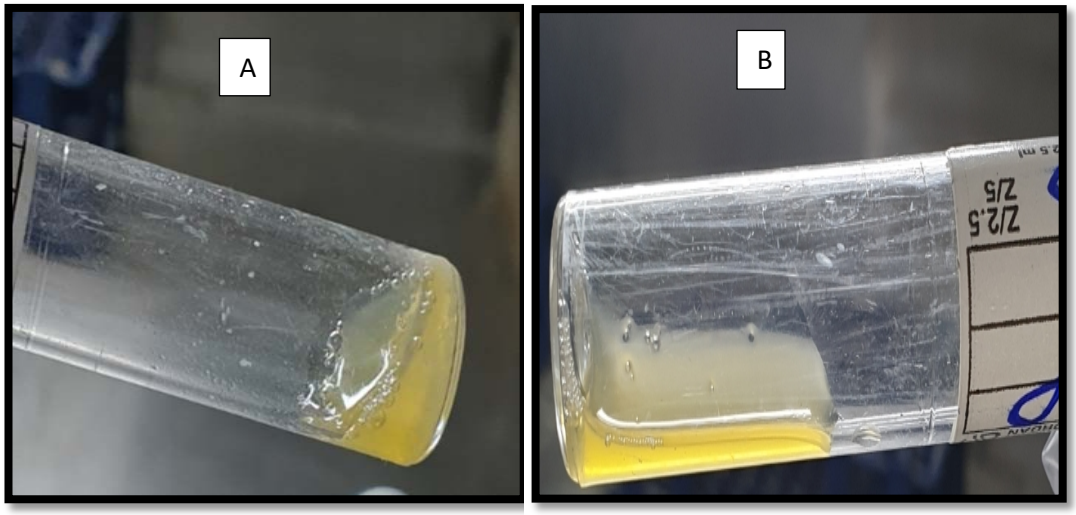

Figure 4-7: A: Clotting (Coagulase test positive). B: No clotting (Coagulase test negative).

4.5.2: Results of Vitek 2 System

A total of 97 urine and 97 semen samples from males were cultured depending on standard laboratory technique and confirmed by the automated Vitek 2 system. Twenty-one was the number of positive growth including four genera of bacteria that were detected in the urine samples while eight genera were detected in the semen samples Table 4-45. The highest infection rate in urine and semen was for the *Staphylococcus aureus* and *Pantoea spp* about 4.12 % and 2.06 % respectively.

Where 4 genera of bacteria were detected in urine distributed following species, *Staphylococcus aureus* (4), *Pantoea spp* (2), *Sphingomonas paucimobilis* (1), *Methylobacterium spp* (1). Besides 13 positive growth were detected concerning the semen samples, where 8 genera were detected, and the frequency for their species is as follow, *Staphylococcus aureus* (4). *Enterobacter aerogenes* (1), *Serratia marcescens* (1), *Pseudomonas putida* (1), *Sphingomonas paucimobili*s (1), *Aeromonas Sobria* (1), *Burkholderia cepacia* (1), *Staphylococcus aureus* (4), *Aerococcus viridans* (1), *Pseudomonas aeruginosa* (1), *Staphylococcus epidermidis* (1).

These results were as same to the findings of Weng *et al*. (2014) and Nasrallah *et al*. (2018) and, who showed that *Staphylococcus aureus* was the most commonly isolated bacteria in semen, and the relationship of semen parameters to bacterial growth shows a slight increase in slow sperm motility progressed in the group with bacterial growth compared to the non-bacterial group, and found that the increases in spermatogenic cells in patients with pyospermia in semen when infected with this bacteria (Kjærgaard *et al*., 1997). The presence of *Anaerococcus* in semen was negatively associated with its quality and semen parameters (Hou *et al*., 2013). Wheres *Pseudomonas and Prevotella* which are predominant in semen, were associated with abnormal semen parameters in male (Weng *et al*., 2014). In a study conducted by *Stamatiou et al*, (2019), they found the

most common bacteria in semen and urine diagnosed by the Vitek 2 system were *E. coli*, coagulase-negative *staphylococci*, *Enterococcus faecalis*, while Iovene *et al*, (2018) who found that the *Enterococcus faecalis* high percentage in semen and urine and *Proteus mirabilis* is a low percentage, but Al-Khafaji and Ali (2021) shows that the highest bacterial infection rate in semen was *Staphylococcus haemolyticus* and lower infection rate for *P. aeruginosa*.

