

**An-Najah National University  
Faculty of Graduate Studies**

**Molecular Determination of Prevalence of  
Certain Urogenital Bacterial Infections  
among Patients with Infertility Problems**

**By  
Intesar Khaleel Hosney Ashour**

**Supervisor  
Dr. Motasem Y. Al-Masri  
Co-Supervisor  
Dr. Ashraf Swafta**

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**This Thesis was Defended Successfully on 2/7/2019, and Approved by:**

**Defense Committee Members**

**Signature**

- |   |       |
|---|-------|
| <b>1. Dr. Motasem Y. Al-Masri \ Supervisor</b>  | ..... |
| <b>2. Dr. Ashraf Swafta \ Co-Supervisor</b>     | ..... |
| <b>3. Dr. Khaled Qabaha \ External Examiner</b> | ..... |
| <b>4. Dr. Amjad Hussein \ Internal Examiner</b> | ..... |

## Dedication

*This thesis is dedicated to:*

*To whom who taught me patience and to the one who worked day and night to give us a moment of joy to who harvested briars from my path to pare the way of education to whom I miss a lot to the enormous heart  
My deceased father Khaleel Ashour*

*To whom the words are not enough to describe her  
To whom who taught me and suffered a lot to make me who I am now*

*To whom I swim in to her sea of terrenes when I feel pain My precious Mother Jehad Ashour*

*To those that support me and gave me love and hope My brothers: Jala, Zaid, Kareem, Qaes, Moamen and sisters:  
Abrar, Amani Ghazl*

*To whom his love flow in to my heart My dear husband  
Bashir zamel*

*To whom Who have tender heart and innocent spirits to my roses My lovely children Rashad, Abdallah and Sara.*

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*This thesis is just the beginning of my journey.*

## الإقرار

أنا الموقعة أدناه مقدمة الرسالة التي تحمل العنوان:

# Molecular Determination of Prevalence of Certain Urogenital Bacterial Infections among Patients with Infertility Problems

أقر بأن ما اشتملت عليه هذه الرسالة إنما هي نتاج جهدي الخاص، باستثناء ما تمت الإشارة إليه حيث ما ورد، وإن هذه الرسالة ككل، أو أي جزء منها لم يقدم من قبل لنيل أية درجة عملية أو لقب علمياً وبحثي لدى أية مؤسسة تعليمية أو بحثية أخرى.

## Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

**Student's name:**

اسم الطالبة:

**Signature:**

التوقيع:

**Date:**

التاريخ:

## List of Abbreviations

<i>M. hominis</i>	<i>Mycoplasma hominis</i>
<i>M. genitalium</i>	<i>Mycoplasma genitalium</i>
<i>U. urealyticum</i>	<i>Ureaplasma urealyticum</i>
<i>U. parvum</i>	<i>Ureaplasma parvum</i>
<i>C. trachomatis</i>	<i>Chlamydia trachomatis</i>
DNA	Deoxyribose Nucleic Acid
rRNA	Ribosomal Ribose Nucleic Acid .
STD	Sexual transmitted disease
dNTP	Deoxynucleoside triphosphate
WHO	World Health Organisation
PID	Pelvic Inflammatory Disease
PCBS	Palestinian central bureau of statistics
PCR	Polymerase Chain Reaction
NGU	Non-ghonorial Urethritis
NCU	Non-Chlamydieal Urethritis
μl	Microlitter
μg	Micro Gram
UV	Ultra Violet
NCBI	National Center for Biotechnology Information
IRB	Institutional Review Board

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**Molecular Determination of Prevalence of Certain Urogenital Bacterial Infections among Patients with Infertility Problems**

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**Abstract**

**Background:** Urogenital tract infections considered an important cause of infertility worldwide affecting both sexes. *Mycoplasma hominis*, (*M. hominis*), *Ureaplasma urealyticum* (*U. urealyticum*) and *Mycoplasma genitalium* (*M. genitalium*) infections are known to have a role in reproductive problems. Genital *Mollicutes* are difficult to diagnose by culturing and thus PCR methods are used for their detection.

**Aim:** PCR technique was used to determine the prevalence of *M. hominis*, *U. urealyticum* and *M. genitalium* genital infections among infertile males and females patients treated at AL-Shunar infertility center in Nablus city.

**Method:** The study included 103 patients visited AL-Shunar clinic and diagnosed with infertility during January 2018 to October 2018. Different specimens (110 specimens) were collected under supervision of a specialist. Specimens were tested by PCR for detection of the studied *Mollicutes*. In addition, DNA sequencing was made for representative PCR products of the positive samples.

**Results:** Out of the 110 examined specimens, 35 (31.8%) were PCR positive for at least one *Mollicutes*, which were 16 (14.6%) *M. hominis*, 11

(10%) *U. urealyticum*, and 8 (7.3%) *M. genitalium*. *Mollicutes* distribution varied among genital and urine specimens, only 18.9% of urine specimens had *Mollicutes* while 31.3% of vaginal swab had *Mollicutes* and 40.4% of semen had *Mollicutes*. Primary and secondary infertile patients had different *Mollicutes* distribution, where primary infertile patients had a higher infection rate than secondary infertile patients.

Males represent 66% of the total patients and had a higher infection rate (41.2%) of the studied *Mollicutes* than females (20%). *Mollicutes* prevalence varied among several demographic and risk factors such as age, occupation and smoking.

Phylogenetic tree of *U. urealyticum* showed that both strains of *U. urealyticum* in the present study were closely related to each other ascending from the same ancestors and both were closest to *U. urealyticum* 23 (serovar 2). In addition of phylogenetic analysis of *M. hominis* and *M. genitalium* showed that *M. genitalium* strain G-3716S was the closest strain to our *M. genitalium* and *M. hominis* strain TO0613 was the closest strain for our *M. hominis*.

Conclusion: Urogenital infections by *M. hominis*, *M. genitalium* and *U. urealyticum* are an important etiological agent of infertility among patients with different age groups, genders and occupations. Thus, more attention by infertility centres and physicians is required in adopting molecular methods for diagnosis of infections by these microorganism.

**Chapter One**  
**Introduction and**  
**Literature Review**

## **1.1 Infertility definition and types**

Infertility is a disease of reproductive system that is defined by failure to achieve a clinical pregnancy after 12 months of regular unprotected sexual intercourse (Zegers-Hochschild *et al.*, 2009). So, infertility is worldwide health problem with a current global infertility of 15% of couples (Alfarrage and Somily, 2017; Mascarenhase *et al.*, 2012). About 70 million couples worldwide were reported to suffer from infertility. The quality of life in couples facing the infertility problem is particularly altered (Alturki, 2015).

World health organization (WHO) guidelines classified infertility into primary and secondary ones. Absence of a prior history of successful pregnancy indicates presence of primary infertility. (WHO, 2015) on the other hand, individuals are diagnosed with secondary infertility when they have a prior pregnancy but are suffering from infertility latter. Furthermore, the condition of infertility can be irreversible and permanent. On the contrary, in conditions into which the probability of spontaneous conception is decreased are called sub-infertility (Row *et al.*, 2000).

## **1.2 Causes of infertility**

Several factors are responsible for impairments of reproductive organs in men and/or women and consequently result in infertility. The most important factors are the congenital and hormonal disorders, lifestyle, environmental hazards and psychological state. Such factors can result in

impairments of genital organs' function, reproductive cells production, semen quality, movement of sperm cell, chemotactic activity of sperm toward oocyte, union of sperm with oocyte and implantation of zygote steps (Abrao *et al.*, 2013; Inoue *et al.*, 2014; Pirkalani *et al.*, 2013; Safarinejad *et al.*, 2010).

Infertility of couples may be attributed to indirect cause. In addition, 20-40% of couples may have multiple factors for infertility (Alfarrage and Somily, 2017).

Depending on the country and the prevalence observed, various etiologic agents have been linked to infertility. Of the most important etiologic agents were infections of reproductive tract, in particular sexually transmitted diseases (STDs), tuberculosis complications, syndrome of polycystic ovary, endometriosis and fibroids (Alturki, 2015).

In males, a healthy urogenital tract is an important requirement to allow normal fertility through production of adequate and functional sperms. Inflammation and infections by different bacterial species have been associated with male infertility (Stojanvo *et al.*, 2018). Female genital infections and pelvic inflammatory disease have been reported to be possible causes of female infertility (Tao *et al.*, 2018).

### **1.2.1 Bacterial infection as cause of infertility**

Several causes are responsible for development of infertility conditions. Urogenital infections seem to play an important role in

infertility and in many studies, reproductive tract infections of both men and women have been shown to impair reproductive function (Ruggeri *et al.*, 2016).

Infection of genital tract is an important cause of male infertility. Such infection affects not only the function of sperm cells but also the whole process of spermatogenesis (Sanocka *et al.*, 2004). Worldwide, from 8 to 35% of male infertility have been associated to genital tract infections and inflammation processes (Elbahar, 2005).

*Chlamydia* and *Mycoplasmas* are discussed as ones of the important etiological factors of male infertility (Golshani *et al.*, 2007). In addition to that their male urogenital tract infections are often asymptotic. Asymptotic presence of these microorganisms in urogenital tracts may have a negative influence on male reproductive health (Winn *et al.*, 2006).

Female genital infections and pelvic inflammatory disease (PID) have been reported to be possible cause of female infertility (Tao *et al.*, 2018). Pelvic inflammatory disease (PID) has multifactorial aetiology and it is strongly reflected on the health of women reproductive tract. Substantial proportion of PID is caused by *Chlamydia trachomatis*. In addition, there are serological evidences for the association of *Mycoplasma genitalium* with PID (Simms *et al.*., 2003).

Moreover, *C. trachomatis*, *Ureaplasma* species, and *Mycoplasma* species are transmitted through sexual intercourse and are associated with

male urethritis and prostatitis, as well as female urethritis, vaginosis, inflammation of the cervix and PID (Holmes *et al.*, 2007) that might affect human fertility (Ikonomidis *et al.*, 2015; Martens *et al.*, 1993). In a study during 2018 in Eastonia including a total of 193 patients, 23.4% were positive for one or multiple sexually transmitted infections (Tjagur *et al.*, 2018).

In both men and women, different tissues along the genital tract can be targeted by sexually transmitted infections and can lead to functional alterations (Ruggeri *et al.*, 2016). Inflammatory alterations may result in pathological conditions that may end in complete infertility. It had been reported that 15% of all cases of infertility in couples were due to undiagnosed or/and untreated urogenital tract infection. Microorganisms pathologically affect human reproduction mainly by metabolic products that cause direct damage to mucosa of the genital tract or by inducing the body to produce pro-inflammatory responses (Sleha *et al.*, 2013).

#### **1.2.1.1 Infertility role of *Mycoplasma* and *Ureaplasma***

The bacterial family *Mycoplasmataceae* includes *Mycoplasma* and *Ureaplasma* genera. Although members of both genera are among the smallest microorganisms, they are self-replicating (Stojanov *et al.*, 2018). From the genitourinary tract mucosal surfaces, two species among *Mycoplasma* genus, commonly referred to as genital mycoplasmas, have been isolated, which were *Mycoplasma hominis* (*M. hominis*), and *Mycoplasma genitalium* (*M. genitalium*) and one from *Ureaplasma* genus,

which was *Ureaplsma urealyticum* (*U. urealyticum*). The infection of both genera occurs via sexual contact (Mariana *et al.*, 2011).

Genitourinary tract infections by *Mycoplasma* and *Ureaplasma* genera were reported to be associated with failure of reproductive organs, neonatal morbidity and mortality and adverse pregnancy out comes. In addition, infection with these genera has been linked to infertility (Strellrecht *et al.*, 2004; Kumar and Singh, 2015).

Both species of *Mycoplasma*, i.e. *M. genitalium* and *M. hominis* are known to colonize genital reproductive tract of females and males. This results in contamination of the semen during ejaculation and sexual intercourse and consequently causing pathologies. It was shown that genital infections of females with *M. genitalium* were associated with inflammation of endometrium and cervix as well as PID and infertility (Stojanov *et al.*, 2018). In males, *M. genitalium* role in acute and chronic drug-resistant non-gonococcal urethritis was confirmed by many studies (Catherine *et al.*, 2011). In addition, *M. genitalium* possess ability to attach to human spermatozoa and thus this microorganism can be carried and transported by motile sperm suggesting an important role in causing infertility (Svenstrup, 2003).

In sexually active women, *M. hominis* is considered as one of the most commonly detected microorganisms into the genital tract. *Mycoplasma hominis* colonization of the genital tract is associated with an increased risk of pathogenic conditions development and certain

abnormalities during pregnancy such as PID, rupture of membranes before time of delivery, chorioamnionitis, and preterm birth (Peerayeh and Sattari, 2006).

Based on their phenotypic and genotypic features, 14 *Ureaplasma* serovars (serotypes) have been classified in two species. The two species were *Ureaplasma urealyticum* (*U. urealyticum*) and *Ureaplasma parvum* (*U. parvum*). Both species are detected and identified separately using polymerase chain reaction assays (Marovt *et al.*, 2014). *Ureaplasma urealyticum* was reported to be associated with cervicitis and vaginitis in non-specific manner, which may lead possibly to nonspecific condition of infertility (Taylor –Robinson and Farr, 2002). Non-gonococcal urethritis is well known to be caused by *U. urealyticum* infections. In addition, infections caused by *U. urealyticum* has been found to be involved in prostatitis, and epididymitis. Some medical reports documented the relationship between presence of *U. urealyticum* in semen and the decrease in sperm density, motility and/or changes in morphology (Peeryah *et al.*, 2008).

*Mycoplasma* and *Ureaplasma* genera lack cell wall, which makes bacteria resistant to  $\beta$ -lactam antibiotics, not stained with Gram stain and make these organisms pleomorphic. In addition, they are fastidious in nature (Bababanagioti and Kyriazopoulou-Dallaina, 2004; Koneman *et al.* 1997; Marovt *et al.*, 2014; Stojanov *et al.*, 2018). These characteristics hinder their laboratory diagnosis of infection by Gram stain and culture.

## **1.3 Literature review**

### **1.3.1 Prevalence of infertility**

There are large differences in prevalence's of infertility among countries that might be due to differences in definitions and epidemiological design (Mascarehanhase *et al.*, 2012).

A study evaluated infertility of women in several populations during a 12-month period showed an estimated overall median prevalence of 9%. The prevalence rate in more developed nations was ranging from 3.5% to 16.7%. On the other hand, in less developed nations, the prevalence ranged from 6.9% to 9.3% (Boivin *et al.*, 2007).

Estimation of the prevalence of infertility during 2010 among women of 20-44 years old (reproductive age) in 190 countries revealed primary and secondary infertility were found to affect 1.9% and 10.5% of evaluated women, respectively. There were regional variations of infertility prevalence. For example, a relatively high prevalence of infertility is noted in different location in Asia continent such as South Asia, Middle East as well as central Asia. Furthermore, relatively high prevalence rate of infertility is also found in Africa and Eastern Europe. (Ruggeri *et al.*, 2016; Mascarenhase *et al.*, 2012). A research carried out in Britain, which defined infertility by unsuccessfully occurrence of pregnancy for a year or longer. In that study, one out of eight women and one out of ten men with age range from 18 to 74 years had suffered from infertility (Datta *et al.*, 2016).

During 2012, infertility prevalence was estimated to be 18.93% in Saudi Arabia. In addition, primary infertility was found to be more common than secondary one (Al-Turki, 2015).

In Palestine, researches evaluating prevalence and causes of infertility are rarely carried out even though the Palestinian central Bureau of statistics (PCBS) extrapolated prevalence of women infertility in Palestine. The PCBS documented that in Palestinian Territory, among married women aged from 14 to 49 years, infertility percentage was reported to be 8.4%. The infertility frequency in the West Bank (8.4%) was very close to that (8.3%) in Gaza strip. Primary infertility in West bank and Gaza strip were 4.5% and 5.2%, respectively. On the other hand, secondary infertility rate among married women in West Bank was 3.9% in comparison to 3.1% in Gaza strip (PCBS, 2011). Due to little information about infertility in Palestinian Territory, more studies should be carried regarding infertility causes and prevalence.

### **1.3.2 General characteristic of *Mycoplasma* and *Ureaplasma***

The class *Mollicutes* is composed of four orders, five families, eight genera, and at least two hundred known species. The four orders include: *Mycoplasmatales*, *Entomoplasmatales*, *Acholeplasmatales*, and *Anaeroplasmatales* (Waites *et al.*, 2003; Tully *et al.*, 1993).

A unique class of bacteria is the *Mollicutes*, which includes simple bacteria that do not possess cell wall and characterized by their small

genome (Bababanagiotti and Kyriazopoulou-Dallaina, 2004; Koneman *et al.* 1997; Marovt *et al.*, 2013). *Mollicutes* are capable of replication outside as well inside the infected host cells and thus are considered facultative intracellular pathogens (Stojanov *et al.*, 2018).

It is expected that *M. hominis* possesses spherical-shaped cells as only a membrane exposed directly to the environment without cell wall binding it. *Mycoplasma hominis* do not ferment glucose or other carbohydrates but uses other sources of energy i.e. mainly arginine (Baczynska *et al.*, 2005). *Mycoplasma hominis* targets the urogenital tract and invasive disease are occasionally caused by this bacterium. However, Extra-genital infections with *M. hominis* occurs mainly in persons suffering from immunosuppression. Gram stain is not used to visualize *M. hominis* due to lack of a cell wall. Culture of *M. hominis* is insensitive approach and requires well trained laboratory personnel to recognize colonies of this bacterium. Although, specific culture for *M. hominis* may be carried out, they are not widely available and are not suitable for diagnostic purposes since they are not rapid methods (Sampath *et al.*, 2017).

The first identification of *M. genitalium* was in 1980, where this bacterium was detected in urethral specimens of two males diagnosed with non-gonococcal urethritis. *Mycoplasma genitalium* represents the smallest free-living self-replicating organism, with the smallest genome size among all known mycoplasmas, *M. genitalium* lacks a rigid cell wall and is non-susceptible to beta-lactams and other antibiotics that target the cell wall

(Fraser *et al.*, 1995). Due to its serologic cross-reactivity and morphologic similarities with *M. pneumoniae*, along with its extremely fastidious growth requirements, the study of *M. genitalium* is challenging, but has made substantial progress after the introduction of PCR assays (Chra *et al.*, 2018).

Studying *M. genitalium* bacterium is faced with several difficulties. Culture of the organism is not applicable for diagnostic purposes as this bacterium is fastidious and culture is difficult. When the organism is successfully isolated, it takes long time (several weeks or even months) to detect growth. The considerable limitation of culture lead to development of serologic methods, but mostly such methods may have a role in epidemiological studies but is reported to have limited value in diagnostic applications. The only available and applicable diagnostic approach for *M. genitalium* is nucleic acid amplification tests (PCR) (Ross and Jensen, 2006; Eastick *et al.*, 2003).

In 1954, *U. urealyticum* was detected in urogenital tract of a male. This prokaryotic microorganism lack cell wall and it's shape is spherical or cocco-bacillary. The diameter of *U. urealyticum* ranges from 0.1 to 1µm in length (Marvot *et al.*, 2014), and this microorganism possesses a unique ability to metabolize urea (Khan and Farzand, 2011). *Ureaplasma urealyticum* has been reported to play an important role in opportunistic infections (Lee *et al.*, 2016). Directly visualization of *U. Urealyticum* bacterium by light microscopy is nearly impossible due to lack of a rigid

cell wall. Though it cannot be stained by Gram stain, it is useful to for ruling out infections by other causative bacteria. It's not easy to culture *U. urealyticum* due to the fastidious nature of this microorganisms, where it's growth requires the supplement of serum, metabolic substrate as well as growth factors such as yeast extract. Several types of media can be used for culture including SP4, Shepard's 10 B and PPLO broth and agar media (Kokkayil and Dhawan, 2015). *Ureaplasma urealyticum* yield energy through urea hydrolysis. Positive culture is indicated through pH changes in liquid culture. However, *U. urealyticum* is known as a bacterium that is difficult to culture because of several factors. This bacterium is very small and lack a cell wall making it very sensitive to surrounding environment. In addition, culture of *U. urealyticum* is insensitive and requires a long time. Recently, several researches have shown that PCR method is more sensitive than culturing *U. urealyticum* (Waites *et al.*, 2011;Sung, 2013).

### **1.3.3 Epidemiology of genital bacterial infection as a possible cause of infertility**

Sexually transmitted diseases (STDs) had been reported by Devroey *et al.* to be the main causes of infertility include pelvic infections (Devroey *et al.*, 2009).

Recently at 2017 in Italy, several bacteria including *U. urealyticum*, *M. hominis* and *M. genitalium* were detected in cervical swaps collected from 1761 females (with vaginitis, cervicitis, history of infertility, pregnant and miscarriage) by PCR. The prevalence of *U. urealyticum*, *M. hominis*

and *M. genitalium* were 9%, 8.6% and 0.5%, respectively (Leli *et al.*, 2017).

In another study by Godura, the frequencies of infections by *Ureaplasma* and *Mycoplasma* were 19.2% , 15.8%, respectively. The highest frequency of infection (15%) was by *U. urealyticum*. On the other hand, *M. hominis* infection was 10.8% and *M.genitalium* was with the lowest rate of infection, which was 5%. Remarkable, more than one species were detected in the 6.7% of the collected semen specimens (Gdoura *et al.*, 2007).

In Riyadh in Saudi Arabia, endocervical specimens were collected from one hundred infertile married women with primary and secondary infertility in addition for fertile women as a control group. Real time PCR assay was performed for detection of *M. genitalium*, which was detected only in 3% of infertile women (Alfarrage and Somily, 2017).