Table 4-42: The Species of Detected Bacteria in Urine and Semen of infected Males and their Frequency.

Method	Species of Bacteria	No. males	Urine Frequent		Semen Frequent		Total Frequent	
			No	%	No	%%	No	%
Vitek2 system	***Staphylococcus aureus****	4	4	40	4	26.67	8	32
	Aerococcus viridans	1	0	0	1	6.67	1	4
	***Methylobacteriun* spp.**	1	1	10	0	0	1	4
	Sphingomonas paucimobilis	1	1	10	1	6.67	2	8
	***Pantoea* spp.▲♥**	2	2	20	0	0	2	8
	***Burkolederia cepacia*♥**		0	0	1	6.67	1	4
	***Aeromonas sobria*▲**		0	0	1	6.67	1	4
	Serratia marcescens	1	0	0	1	6.67	1	4
	Pseudomonas putida	1	0	0	1	6.67	1	4
	Enterobacter aerogenes	1	0	0	1	6.67	1	4
	Pseudomonas aeruginosa	1	0	0	1	6.67	1	4
	Staphylococcus epidermidis	1	0	0	1	6.67	1	4
	Total= 12 species	14	8	80	13	86.67	21	84
PCR	***lactobacillus jensenii***	1	0	0	1	6.67	1	4
	***Holomonas* spp.**	1	1	10	1	6.67	2	8
	Unculturable bacteria	1	1	1	0	0	1	4
	Total= 3 species	3	2	20	2	13.33	4	16
Total	**15 species**	17	10	100	15	100	25	1

4.5.3: Results of Polymerase Chain Reaction (PCR)

This study included the diagnosis of three Genera of bacteria in semen and urine in male using the Polymerase Chain Reaction (PCR) by using the *16S rRNA* gene and pairwise sequence alignment of the sequence data was obtained by using NCBI-BLAST (Nucleotide

Blast), the result represented in (Table 4-46). PCR is a useful tool for identifying bacteria with high levels of accuracy, sensitivity, and specificity (Srinivasan *et al*., 2015), a two percentage of agarose gel electrophoresis of PCR products of *16S rRNA* gene with a size of 1250 bp (Figure 4-9) (Figure 4-10).

Table 4-43: Bacterial Identification based on *16S rRNA* sequencing data.

Bacterial strain	Maximum score	Total score	Query coverage	E. value	Identity percentage	Accession No.
Holomonas spp	335	335	52%	5e-87	93.04%	MZ097524.1
Lactobacillus jensenii	204	204	22%	4e-47	79.44%	AY262350.1
Unculturable bacteria	97.1	97.1	11%	6e-15	83.64%	HM707169. 1
Holomonas spp	200	200	19%	3e-46	94.66%	EU373440.1