In a study carried out in Japan two species of *Ureaplasma*, which included *U. urealyticum* and *U. parvum* as well as *M. hominis* and *M. genitalium* were identified applying phylogenetic analysis. Among these four species only *M. genitalium* was significantly more frequent in men with non-gonococcal urethritis (NGU) or Chlamydia negative NCU than asymptomatic control (Yoshida *et al.*, 2002).

In Italy, foreign patients living in Italy were evaluated for urogenital pathogens prevalence by multiplex PCR. The specimens included urine

and seminal fluids as well as swabs from cervix and urethra. *U. urealyticum* possessed the highest infection rate (7.7%) followed by *M. hominis* (4.9%) and then *M. genitalium* (1.1%). Concomitant infection was detected in several patients (Brunelli *et al.*, 2012).

In a prospective study performed in Greece, prevalence of *Chlamydia trachomatis* (*C. trachomatis*), *Ureaplasma* and *Mycoplasma* species were determined in a total of 301 patients. The patients were both men and women and specimens were examined by PCR. *Chlamydia. Trachomatis* infection was diagnosed in only 0.7% of the patients and no patient was found to be infected by *M. genitalium*. Higher infection rate (3.65%) was detected for *M. hominis*. *Ureaplasma* spp. infection was the highest (25.5%) and was found to occur at higher rate in women (41.18) than in men (13.45%) (Ikonomidis *et al.*, 2015).

A total of 337 specimens including urine and vaginal swabs obtained from infertile women from Pakistan were examined for detection of *Mycoplasmatales*. The bacterium *U. urealyticum* was detected in 162(48.07%) cases and *M. hominis* in 89 (26.40%) cases. Among the women who have concomitant infection by *Mycoplasmatales* species, 38% were those with nonspecific cervicitis and 51% were the cases of with nonspecific vaginitis. On the other hand, no apparent signs of urogenital disease were shown in 11% cases of concomitant infection (Khan and Farzand, 2011).

In Los Angeles, 84 patients gave cervical and vaginal swap, urine, and semen, 28 (33%) were PCR positive, *Ureaplasma spp.* were detected in 23 cases and *M. hominis* in 3 cases. In addition, both of species were detected in 2 cases (Strellrech *et al.*, 2004).

A research carried out in Poland to evaluate the incidence of *M. hominis*, *U. urealyticum* and *C. trachomatis* infection in women with urogenital disease. In the studied female cases, infection by *U. urealyticum* (29.8%) was much higher than that *M. hominis* (3.7%). The bacterium *U. urealyticum* was detected with *C. trachomatis* and with *M. hominis* in 13.9% and 10.1% of the cases, respectively. *Mycoplasma* recovered by culture was mostly from infertile women (Zdrodowska-Stefanow *et al.*, 2006).

In Iran, the technique of multiplex PCR was applied for detection of *M. hominis*, *U. urealyticum* and *C. trachomatis* in semen of 200 infertile men. At least one bacterium was detected in 33% of specimens. A significant correlation ( $p < 0.0001$ ) was found between the presence of bacteria in semen and the frequencies of non- motile and morphologically abnormal sperms (Golshani *et al.*, 2007).

Among 541 women from gynecological and sexually transmitted disease out-patient clinics in Poland, 161 (29.8%) had *U. urealyticum*. The predominant symptom in women infected by *U. urealyticum* was suffering from vaginal discharge that was reported in 43.3% of the cases (Zdrodowska-Stefanow *et al.*, 2006).

In a research carried out in Iran, a total of 377 endocervical swabs were collected from infertile women. The swabs were examined by PCR for a number of sexually transmitted pathogens. Out of the examined swabs, 30.7% were PCR positive for *U. urealyticum* and/or *M. hominis*. Considerable percentages (29%) of infected women showed signs or symptoms such as vaginal discharge, cervicitis and/or abortion (Peerayeh and Satri, 2006).

The variation in results of different researches could be attributed to several factors. One of the most important factor is the study design, which determines the selection of studied population and the criteria for the acceptance or rejection of the specimen. Type of sample is also important factor. A very important factor is the applied diagnostic method like serologic method, culture or polymerase chain reaction (PCR). Due to limitation of other methods, PCR appears to be the most suitable method (Capoccia *et al.*, 2013).

#### **1.3.4 Role of bacterial infection in infertility**

The exact role of *U. urealyticum* in causing infertility in males is still controversial among scientists. In some researches, detection of *U. urealyticum* in seminal fluid has been found to associate with infertility diagnoses. For example, a study included 106 individuals was carried in Turkey. This study divided males into three groups: infertile group included 41cases, 33cases included a group suffering from lower urinary tract symptoms, and 32 fertile cases were considered as the control group.

Of the important criteria of the inclusion of patients in infertile group that they do not had a story of varicocele, testicular torsion, hydrocele, undescended testes, and hormonal disorders. The bacterium *U. urealyticum* was isolated from 17 out of the infertile group and 15 of the group with lower urinary tract symptoms. In addition, *U. urealyticum* was isolated from 6 of control group (Taken *et al.*, 2015).

Spermatozoa collected from 31 healthy donors were washed and exposed to *U. urealyticum* to detect possible in-vitro effects on motility, membrane integrity and morphology. Clearly, motility of sperms was decreased. This decrease in motility was significant ( $p < 0.001$ ) when the contact time was increased to 24 hours (Calonge *et al.*, 1998).

A study included 130 hospitalized pregnant women with evidence of preterm delivery and pre-mature rupture of membrane. The bacterium *U. urealyticum* was isolated in 69(53.08%) of cases (Randelovic *et al.*, 2006).

Using PCR, the frequency of detection of DNA of *U. urealyticum* in semen specimens from varicocele patients was higher (21%) than that (3%) of healthy men. In addition, the detection of *U. urealyticum* was associated in lowering with semen volume, spermatozoa count, and increasing morphological abnormality (Peerayeh *et al.*, 2008).

In a study performed in Kurdistan including 104 women in reproductive age and suffering from infertility. Cervical swabs were collected from women and examined by multiplex-PCR for detection of

genital *Mycoplasmas* and *Ureaplasma*. *U. urealyticum* was the most predominant (37.5%) followed by *M. genitalium* (2.9%) and *M. hominis* (2.9%). Furthermore, co-infection was diagnosed in 3.8% of the examined patients (Mousavi *et al.*, 2014).

#### **1.4 Aims of the study**

This study was carried out to investigate the prevalence of *M. hominis*, *M. genitalium* and *U. urealyticum* infections among men and women with infertility and treated at Al- Shunar center in Nablus. Detection of infection by these pathogens was done using PCR method.

# **Chapter Two**

# **Materials and Methods**

## **2.1 Specimen collection**

A total of 110 specimens (16 vaginal swabs, 57 semen, and 37urine) were collected from 103 patients at Al-Shunar center. At least one specimen per patient was obtained independently of specimen type. Specimen collection process was carried out from January 2018 to October 2018

Male patients were not included in the study if they suffered from varicocele, testicular torsion, hydrocele, undescended testes, hormonal disorders, or genetic syndrome. Females were not included if they have hormonal disorders or genetic syndrome.

Sample collection and preservation was done according to instruction of kit that was used for DNA extraction from semen i.e. EZ-DNA kit (Biological Industries Israel Beit Haemik Ltd, Israel).

### **2.1.1 Semen**

The semen specimens were collected into a sterile container and stored at 4°C until it was transported in to the laboratory and placed in sterile eppendorf tube and stored at -20°C until time of DNA extraction (repeated freezing was avoided).

### **2.1.2 Vaginal swabs**

A sterile cotton swab was used to collect a sample from vagina. The sample then was placed in 3 ml sterile normal saline and stored at 4°C until

it was transported in to the laboratory and placed in sterile eppendorf tube and stored at -20°C until used.

### **2.1.3 Urine**

The first voided urine sample was collected and placed in sterile container and then transported to the laboratory and directly DNA was extracted.

## **2.2 DNA Extraction**

### **2.2.1 Semen**

EZ-DNA kit (Biological Industries Israel Beit Haemik Ltd, Israel) was used for DNA extraction from semen specimens. Briefly, 0.25 ml sample was gently, but thoroughly, homogenized with the 1.25 ml of EZ-DNA lysing reagent. Homogenization was achieved by repetitive pipetting with a sterile pasture pipette and until sample became viscous. Then sample was held at room temperature for 5 minutes. The mixture was centrifuged at 10,000g for 5 min at room temperature and the supernatant was aspirated to sterile eppendorf tubes. After that DNA precipitation was achieved by adding 1ml of absolute ethanol per 1 ml of the supernatant and mixing by inverting the tube 10 times (homogeneous solution). The sample was stored at room temperature for 3 minutes then centrifuged at 5000g for five minutes. The resulted DNA pellet was washed by adding 1ml of 75% ethanol. Then the DNA was settle at the bottom by centrifugation at 1000g for 10 minutes. The last step was DNA

solubilization by removing the remaining ethanol wash and the DNA pellet was air- dry for 5 minutes. Obtained DNA was dissolved in freshly prepared 8 mM NaOH.

### **2.2.2 Urine and Vaginal swabs**

A volume of 1ml of the sample was subjected to centrifugation at 12000 ×g for 10 min. The supernatant was discharged and the pellet was washed with Phosphate buffered saline (pH 7.4) and resuspended in 50µl of distilled water. After boiling for 10 min, an aliquot of extract was used directly in PCR experiments (Peerayeh and sattari, 2006).

### **2.2.3 DNA quantification**

DNA was quantified and analysed by using Spectrophotometer ( in order to standardized DNA concentration for PCR reaction mixture.

Specimen was diluted 1:100 (20 µl DNA sample was added 1980µl sterile distilled water) and mixed by using vortex and then the mixture was placed in quartz cuvette, which was placed in spectrophotometer (Jenway7315, England) and light absorption was read then the concentration was calculated according to the following equation:

DNA concentration µg/ml= optical density at 260 X Dilution factorX50

After DNA quantification the samples were diluted in order to have 100 ng/50 µl for each sample.

### 2.2.4 Polymerase chain reaction to detect *U. urealyticum*, *M. genitalium* and *M. hominis*

The applied oligonucleotide primers sequences corresponding to sequences of 16S rRNA gene (16S rDNA gene) within the *M. genitalium*, *U. urealyticum* and *M. hominis*, which are shown in Table 2.1.

**Table (2.1): DNA sequences and targets of applied primers**

Primer	DNA sequence	Target bacteria	Reference
16SUu-AS-	5'-ACTATATTTCTA TAG CGTCGCAA-3	<i>U. urealyticum</i>	Aguilera-arreole <i>et al.</i> , 2014
16SUu-S-	5'-TACCCTTAAGTT GG GGATAA-3'	<i>U. urealyticum</i>	Aguilera-arreole <i>et al.</i> , 2014
16SMh-S	5'-ACCCATTGGAA ACAATGGCTAATG CCGGATACG-3'	<i>M. hominis</i>	Aguilera-arreole <i>et al.</i> , 2014
16SMh-AS	5'-ATAGACCCAGT AAGCTGCCTTCGC CT-3'	<i>M. hominis</i>	Aguilera-arreole <i>et al.</i> , 2014
16SFG2	5'-CCT TAT CGT TAG TTA CAT TGT TTA A-3'	<i>M. genitalium</i>	Eastick <i>et al.</i> ,2003
16SRG	5'-TGA CAT GCG CTT CCA ATA AA-3'	<i>M. genitalium</i>	Eastick <i>et al.</i> ,2003

Each PCR reaction (50µl) for one sample consisted of 0.1 µM Forward primer, 0.1 µM Reverse primer, 100 ng/50 µl DNA Template, 0.4 mM dNTP, 2mM MgCl<sub>2</sub> and 2.5-unit Taq DNA polymerase (Aguilera-arreole *et al.*, 2014; Eastick *et al.*,2003). The components of PCR reaction were obtained from Sigma (USA) and Invitrogen (USA).

Cycling began with an initial denaturation step of five minutes at 94°C, then 41cycles of denaturation at 94°C for 1-minute, suitable

annealing temperature for 30 seconds and extension for 2 minutes at 72°C. This was followed by a final extension step of five minutes at 72°C. The annealing temperatures for *U. urealyticum*, *M. genitalium* and *M. hominis* were 46, 46 and 48 °C, respectively. Products were electrophoresed in 1.5% agarose (Invitrogen, USA) alongside a 100 b.p ladder and stained with ethidium bromide. UV trans-illumination was used to observe DNA bands.

After detection of each pathogen by PCR. The positive samples were used as a positive control in each PCR and all negative results were retested again with positive results. Some of the positive samples were also confirmed by DNA sequencing. In addition, each run of PCR included a negative control.

### **2.2.5 DNA sequencing and alignment**

DNA sequencing was done in biological laboratory in Bethlehem University. Nucleotide sequences were determined for representative PCR products to confirm identity of studied *Mollicutes* (*M. hominis*, *M. genitalium* and *U. urealyticum*). The resulting DNA sequence was examined by nucleotide blast of National Center for Biotechnology Information (NCBI) data base. In addition, using Clustal Omega (EMBL-EBI Hinxton), the DNA sequences of the present study strains were aligned with that of other strains available at NCBI and a phylogenetic tree was made.

### 2.3 Statistical Analysis

Statistical analysis was conducted by a specialist using SPSS 20 for windows. These analysis were performed to determine relation between variables (gender, age, previous pregnancies, miscarriage, period of infertility, smoking tubaco and bubbly) and the presence of *Mollicutes* infection in included patients. In addition, presence of co-infection was also statistically evaluated. A *P* value <0.05 was considered statistically significant.

### 2.4 Instrumentation:

**Table (2.2): Instrumentations used in this study**

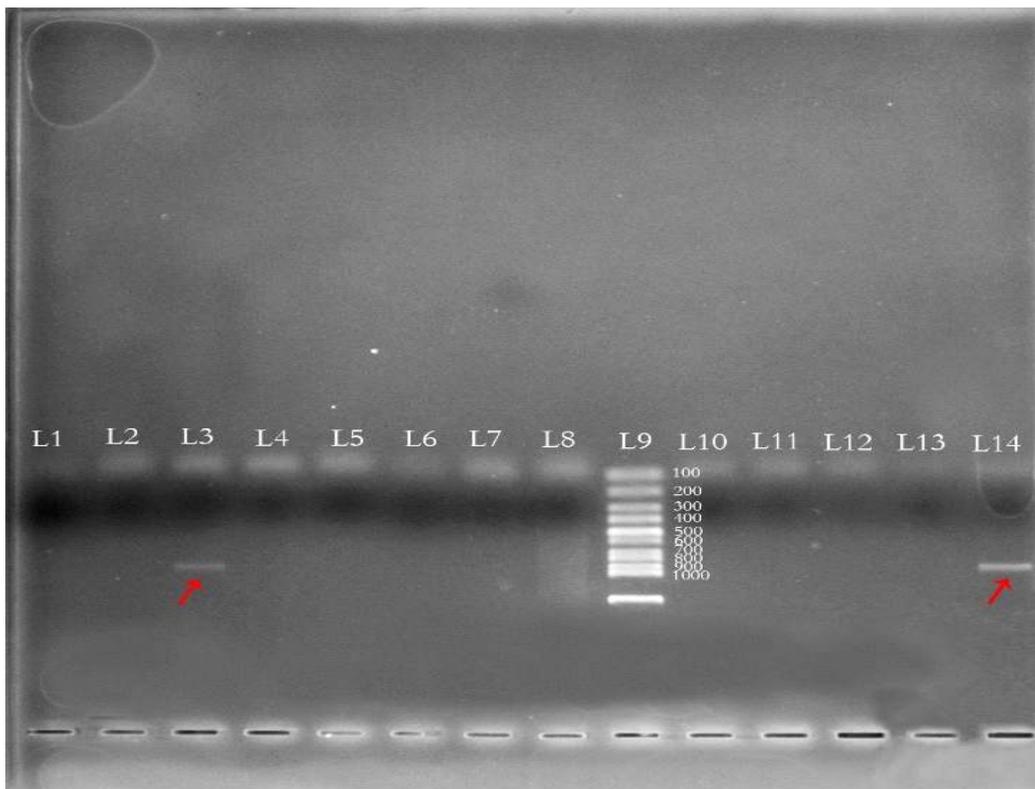
<b>Instrumentations</b>	<b>Manufacturer</b>	<b>Country</b>
1. Balance	Radwag, max 220g	Poland
2. Ultra violet trans illuminator	Ariston	Italia
3. Refrigerator	Beko	Turkey
4. Vortex	VELP Scientifica	Europe
5. Autocleave	Mrc-lab-equipment	Palestinian occupied territories
6. Termocycler	Biometra	Germany
7. Centrifuge	Hettich Zentrifugen	
8. Incubator	Nuve	Turkey
9. PH meter	Jenway	UK
10. Spectrophotometer	Jenway 7315	England

# **Chapter Three**

## **Results and Discussion**

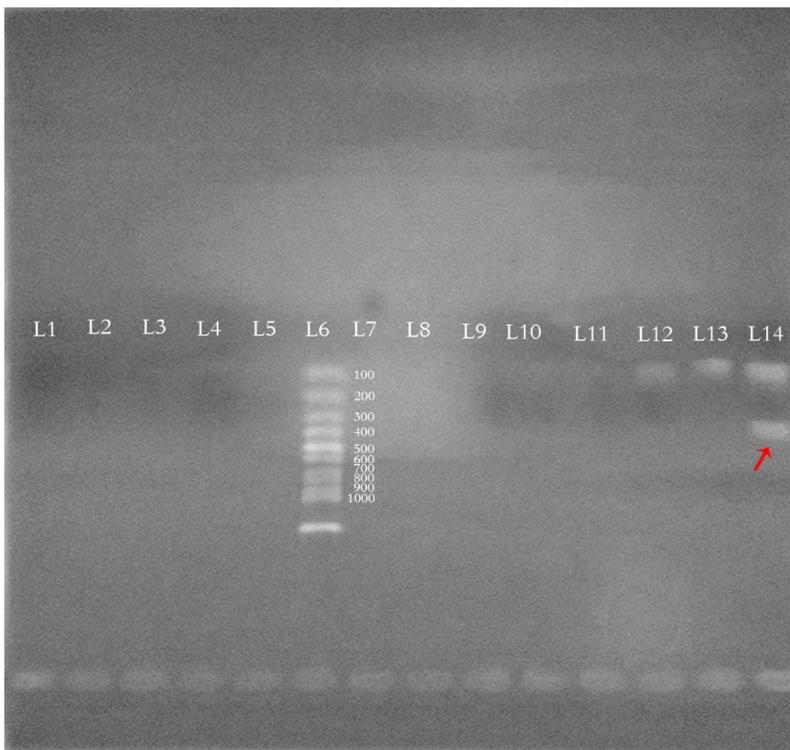
### 3.1 Polymerase chain reaction results

Among 110 examined specimen, 35 were PCR positive for at least one *Mollicutes* included in the study, which were *Ureaplasma urealyticum* (*U.urealyticum*), *Mycoplasma genitalium* (*M.genitalium*) and *Mycoplasma hominis* (*M. hominis*). These 35 specimens showed the expected band as demonstrated in Figure 3.1- 3.3. *Ureaplasma urealyticum*, was detected in 11 specimens (10%), where the expected band, which was 898 b.p was detected in all positive cases. In addition, two positive PCR products were confirmed by DNA sequencing.



**Figure (3.1):** Agarose gel electrophoresis of PCR products of *Ureaplasma urealyticum* (898 bp) stained with Ethidium bromide. Lane (L) 1, negative control; L 2,4,5,6,7,8,10,11,12 &13 negative samples; L 3 &14, positive sample; L 9,100 bp ladder.

Figure 3.2 showed the expected band of 343 b.p detected by electrophoresis of PCR products of *M. genitalium*. The DNA of *M. genitalium* was detected in 8 (7.3%) out of 110 specimens. The DNA sequence of one of the PCR products was sequenced to confirm its identification



**Figure (3.2):** Agarose gel electrophoresis of PCR products of *Mycoplasma genitalium* (343bp) stained with Ethidium bromide. Lane (L) 1, negative control; L 2,3,4,5,7,8,9,10,11,12 &13 negative samples; L 14, positive sample; L 6, 100 bp ladder.

*Mycoplasma hominis* was detected in 16 (14.6%) out of 110 specimens, where the expected band (603 b.p) was found in gel electrophoresis of the PCR as shown in figure 3.3. DNA sequence was made for one PCR product and confirmed identification of *M. hominis*.



**Figure (3.3):** Agarose gel electrophoresis of PCR products of *Mycoplasma hominis* (603 b.p) stained with Ethidium bromide. Lane (L) 1, negative control; L 2,4,5,6,7,9,10,11,12 & 14 negative samples; L 13, positive sample; L 8,100 bp ladder.

According to some authors, *M. hominis* is a major pathogen in pelvic inflammatory diseases, being detected in 10-40% of cases (Waites, 2002; Taylor-Robinson *et al.*, 2002; Hong, 2002). In other studies, *M. hominis* and *U. urealyticum* were detected in 5-13% and 11-35%, respectively in patients with infertility problems (Rodriguez *et al.*, 2001; Rosemond *et al.*, 2006). The prevalence of *M. genitalium* was reported to range from 3% to 13.4%, (Alfarrage and Somily, 2017; Ikonomidis *et al.*, 2015; C Anagrius, 2009).