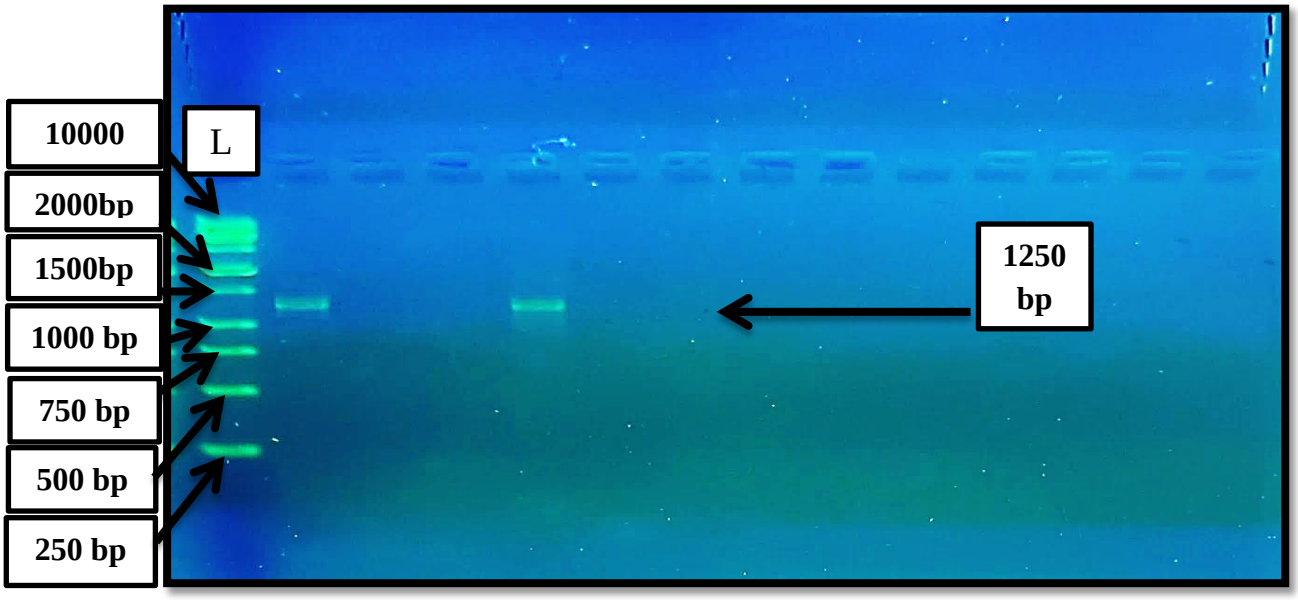

Figure 4-8: Sample Urine. PCR product size 1250 bp Universal primer *16S rRNA*. The product was electrophoresed in 2% agarose at 5 volts/cm^2. 1x TBE buffer for 1 hour. L: DNA ladder (1000 bp).

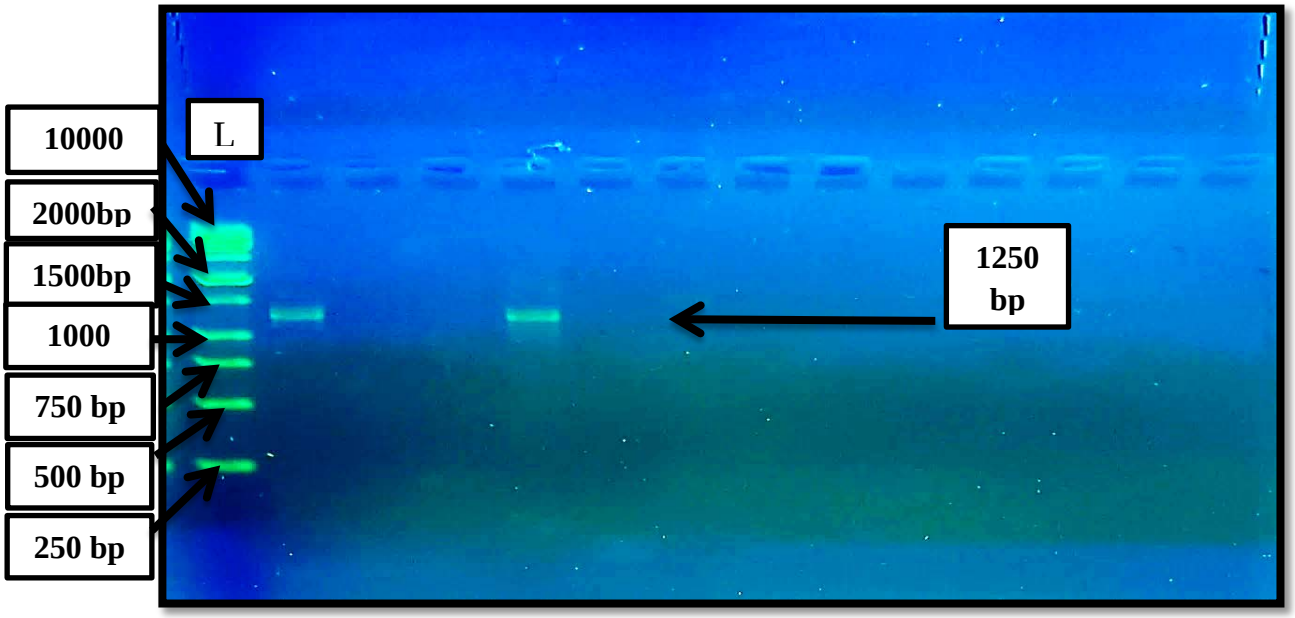

Figure 4-9: Sample Semen. PCR product size 1250 bp Universal primer *16S rRNA*. The product was electrophoresed in 2% agarose at 5 volts/cm^2. 1x TBE buffer for 1 hour. L: DNA ladder (1000 bp).

In a study conducted by Lacroix *et al*. (1996) on semen and urine by using the *16S rRNA* gene on bacteria associated with genito-urinary tract infections, where they found a high infection rate for *E coli* and lower for *C. trachomatis*, while Monteiro *et al*. (2018), they found a high prevalence of *Staphylococcus*, *Anaerococcus*, *Corynebacterium*, *Peptoniphilus*, and *Propionibacterium* in semen but the *Lactobacillus* bacteria were predominant in the semen. on the other hand, Pellati *et al*. (2008) where found about 15% of bacterial infections of the male genitourinary system like *Escherichia coli* most frequently in semen, and aerobic cocci which present in about 50% of semen of male partners in infertile couples such as *Enterococcus faecalis.* Brüggemann and Al-Zeer (2020), where they are found a high prevalence of the genera *Corynebacterium*, *Staphylococcus*, and *Streptococcus* in urine and a lower prevalence of *Streptococcus anginosus, Anaerococcus spp*.

Chapter Five

CONCLUSIONS

AND

RECOMMENDATIONS

Conclusions

1. The asymptomatic of trichomoniasis among males and the negative result of the microscopic examination does not mean is the real result, but rather a molecular examination using PCR, must be performed to ensure that there is no infection.

2. The wet mount is useless for the diagnosis of trichomoniasis infection in the urine and semen of the males.

3. PCR is the golden tool for the diagnosis of *T. vaginalis* in males. which is highly recommended for asymptomatic infection in males.

4. *Staphylococcus aureus* and *Pantoea spp* were the most prevalent bacteria in males urogenital tract.

5. Using PCR to test unculturable bacteria or negative samples.

6.There is a significant effect of *T. vaginalis* or bacteria-*T. vaginalis* interaction in males which finally affects fertility by an effect on some parameters of the quantity and quality of the semen and spermatozoa.

7. The RT-qPCR has higher sensitivity than other techniques such as WM, RADT, and PCR in detecting *T. vaginalis* in urine and semen samples.

Recommendations

1. We strongly recommend replacing the methods of diagnosis of *T. vaginalis* represented by wet mount and microscopic examination by PCR.

2. To detect male infertility we recommend exchanging the wet mount and microscopic method for a rapid antigen detection test (RADT) which had moderate sensitivity.

3. Conduct an expanded study about the association of *T. vaginalis* with other microorganisms other than bacteria.

Chapter Six

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Appendices

Appendix (A): The Questionnaire paper used in the current study in the English language:-

Sample code

A questionnaire form to study the infection rate of *T. vaginalis* and bacterial co-infection among men in Maysan Governorate, southern Iraq.

Name:-	**Age:-**
Region:-	**Education level:**
Social status:-	**Occupation:-**
Is the husband polygamous?	**Number of Children:**
Disease history / history of marriage / history of treatment / type of treatment/	
The presence of symptoms such as / burning / itching / secretions / urinary incontinence prostate enlargement//	
Urine color /	**is there pain in the urethra/**
Type of washing water /	**Extent of using others' tools/**
Acidity of urine and semen/	
Notes /	