Table 3.1 shows results of PCR without differentiating between specimen types collected from infertile men and women. Among 110

examined specimens, 35 (31.8%) were positive for at least one genital pathogen. Frequency of detection of *M. hominis* (14.6%) was the highest. Remarkably, *M. hominis* was significantly associated with *U. urealyticum* (3.6%) ( $P= 0.044$ ) and *M. genitalium* (3.6%;  $P=0.005$ ). However insignificant association between *U. urealyticum* and *M. genitalium* (1.8%) was found ( $P= 0.172$ ). Close to our results, Stellrecht *et al.* (Stellrecht *et al.*, 2004), reported that out of 84 specimens (cervical, vaginal swabs,

**Table (3.1): PCR results from 110 specimens (Semen, urine and vaginal swabs)**

Pathogen	Single PCR positive result	
	Number	Percentage (%)
<i>M. genitalium</i>	8	7.3
<i>M. hominis</i>	16	14.6
<i>U. urealyticum</i>	11	10
Total	35	33.8
Multiple PCR positive result		
<i>M. hominis</i> + <i>M. genitalium</i>	4	3.6%
<i>M. hominis</i> + <i>U. urealyticum</i>	4	3.6%
<i>U. urealyticum</i> + <i>M. genitalium</i>	2	1.8%
<i>M.hominis</i> + <i>M.genitalium</i> + <i>U.urealyticum</i>	1	0.9%

semen and urine), 28 (33%) were positive for at least one of *U. urealyticum*, *M. hominis* and *M. genitalium*. In addition, *M. hominis* was also detected in association with *U. urealyticum* in 2 cases. Mousavi *et al* (Mousavi *et al.*, 2014) showed that out of 104 patients, 39 cases (37.5%) were infected with *U. urealyticum*, *M. genitalium* and/or *M. hominis* among infertile women. Co-infections were seen in 3.8% of the patients. Godura *et al.* (Godura *et al.*, 2007) also reported that mixed species of *Mycoplasmas* and *Ureaplasmas* were detected in 6.7% of semen sample. Clinical studies showed that *Mycoplasma* incidence is raised in the

presence of an anaerobic primer pathogen. In addition, the growth of this microorganism increases in anaerobic condition (Serin *et al.*, 2013). *Mycoplasmas* can grow in the stress environment created by primary pathogen. Notably, these microorganisms colonize in numerous numbers in sexually active adults (Schlicht *et al.*, 2004).

Remarkably, the specimen in which the 3 types of bacteria were detected as shown in Table 3.1 was collected from a male diagnosed to have symptoms of bacterial infection and his wife had symptoms of infection with repeated (3 times) miscarriage.

Seven patients (4 females and 3 males) gave two types of samples. One male was negative for all examined types of bacteria. In 2 males, *M. genitalium* was detected in semen and urine in one of them, while *M. hominis* was detected in both specimen of one patient and only in semen of the other. Out of the 4 females only one had *M. hominis* in both vaginal swab and urine sample.

### **3.2 *Mollicutes* detection frequency in different specimen types**

Table 3.2 compares the frequency of detection of the studied *Mollicutes* (*M. genitalium*, *M. hominis* and *U. urealyticum*) in different sample types. PCR detected *Mollicutes* in semen specimens in 23 (40.4%) out of 57 specimens, while in urine specimens 7 out of 37 specimens (18.9%) were with positive PCR results for *Mollicutes*. In addition, PCR detected *Mollicutes* in 5 out of 16 vaginal swabs (31.3%). Remarkably,

the percentage of detection of *Mollicutes* in semen (40.4%) was significantly ( $P=0.023$ ) higher than that in urine (18.9%) and insignificantly higher than that in vaginal swab (31.3%). Higher frequencies of detection of *Mollicutes* in specimens collected from genital tract (semen and vaginal swab) than in urine specimen collected from urinary tract may reflect the tissue tropism of *Mollicutes* toward the genital system. *M. hominis* was found to be the most predominant in semen and urine specimens, which were 21.1%, 8.1%, respectively. In vaginal swabs specimens, *M. hominis* (6.3%) was found to be less predominant than *U. urealyticum* and *M. genitalium*, which had the same percentage (12.5%) as shown in Table 3.2

**Table (3.2): Detection of *U. urealyticum*, *M. genitalium*. and *M. hominis* in different types of specimens**

Specimen	Number (%out of specimens)	<i>U. urealyticum</i> (%)	<i>M. genitalium</i> (%)	<i>M. hominis</i> (%)	Total number of pathogen (%)
Semen	57(51.8)	7(12.3)	4(7)	12(21.1)	23(40.4)
Urine	37(33.6)	2(5.4)	2(5.4)	3(8.1)	7(18.9)
Urine male	17(15.5)	2(11.8)	1(5.9)	2(11.8)	5(29.4)
Urine female	20(18.2)	0(0)	1(5)	1(5)	2(10)
Vaginal swab	16(14.6)	2(12.5)	2(12.5)	1(6.3)	5(31.3)
Total	110 (100)	11(10)	8(7.3)	16(14.6)	35(31.8)

Association of both *M. hominis* and *M. genitalium* infection (co-infection) in semen sample was found to be significant ( $P=0.000$ ), and the co-infection of *M. hominis* and *U. urealyticum* in urine sample was also significant ( $P=0.013$ ).

The prevalence of *Mollicutes* in urine samples from males was higher than that from females, where in the present study, in male samples 5 out of 17 (29.4%) were PCR positive for the presence of at least one *Mollicutes*. In more details, *U. urealyticum* and *M. hominis* had the same detection percentage (11.8%) followed by *M. genitalium* (5.9%). On the other hand, in female urine samples, 2 out of 20 (10%) were PCR positive for the presence of at least one *Mollicutes* out of them *M. hominis* and *M. genitalium* had the same percentage (5%) and *U. urealyticum* was not detected. Higher detection of *Mollicutes* in male urine samples may be due to anatomical difference between the two genders, where in males the urethra is longer and an exist for urine and during ejaculation for semen.

In a previous study (Gdoura *et al.*, 2007), among 120 semen samples examined by PCR, the frequency of genital *Ureaplasmas* and *Mycoplasmas* detected in semen samples of infertile men were respectively 19.2% and 15.8%. In more details, the frequency of *U. urealyticum* (15%) was higher than that of *M. hominis* (10.8%) and *M. genitalium* (5%). In another previous study, out of 84 specimens collected from infertile in Scotland, 28 were PCR-positive for *Mollicutes*, where *Ureaplasmas* was detected in 23(82%) samples, *M. hominis* was detected in 3 (11%) and both were detected in 2 (7%) (Sterllrech *et al.*, 2004). Also, McIver *et al* (McIver *et al.*, 2009) studied non-gonococcal agents in 233 cervical swaps, *M. hominis* was detected in 13.7% of specimens, followed by *U. urealyticum* (6.1 %) and *M. genitalium* (1.3%). Campos *et al* (Campos *et al.*, 2015) reported that the molecular finding among 302 women of vaginal swab of *M.*

*hominis* and *M. genitalium* were 31.8%, 28.1% respectively and co-infection of both *Mollicutes* was 4.97%. The report of Takahashi *et al* (Takahashi *et al.*, 2006) in Japan, which included PCR examination of first-voided urine specimens collected from 100 male recorded detection of *M. genitalium*, *M. hominis* and *U. urealyticum* in 1%, 4% and 12%, respectively.

### **3.3 *Mollicutes* distribution among primary and secondary infertile patients**

The data of 103 patients suffering from infertility were analysed as shown in Table 3.3. Among these patients the overall prevalence of primary infertility was 65%, which was much higher than secondary infertility (35%). In parallel to our study, Al-Turki (2015) found that among male and female patients suffering from infertility, 78.9% were diagnosed to have primary infertility. In Jezira area in Sudan, primary infertility was found to represent 77.4% of infertility cases (Abdalla, 2011).

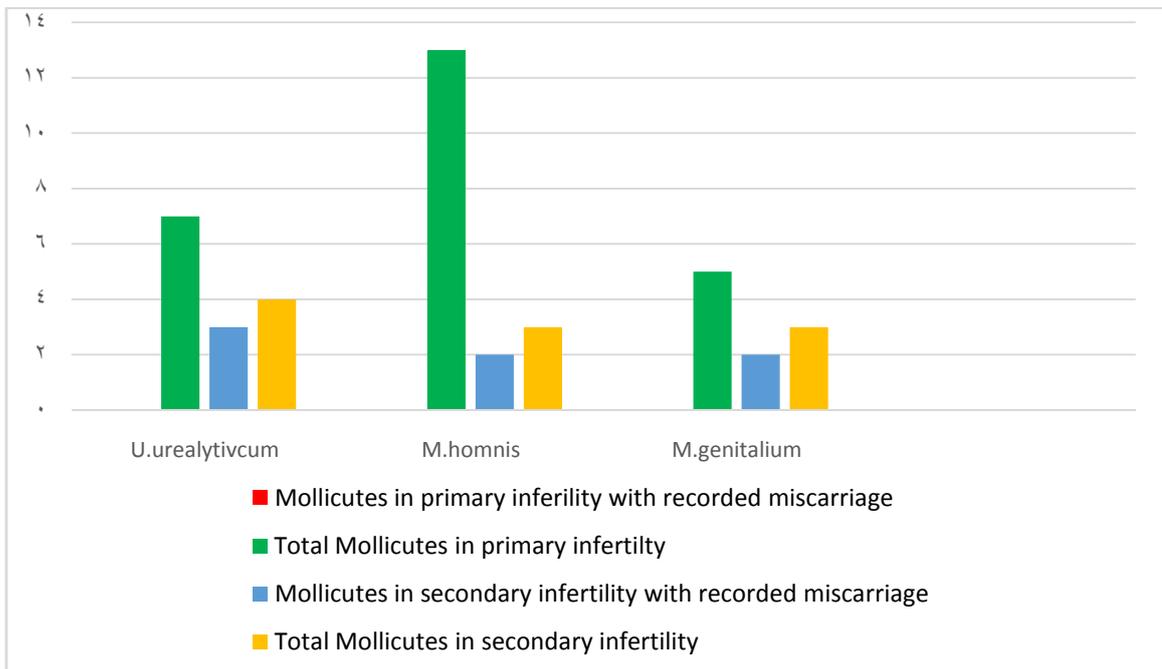
In our study, the average age of patients with primary infertility ( $31.5 \pm 8.1$  years) was insignificantly lower than that of patients with

**Table (3.3): Comparison between primary and secondary infertility in relation to *Mollicutes* infections**

Parameter	Primary infertility	Secondary infertility
Number of patients	67(65%)	36(35%)
Mean age (years)	31.5±0.9	33.7±1.3
Mean period of infertility (years)	5.5±0.5	7.7±0.9
Cases recorded of miscarriage	3 (21.9%)	20(64.5%)
<i>M. genitalium</i>	5 4.9%	3 2.9%
<i>M. hominis</i>	13 12.6%	3 2.9%
<i>U. urealyticum</i>	7 6.8%	4 3.9%

secondary (33.7±8.4%) infertility. No correlation was found between age group and type of infertility ( $P=0.503$ ). Our results showed a significant association between period of infertility and type of infertility, the patients who suffered from secondary infertility had a significantly ( $P=.028$ ) longer period (7.7± 5.7 years) than who suffered from primary infertility (5.5±4.3). In addition, strong significant ( $P=.000$ ) association between secondary infertility and abortions (miscarriage) was found in the present study. In more detail, among 23 cases of abortions, secondary infertility was recorded in 20 cases. The abortion conditions significantly associated with patients who had a previous pregnancy then they had suffered from infertility problem and unable to give child again and the possible reason is having infections after the first pregnancy with one of the studied *Mollicutes*. Roles of *Mycoplasma* and *Ureaplasma* in causing infertility may be by causing premature rupture of the membranes and induction of abortion by an inflammatory response by microbial colonization as suggested by many clinical and experimental studies and

researches (Capoccia *et al.*, 2013; Farhadifar *et al.*, 2016; Lamont *et al.*, 1987; Randelovic *et al.*, 2006). The relation between patients diagnosed with infertility and *Mollicutes* are shown in Figure 3.4. Genital colonization with *M. hominis* and *U. urealyticum* may predispose to spontaneous abortion (Bayraktara *et al.*, 2010). A previous study by Maleki *et al.* (2006) revealed direct strong relation between *M. hominis* and *U. urealyticum* and habitual abortion as well as urogenital infections. Remarkably, it was found that presence of bacterial vaginosis at the beginning of pregnancy was strongly associated with subsequent early pregnancy loss. However, *M. hominis*, and *U. urealyticum* but not other microorganisms remained associated with an increased risk of miscarriage (Donders and Van, 2000).



**Figure (3.4): *Mollicutes* distribution among primary and secondary infertile patients**

In the present study, in patients with primary infertility, analysis revealed that the highest prevalence of *Mollicutes* infection was that of *M.*

*hominis* (12.6%) followed by *U. urealyticum* (6.8%) and *M. genitalium* (4.9%), whereas *U. urealyticum* (3.9%) possessed the highest prevalence of *Mollicutes* among patients with secondary infertility followed by *M. genitalium* and *M. hominis* (2.9%). The primary infertile patients who had miscarriage history were negative *Mollicutes* infections as shown in Figure 3.4. This result was in agreement with previous report on recurrent (habitual) abortion in the presence of these *Mollicutes* when Maleki *et al.* (Maleki *et al.*, 2006) reported the relation of recurrent abortion with the presence of the studied *Mollicutes* in either urine or genital samples of women was as the following: the prevalence of *Mollicutes* were considerably higher in urogenital samples with women suffering from recurrent abortion history in comparison with those without such condition. Bayoumi *et al* (Bayoumi *et al.*, 2006) also reported that *M. hominis* was detected in 30.4% of women with repeated pregnancy loss and not in pregnant women (as control) and the presence of *M. hominis* was observed more frequently in women with repeated abortions. In another study, Mousavi *et al* (Mousavi *et al.*, 2014) reported that out of 104 married infertile women, *Mollicutes* were detected by PCR in 39 cases. In addition, out of 62 women with primary infertility, 23(37.1%) cases were PCR positive for *Mollicutes* and 42 women with secondary infertility had 16 (38.1%) cases of *Mollicutes* infection ( $P=.950$ ). The same research also recorded that there was no statistically association between the infections and history of abortion, type of infertility and infertility duration.

In a previous research (Marten *et al.*, 1993), cervical swabs were collected from 80 female patients, *Mollicutes* were detected including *M. hominis* (6%) and *U. urealyticum* (28%). In addition, among all female participants, 18 cases were recorded for miscarriage in and the mean duration period of primary infertility was 36.3 months.

In our study, *M. genitalium* infection was significantly associated with females (and not males or both, males and females) diagnosed with secondary infertility, where among 25 female patients with secondary infertility all the 3 PCR positive result were *M. genitalium* ( $P=.018$ ). Furthermore, *M. genitalium* had significant association ( $P=.025$ ) among females with a history of miscarriage, where 3 out 11 (27.3%) infertile females suffered from miscarriage were 3 PCR positive for *M. genitalium*. In contrast, no *M. genitalium* was detected in women with no history of miscarriage. *M. genitalium* was considered an important possible etiological agent of infertility particularly tubal infertility and pelvic inflammatory disease in women (Maharat *et al.*, 2003; Svenstrup *et al.*, 2008).

### **3.4 *Mollicutes* infections variation among infertile men and women**

The results presented in Table 3.4 below showed that there was a different distribution of *Mollicutes* among males and females. Specimens collected from males represent 66% of total specimen. Among the 68 males 28 (41.2%) possessed PCR positive results. *Mycoplasma hominis* had the highest prevalence (20.6%) followed by *U. urealyticum* (13.2%) and *M. genitalium* (7.5%). Females represented 34% of the patients.

**Table (3.4): Distribution of *Mollicutes* infection in the 2 genders**

Gender	Number (%)	<i>M. hominis</i> (%)	<i>U. urealyticum</i> (%)	<i>M. genitalium</i> (%)	<i>Mollicutes</i> (%)
Female	35(34)	2(5.7)	2(5.7)	3(8.6)	7(20)
Male	68(66)	14(20.6)	9(13.2)	5(7.4)	28(41.2)

The total number of *Mollicutes* among females was 7 (20%). Furthermore, the results represented in Table 3.4 show that male have the highest number of each studied pathogen except for *M. genitalium* regardless of specimen types. However only *M. genitalium* was more predominant in females. This high prevalence may be a consequence of histological features of female's vaginal epithelial cells, which may reflect a reduced host immune response to *M. genitalium* (Mobily *et al.*, 2012). Both *M. hominis* and *U. urealyticum* inhabit male urethra and contaminate semen through ejaculation playing an important roles in male infertility (Andrade-Rocha, 2003; Wang *et al.*, 2006).

A total of 28 out of 35 of PCR positive results belong to the infertile men while infertile women have 7 out of 35 of PCR positive result for the studied pathogen regardless of specimen type (vaginal swab or urine). The variation of infections among males and females suggested a different susceptibility to infections, probably due to biological differences.

*Mollicutes* distribution among the 2 genders in our study was different from other research, where *Mollicutes* were detected in males more than in females. In another study carried out by Foschil *et al.* (2018), *Mollicutes* infection in females turned to be twice as often as men (33.3% vs 17.8%). Maleki *et al.* (Maleki *et al.*, 2006) revealed that the highest prevalence of *M. hominis* (71.4%) and *U. urealyticum* (60%) were in

females. These differences may be attributed to cultural and religious variations in different regions.

There was no significant association of *U. urealyticum* or *M. genitalium* with gender ( $P=0.242$  and  $P=0.827$ , respectively), where males had 9 (13.2%) cases of *U. urealyticum* infection and 5 (7.4%) of *M. genitalium* infection. On the other hand, females had 2 (5.7%) and 3 (8.6%) of infection by *U. urealyticum* and *M. genitalium*, respectively. There was a significant association ( $P=.048$ ) of *M. hominis* with gender where *M. hominis* was found in higher percentage in males (20.6%) in comparison with females (5.7%).

### **3.5 Prevalence of *Mollicutes* infections in relation to different demographic and risk factors**

The prevalence of *Mollicutes* varied in patients who were between 18 and 62 years old. Patients aged between 40-50 years old and 29-39 years old had the highest frequencies of positive results, which were 58.3% and 31.9%, respectively. *Mollicutes* were also detected in considerable rates among 18-28 age groups as shown in Table 3.5. *M. hominis* (17%) and *U. urealyticum* (12.8%) were more predominant in patients between 29 and 39 years old as shown in Figure 3.5 and Figure 3.6, respectively, where *M. genitalium* (10%) had been more predominant in patients between 18-28 years as shown in Figure 3.7. However, no significant correlation was found between the studied *Mollicutes* and patients with different age groups with the exception of females who aged between 18-28 years,

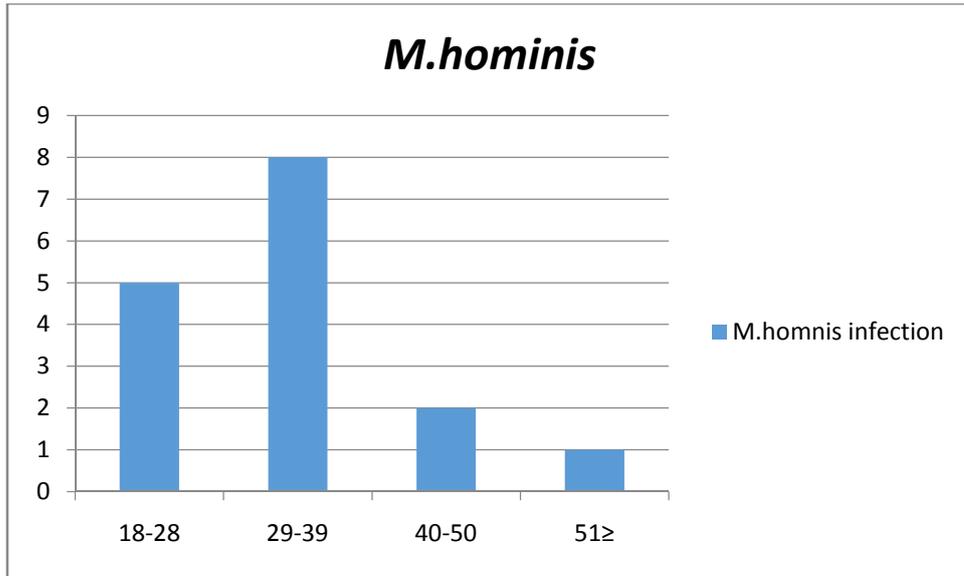
where a significant association ( $P=.003$ ) of *M. genitalium* infection was found with them. Its high prevalence could be due to the increase of the genital mycoplasma colonization after puberty. In addition the prevalence of genital mycoplasma increase after the first sexual contact as a main reason (Gupta *et al.*,2009). This is consistent with our community where the sexual contact as a result of marriage is predominant at age of 18-28 years old.