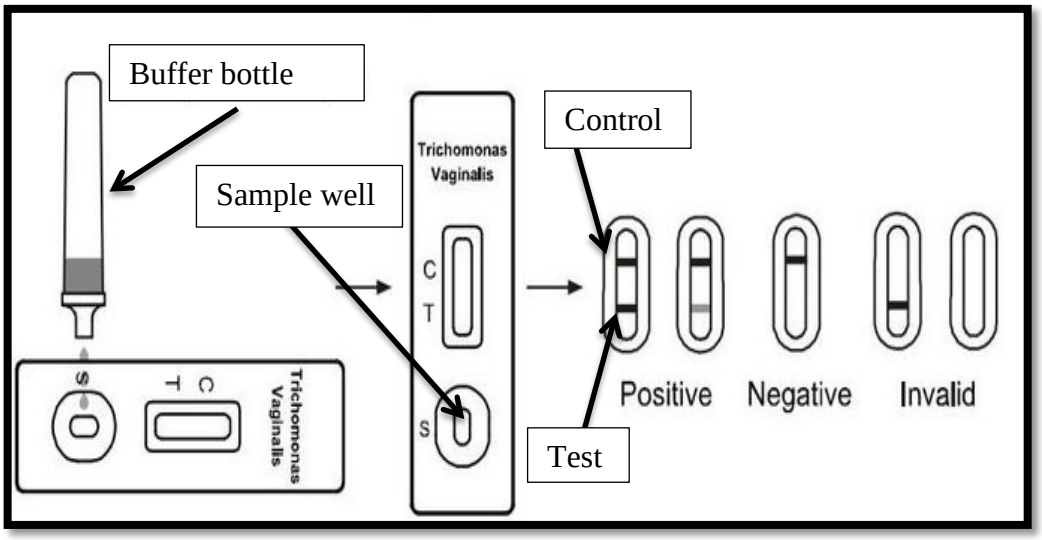
Result ( )

Research student/ Udai Aziz Jabr Al-Rubaie

Date ( )

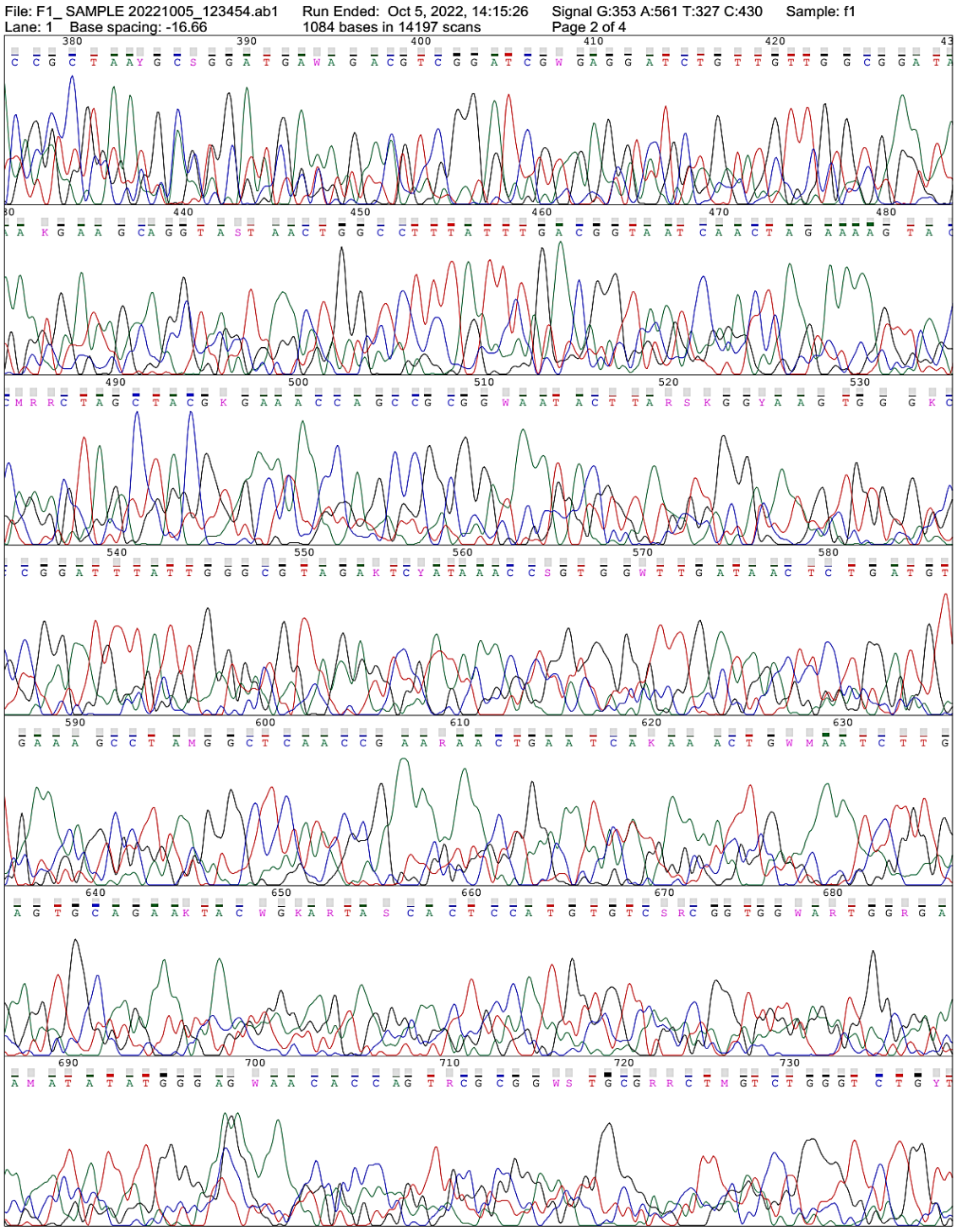
Appendix (B):