**Table (3.5): Frequencies of *Mollicutes* infection in relation to different factors**

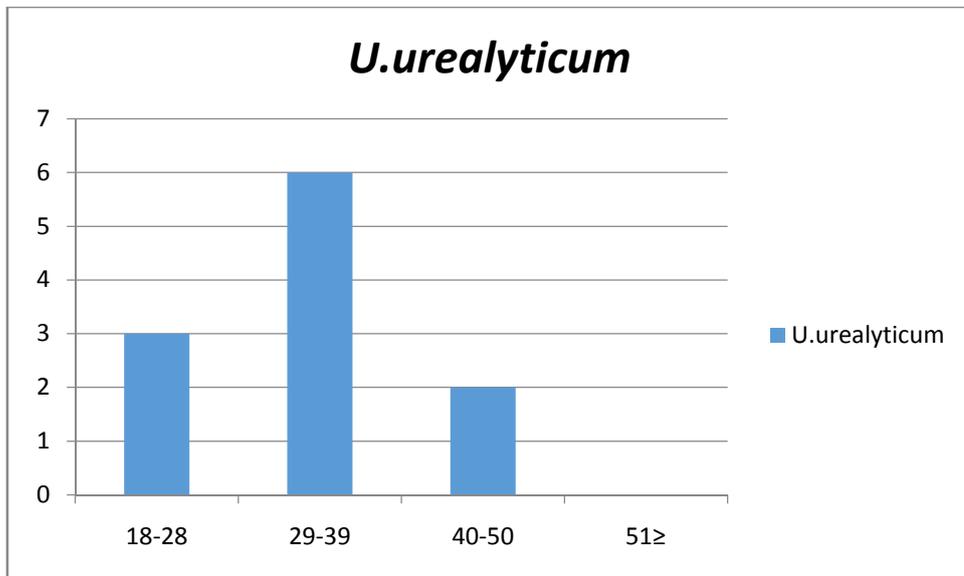
Variable	Number (%)	<i>M. hominis</i> (%)	<i>U. urealyticum</i> (%)	<i>M. genitalium</i> (%)	Total Number of <i>Mollicutes</i> (%)
Age(years)					
18-28	40(38.8)	5(12.5)	3(7.5)	4(10)	12(30)
29-39	47(45.6)	8(17)	6(12.8)	1(2.1)	15(31.9)
40-50	12(11.7)	2(16.7)	2(16.7)	3(25)	7(58.3)
51≥	4(3.9)	1(25)	0(0)	0(0)	1(25)
Occupation					
Employee*	34(33)	5(14.7)	3(8.8)	1(2.9)	9(26.5)
Workers*	39(37.9)	9(23)	5(12.8)	4(10.3)	18(46.2)
House wife	28(27.2)	2(7.1)	2(7.1)	3(10.7)	5(17.9)
Out of work	2(1.9)	0(0)	0(0)	0(0)	0(0)
Smoking					
Yes	48(46.6)	14(29.2)	7(14.6)	5(10.4)	25(54.2)
No	55(53.4)	2(3.6)	4 (7.3)	3(3.4)	9(16.4)
Bubbly					
Yes	72(69.9)	12(16.7)	9(12.5)	4(5.6)	26(34.7)
No	31(30.1)	4 (12.9)	2(3.2)	4 (12.9)	10(32.2)

\* Employee, who works at offices without physical efforts such as lawyer & marketing manger;

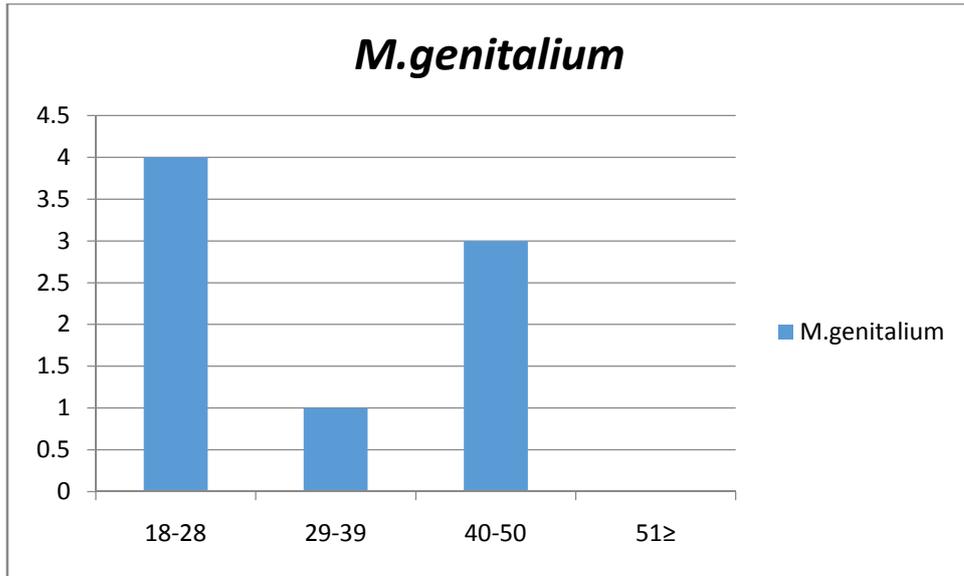
\* Workers, who works out offices with high physical efforts such as builders & painters.



**Figure (3.5): *Mycoplasma hominis* distribution in different age group**



**Figure (3.6): *Ureaplasma urealyticum* distribution in different age groups**



**Figure (3.7): *Mycoplasma genitalium* distribution in different age groups**

Our finding was in parallel to the results of Verteramo *et al* (Verteramo *et al.*, 2013) who reported that *M. hominis* was more predominant in older patients and the highest infections was at age of 30-39 years followed by patients  $\geq 40$ . The same author recorded that the prevalence of *U. urealyticum* infections increases with increasing age until the age of forty. Zdrodowska *et al* (2009) found higher prevalence of *M. hominis* (50%) and *U. urealyticum* (29.2%) infections in women aged 26-30 years old. In other research (Andersen *et al.*, 2007), *M. genitalium* infection in female aged 18-28 was 3.2%, which was higher than the prevalence of infection in men of the same age which was 1.1%.

The numbers and percentages of occupations of the patients and prevalence of *Mollicutes* among them also shown in Table 3.5. Workers who represent 37.9% of the patients have the highest PCR positive results for *Mollicutes* (46.2%) followed by employee (26.5%), house wives (17.9%) and people without job (0%). As shown in Table 3.5, the

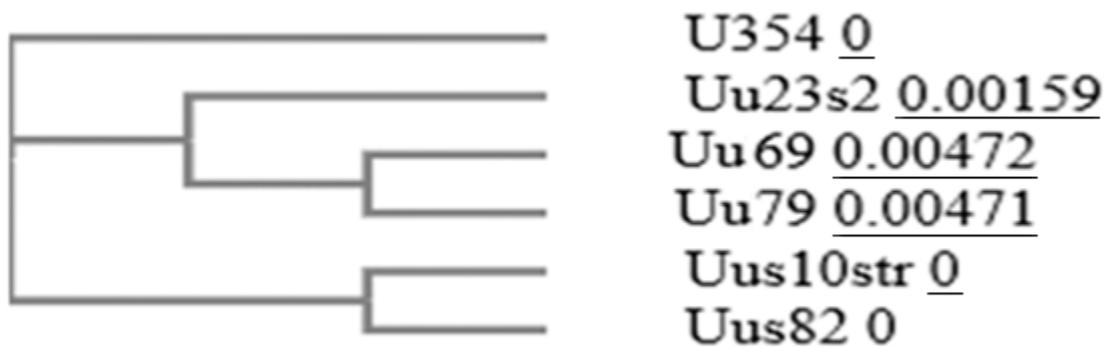
prevalence of *M. hominis* was the highest followed by *U. urealyticum* and *M. genitalium* in both workers and employees. Equal distribution of both *M. hominis* and *U. urealyticum* was found among housewives (7.1%), and equal distribution of each *Mollicutes* was found among unemployed patients (0%). Statistical analysis showed that there was no significant association between patient occupations and frequency of infection by *M. hominis*, *M. genitalium* or *U. urealyticum*. Our results were consistent with a previous research, which recorded that the prevalence among 100 participants were 42% and 15% for *U. urealyticum* and *M. hominis*, respectively and no significant association of various occupation types with the infected participants was found (Simbulan *et al* 2001). The negative correlation of *Mollicutes* infection may refer to the vulnerability to have this microorganism is increased with sexual activity and risk behaviours and not to economic life and work stress (Simbulan *et al.*, 2001).

Furthermore *Mollicutes* distributions among smokers (tobacco and bubbly) varied, where 48 tobacco smokers had 7 cases (14.6%) of *U. urealyticum* infection, 14 (29.2%) cases of *M. hominis* and 5 cases (10.4%) of *M. genitalium* compared with bubbly smokers who had 12 cases (16.7%) *M. hominis* infection, 9 cases (12.5%) of *U. urealyticum* and 4 cases of (5.6%) *M. genitalium*. Smoking appeared to influence the infection rate of 2 of the studied *Mollicutes*, i.e. *M. hominis* and *U. urealyticum*. Significant association between tobacco smokers and infection of *M. hominis* ( $P=.048$ ) and between bubbly smoker and *U. urealyticum* ( $P=.021$ ) were found. In another study Verteramo *et al*

(Verteramo *et al.*, 2013) reported a significant association between *U. urealyticum* and smoking ( $P=0.045$ ) in contrast with *M. hominis* infection, which possessed no association with smoking. However Huang *et al* (Huang *et al.*, 2015) reported that there is no significant association between smoking and each *Mycoplasma* and *Ureaplasma* genital infection.

### 3.6 Phylogenetic analysis

Two PCR products (898 b.p) of *U. urealyticum*, were sequenced. The alignment of 545 and 562 bp. results showed 98 to 99% similarity to a number of *U. urealyticum* strains such as *U. urealyticum* 23 *servoar*2 as shown in phylogenetic tree below (Figure 3.8). Both strains of our research (Uu69 and Uu79) appeared to be more closely related than to other strains (Figure 3.8).



**Figure (3.8):** Phylogenetic tree of *Ureaplasma urealyticum*. The tree is based on analysis of 564 rRNA genes showing our *Ureaplasma urealyticum* number 69 and 79 in comparison with different *Ureaplasma* strains. The numbers underlined correspond to a sequence distance measure.

The phylogenetic tree represent evolutionary relationship of the same *Ureaplasma* species constructed by comparing 16SrRNA which is

indicative of the evolutionary distance between the sequences; a clade is a group of organisms believed to have evolved from a common ancestor and it just represent a hypothesis about actual evolutionary history (NCBI). In our phylogenetic tree (cladogram) *Ureaplasma urealyticum* 23 (serovar 2) represent the closest internal node to our studied *Ureaplasma* strains, which means it possess common ancestors to those descendants.

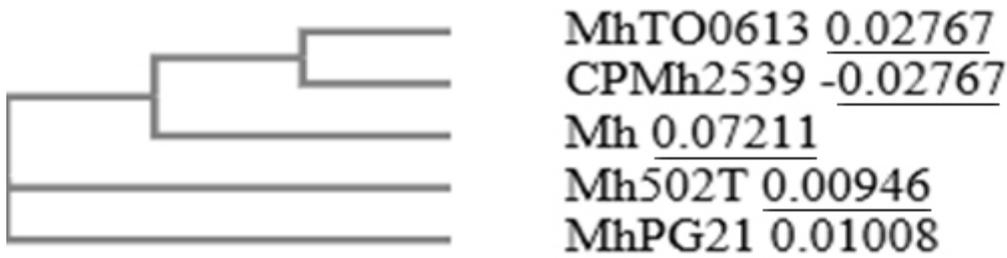
The DNA sequence (305 b.p) of *M. genitalium* of one case was confirmed by DNA sequencing, which resulted in 86% similarity with *M. genitalium* strain G-3716S. Figure 3.9 demonstrates the phylogenetic tree of the sequenced strain and sequences of other strains obtained from NCBI. Our phylogenetic tree (cladogram) below showed that *M. genitalium* G-3716S was the closest strain to our studied *M. genitalium* (Mg).