The kit of RADT(Laboquick)



The producers of the RADT.

Appendix (C): *16S rRNA* sequencing data



Sequence	Type of bacteria
AACGAAGCAGGCGCAGCTACCATGCAGTCGAGCGGTAACAGGAGGTGCTT GCCCCCGCTGACGAGCGGCGGACGGGTGAGTAATGCGTAGGAATCTGCCC GATAGTGGGGGATAACCTGGGGAAACCCAGGCTAATACCGCATACGTCCT ACGGGAGAAAGGGGGCTTCAGCTCCCGCTATCGTATGATCCTATGTCAGA TTATCTGGTTGGTGATGTAAGGGCTCCCCTCCGCGACCATCCGTCCCTCC TTCGTGAGGATGAGATCCCCCCCCGCGACTCTGAAACCGCCCCCATATCCG TTCGGACGCTCCGCTATCGAATATTCGCAACGCACACCAGTCCGCCTCAC CCGCCCCGCATCTGCTGAATAAAAAGCCTTTTTTTCGTCTCTCACTTAAG TGAGGGATAACTTGATTAAACATTCTGTCAT	Holomonas spp
GCCGAAGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTTGCCTA TAGAAGTTCT GCCGAAGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTTGCCTA TAGAAGTTCT	*Lactobacillus jensenii*
AGAACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGCTCGTAG CAATACGGGA AGAACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGCTCGTAG CAATACGGGA	Uncultured bacterium

الخلاصة

ان المشعر المهبلي *T. vaginalis* هو طفيلي مسوط من الأوالي أجباري المعيشة خارج الخلايا. وهو شائع وواسع الانتشار في جميع أنحاء العالم. يعد أحد مسببات الأمراض التي تنتقل عن طريق الاتصال الجنسي في كل من الرجال والنساء ويسبب مرضًا يعرف بداء المشعرات المهبلية. أجريت الدراسة الحالية لتشخيص طفيلي المشعرات المهبلية والبكتريا المصاحبة له في الجهاز البولي التناسلي وتأثيراتهما على خصوبة الرجال المراجعين الى العيادات الخاصة في محافظة ميسان. تم جمع سبعة وتسعون عينة من البول والسائل المنوي من٩٧ رجلاً وافقوا على المشاركة في هذه الدراسة تراوحت أعمارهم بين ١٨ إلى ٥٠ عاماً، من العيادات الخاصة في مدينة العمارة محافظة ميسان جنوب العراق خلال الفترة من ٢٣ كانون الثاني إلى ٢٠ تشرين الثاني. ، ٢٠٢٢.

تم تسجيل المعلومات الخاصة بكل رجل مشارك في ورقة استبيان، بما في ذلك اسم المريض، عمره، الحالة الاجتماعية، المستوى التعليمي، مكان الإقامة. تم استخدام تقنية المسحة الرطبة والكشف السريع عن المستضد والتقنيات الجزيئية للتعرف على المشعرات المهبلية في عينات البول والسائل المنوي التي تم جمعها.

في السائل المنوي والبول، وجدت هذه الدراسة أن معدل الإصابة بالمشعرات المهبلية بين الذكور كان ٣١,٨%، وكان معدل الإصابة بالبكتيريا بين الذكور ١٧,٥٣%. أظهرت هذه الدراسة أن للمثقبية المهبلية تأثير ذو دلالة إحصائية على العدد الكلي للحيوانات المنوية، ومتوسط عدد الحيوانات المنوية في المليلتر، ولزوجة السائل المنوي، وخلايا القيح في السائل المنوي، وحجم السائل المنوي، في حين لم يكن هناك تأثير ذو دلالة إحصائية على زمن تسييل السائل المنوي ، سرعة الحيوانات المنوية، موت الحيوانات المنوية، الحيوانات المنوية الخملة، تباطؤ الحيوانات المنوية، كرات الدم الحمراء في السائل المنوي، خلايا القيح في البول، عدد كرات الدم الحمراء في البول،وكان للبكتيريا تأثير ذو دلالة إحصائية على العدد الكلي للحيوانات المنوية، متوسط عدد الحيوانات المنوية لكل مل، لزوجة السائل المنوي، في حين لم يكن لها تأثير ذو دلالة إحصائية على زمن تميع السائل المنوي، سرعة الحيوانات المنوية، موت الحيوانات المنوية، الحيوانات المنوية الخملة، تباطؤ الحيوانات المنوية، السائل المنوي. خلايا القيح، كرات الدم الحمراء في السائل المنوي، حجم السائل

المنوي، ومع ذلك، فإن التداخل بين المشعرات المهبلية والبكتيريا كان له تأثير ذو دلالة إحصائية على خلايا القيح في السائل المنوي. بدلا من ذلك، لا يوجد تأثير ذو دلالة إحصائية على العدد الكلي للحيوانات المنوية، كثافة الحيوانات المنوية / مل من السائل المنوي، لزوجة السائل المنوي، سرعة الحيوانات المنوية، موت الحيوانات المنوية، الحيوانات المنوية الخملة، تباطؤ الحيوانات المنوية، كرات الدم الحمراء في السائل المنوي، خلايا القيح في البول، كرات الدم الحمراء في البول.

تم تشخيص البكتيريا بنظام الفايتك وتقنية البلمرة المتسلسل في البول وكانت النتائج موزعة كالتالي :

أربع عزلات *Staphylococcus aureus*، عزلتان *Pantoea spp* ، عزلة واحدة لكل من الانواع البكتيرية *Sphingomonas paucimobilis* ، *Methylobacterium spp* ، *Halomonas spp* ، بينما توزعت النتائج في السائل المنوي كالتالي:

أربع عزلات *Staphylococcus aureus* ,عزلة واحدة لكل من الانواع البكتيرية *Enterobacter aerogen* ، *Serratia marcescens, Pseudomonas putida,* , *Sphingomonas paucimobilis, Aeromonas Sobria, Burkholderia cepacia, Aerococcus viridans, Pseudomonas aeruginosa, Staphylococcus epidermidis , Lactobacillus jensenii, Halomonas* spp.

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قسم علوم الحياة

تشخيص طفيلي المشعرات المهبلية والبكتريا المصاحبة له في الجهاز البولي التناسلي وتأثيراتهما على خصوبة الرجال المراجعين الى العيادات الخاصة في محافظة ميسان.

رسالة مقدمة

الى مجلس كلية العلوم/قسم علوم الحياة

وهي جزء من متطلبات نيل درجة الماجستير علوم في علوم الحياة

من قبل

عدي عزيز جبر

بكالوريوس علوم حياة/جامعة ميسان (٢٠١٥)

بأشراف

أ.د. حسين علي مهوس الساعدي　　أ.د. زاهد سعدون عزيز

آب ٢٠٢٣م　　محرم الحرام ١٤٤٥هـ