**Figure (3. 9): Phylogenetic tree of *Mycoplasma genitalium*. The tree is based on analysis of part of rRNA gene showing our *M. genitalium* (Mg) in comparison with different *M. genitalium* strains. The numbers underlined correspond to a sequence distance measure.**

One of the *M. hominis* positive results were confirmed by DNA sequencing, which resulted in 97% similarity with *M. hominis* strain MhT0613 16S ribosomal RNA gene, partial sequence of 238 bp sequence.

In addition, the phylogenetic tree is shown in figure 3.6 below. Where *M. hominis* (Mh) of the present study was closest to *Mycoplasma hominis* strain MhT0613.



**Figure (3.10):** Phylogenetic tree of *Mycoplasma hominis*. The tree is based on analysis of part of rRNA gene showing our *M. hominis* (Mh) in comparison with different *M. hominis* strains. The numbers underlined correspond to a sequence distance measure.

### 3.7 Conclusion

Interestingly, *M. hominis*, *U. urealyticum* and *M. genitalium* appeared to play an important role in infertility among males and females in the studied patients. Although *Mollicutes* infection was detected in all age groups, *Mollicutes* infections rate were the highest in the age range 40-50 years. *Mollicutes* were detected in both genders where males had higher *Mollicutes* infections than females, workers had the highest infection rate followed by employee and house wives. Smoking appears to influence two *Mollicutes* (*U. urealyticum*, *M. hominis*) infection rates.

### 3.8 Recommendations

Almost all of the patients with *Mollicutes* infection are not aware of their infections (in any of the studied *Mollicutes*) because of unavailability of detection technique in our region. Treatment of *Mollicutes* infection

becoming more and more difficult and limited due to abuse of antibiotic use that lead to antibiotic resistance and the nature of this *Mollicutes*, which lack cell wall making them unsusceptible to penicillin and cephalosporine and difficulty to culture these microorganisms in order to test their susceptibility to different antibiotics. It's strongly recommended to examine patients suffering from infertility for the presence of *Mollicutes* infection. Such examination may provide a better diagnosis and treatment of the infertility cases. It's recommended to carry out follow up of the patient treated for *Mollicutes* infection and detect resistance profile of *Mollicutes* by the suitable antibiotic test or genetic method.

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# Appendices

## Appendix A

An-Najah  
National University  
Health Faculty of medicine &  
Sciences  
IRB



جامعة النجاح  
الوطنية  
كلية الطب وعلوم الصحة  
لجنة أخلاقيات البحث العلمي

### RB Approval Letter

**Study Title:**

"Molecular determination of prevalence of certain genital bacterial infections among patients with infertility problems"

**Submitted by:**

Intesar Khaleel Ashour

**Supervisor:**

Dr. Motasem Almasri  
Dr. Ashraf Sawafta

**Date Reviewed:**

13<sup>th</sup> Nov. 2017

**Date Approved:**

25<sup>th</sup> Feb 2018

Your Study titled "Molecular determination of prevalence of certain genital bacterial infections among patients with infertility problems" with archived number (11) Feb 2018 was reviewed by An-Najah National University IRB committee and was approved on 25<sup>th</sup> Feb 2018.

Hasan Fitian, MD  
IRB Committee Chairman  
An-Najah National University



نابلس - ص ب 7 أو 707 || هاتف (970) (09) 2342902/4/7/8/14 || فاكس (970) (09) 2342910

Nablus - P.O Box: 7 or 707 | Tel (970) (09) 2342902/4/7/8/14 | Faximile (970) (09) 2342910 | E-mail : hgs@najah.edu

## Appendix B

### Infertility Questionnaire

The following questionnaire is designed to help the physician to evaluate your infertility. Please the appropriate answer. If you have any questions or additional comments, write them in the space below.

#### A. General Information

Date:

2. Name and number: \_\_\_\_\_

\_\_\_\_\_ Age: \_\_\_\_\_

3. Wife's/Partner's Name and number: \_\_\_\_\_

Age: \_\_\_\_\_

4. Occupation: \_\_\_\_\_

5. Wife's/Partner's Occupation: \_\_

#### B. Fertility History

1. what is the duration of your current marriage/relationship?

\_\_\_\_\_

2. How long have you been attempting to initiate a pregnancy?

\_\_\_\_\_

3. Have you been involved in any previous pregnancies in this relationship? Yes No
- 

4. Have you been involved in previous pregnancies in any other relationships? Yes No

5. Have you been previously evaluated or treated for infertility? Yes  
No If yes: When:

Has your wife/partner been evaluated for infertility? Yes No

6. Do you have any problems with erection? Yes No

c. Medical History

1. Do you have any medical problems? Yes No

If yes, what are they? \_\_\_\_\_

---

2. Do you take any medication? Yes No

d. Infections

1. Have you had any previous infections =- (If yes, please list dates and describe treatment below)
- 

e. Social History



جامعة النجاح الوطنية  
كلية الدراسات العليا

# التحديد الجزيئي لمدى انتشار بعض أنواع الإلتهابات البكتيرية في الجهاز البولي والتناسلي لدى المرضى الذين يعانون من مشكلة العقم

إعداد

إنتصار خليل حسني عاشور

إشراف

د. معتصم المصري

د. أشرف صوافطة

قدمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في العلوم  
الحياتية بكلية الدراسات العليا في جامعة النجاح الوطنية في نابلس، فلسطين.

2019م

ب

التحديد الجزيئي لمدى انتشار بعض أنواع الإلتهابات البكتيرية في الجهاز البولي والتناسلي  
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د. أشرف صوافطة

الملخص

**الخلفية العلمية:** تعد إلتهابات الجهاز التناسلي من أهم العوامل والأسباب التي تؤدي إلى مشاكل العقم حول العالم، والتي تؤثر علا كلا الجنسين، وتعد بكتيريا المفطورة البشرية، وبكتيريا الميورة الحالة لليوريا، والمايكوبلازما التناسلية عاملا في مشاكل الجهاز التناسلي، حيث يصعب تشخيصها وزراعتها ويعد تفاعل البوليمريز من أهم الطرق في الكشف عن وجود هذه البكتيريا

**الأهداف:** هدفت هذه الدراسة إلى الكشف عن مدى تواجد كل من بكتيريا المفطورة البشرية، وبكتيريا الميورة الحالة لليوريا، والمايكوبلازما التناسلية لدى المرضى الذين يعانون من مشاكل العقم بإستخدام تقنية تفاعل البوليمريز، في مركز الشنار في مدينة نابلس.

**الطريقة:** تضمنت الدراسة مئة وثلاث مرضى زاروا عيادة الشنار وشخصوا بالعقم خلال الفترة ما بين كانون الثاني وتشرين أول من سنة 2018. جمعت العينات (110 عينة) بإشراف مختص وتحت إشراف الطبيب. خضعت العينات للفحص الجزيئي باستخدام تقنية البوليمريز للكشف عن وجود بكتيريا المفطورة البشرية، وبكتيريا الميورة الحالة لليوريا، والمايكوبلازما التناسلية. وقد استخلص الحمض النووي من العينات المختلفة ثم أخضع لتفاعل البوليمريز، بالإضافة إلى عملية الكشف عن تسلسل القواعد النيتروجينية للحمض النووي المستخرج من بعض العينات التي كانت نتيجتها إيجابية في تفاعل البوليمريز، ومن ثم المعلومات الناتجة حلت بواسطة برنامج إحصائي.

**النتائج:** من بين مئة وعشر عينات خضعت للفحص 35 (31.8%) كانت نتائجها إيجابية لنوع واحد من البكتيريا المدروسة على الأقل، بحيث كانت نسبة بكتيريا الميورة الحالة لليوريا 11 (10%)، وكانت نسبة بكتيريا المفطورة البشرية 16 (14.6%)، بينما كانت نسبة المايكوبلازما التناسلية 8 (7.3%).

اختلف وجود الإلتهاب البكتيري لهذه البكتيريا عديمة الجدار بين عينات الجهاز التناسلي وعينات الجهاز البولي بحيث تواجد 18.9% منها في عينات الجهاز البولي (بول) وفي عينات المسحات المهبلية (31.3%) والسوائل المنوية (40.4%)، ومن الجدير بالذكر أن تواجد البكتيريا أنفة الذكر في السائل المنوي كان ذا دلالة إحصائية ( $P=0.023$ ).

اختلف توزيع البكتيريا المدروسة بين المرضى الذين يعانون من عقم أولي والمرضى الذين يعانون من عقم ثانوي، بحيث كانت نسبة تواجد جميع أنواع البكتيريا المدروسة عند المرضى الذين يعانون من عقم أولي أكبر من نسبتها عند المرضى الذين يعانون من عقم ثانوي.

شكل الذكور ما نسبته 66% من كلي عدد المرضى، وكانت نسبة الإصابة بالإلتهاب بكتيري للذكور (41.2%) أعلى منه للإناث (20%) وقد تواجدت بكتيريا المفطورة بنسبة أكبر لدى جميع الفئات العمرية بالمقارنة مع الميورة الحالة لليوريا والمايكوبلازما التناسلية، ولدى المرضى بمختلف أنواع المهن، بالإضافة إلى تواجدها بنسبة أكبر لدى جميع المرضى المدخنين.

أظهرت شجرة النشوء والتطور لبكتيريا الميورة الحالة لليوريا أن سلالتنا هذا البحث تنتمي إلى سلالتين قريبتين وتتحدران من نفس المنشأ، وقد كانت أقرب إلى سلالة *Ureaplasma urealyticum* 23 (serovar 2)، بالإضافة إلى ذلك أظهرت شجرة النشوء والتطور لكل من بكتيريا المفطورة البشرية وبكتيريا المايكوبلازما التناسلية، بحيث كانت عزلة المايكوبلازما أقرب إلى *Mycoplasma genitalium strain G-37*، وكانت عزلة المفطورة البشرية أقرب إلى سلالة *Mycoplasma hominis strain TO0613*.

**الاستنتاج:** الإلتهابات التناسلية البولية بسبب كل من المفطورة البشرية والميورة الحالة لليوريا  
والمايكوبلازما التناسلية تلعب دورا مهما كمسبب للعقم للمرضى بمختلف أعمارهم، وبغض  
النظر عن جنسهم ونوع وظيفتهم، لذا مزيد من الاهتمام والتركيز يجب أن يتبنى من مراكز  
العقم و المختصين، بحيث نوظف التقنيات الجزيئية في التشخيص للكشف عن هذه الأنواع من  
البكتيريا.