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Chlamydia trachomatis, Mycoplasma hominis, Mycoplasma genitalium, and *Ureaplasma urealyticum* in Patients with Sterile Pyuria in Gaza - Palestine

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بناءً على موافقة عمادة الدراسات العليا بالجامعة الإسلامية بغزة على تشكيل لجنة الحكم على أطروحة الباحث/ **فضل أحمد جبر نصار** لنيل درجة الماجستير في **كلية *العلوم*/ قسم العلوم الحياتية – أحياء دقيقة** وموضوعها:

Chlamydia trachomatis, Mycoplasma hominis, Mycoplasma genitalium, and Ureaplasma urealyticum in Patients with Sterile Pyuria in Gaza - Palestine

وبعد المناقشة العلنية التي تمت اليوم الثلاثاء 14 محرم 1429هـ، الموافق 2008/01/22م الساعة الواحدة ظهراً، اجتمعت لجنة الحكم على الأطروحة والمكونة من:

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وبعد المداولة أوصت اللجنة بمنح الباحث درجة الماجستير في **كلية *العلوم*/ قسم العلوم الحياتية-أحياء دقيقة**.

واللجنة إذ تمنحه هذه الدرجة فإنها توصيه بتقوى الله ولزوم طاعته وأن يسخر علمه في خدمة دينه ووطنه.

والله ولي التوفيق ،،،

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Signature	**Name**	**Date**
Fadel	*Fadel Ahmed Nassar*	*December, 2007*

Chlamydia trachomatis, Mycoplasma hominis, Mycoplasma genitalium, and *Ureaplasma urealyticum* in Patients with Sterile Pyuria in Gaza - Palestine

Abstract

Objectives. To determine the occurrence of *Chlamydia trachomatis, Mycoplasma hominis, Mycoplasma genitalium, and Ureaplasma urealyticum* in patients with sterile pyuria.

Design. Sterile pyuria urine samples collected during the period from February 2006 to April 2007 were tested by polymerase chain reaction (PCR) for the presence of *C. trachomatis, M. hominis, M. genitalium,* and *U. urealyticum* using specific primers for each species.

Samples. Urine samples were collected from 200 patients (96 male, 104 female) aged ≥ 18 years attending the genitourinary clinic at Al-Shifa hospital, Gaza.

Results. The PCR amplified DNA from 38 of the 200 urine samples (19%) from patients with sterile pyuria. *C. trachomatis* was detected in 20 samples (10%), *U. urealyticum* in 10 samples (5%), *M. hominis* in 6 samples (3%) and *M. genitalium* in 2 samples (1%). The difference in occurrence of *C. trachomatis* was statistically insignificant between males and females (P=0.509) but, it was significant (P=0.008) for *U. urealyticum* . *M. hominis* was detected only in samples collected from female patients. On the other hand, *M. genitalium* was detected only in men

Conclusion. PCR testing of sterile pyuria showed a significant number of *C. trachomatis*, *Mycoplasma* , and *Ureaplasma* infections. Consequently PCR is recommended for the detection of those microorganisms in the urine samples of sterile pyuria patients.

Key words

Sterile pyuria, Polymerase Chain Reaction, *Chlamydia trachomatis*, *Mycoplasma hominis, Mycoplasma genitalium, Ureaplasma urealyticum*, Gaza.

الكشف عن بكتيريا كلاميديا تراكوماتس و الميكوبلازما هومينيس و الميكوبلازما جينيتاليوم و اليوريابلازما يورياليتكم في عينات البول سالبة المزرعة البكتيرية.

الملخص العربي

تهدف هذه الدراسة إلي التعرف على نسبة تواجد بكتيريا كلاميديا تراكوماتس و الميكوبلازما هومينيس و الميكوبلازما جينيتاليوم و اليوريابلازما يوياليتكم في عينات بول سالبة المزرعة البكتيرية التي أخذت من أشخاص يعانون من أعراض مرضية في المسالك البولية والتناسلية حيث جمعت العينات في الفترة من بداية شهر فبراير 2006 حتى نهاية شهر أبريل 2007 من المرضى الذين يترددون على عيادة المسالك البولية و التناسلية في مستشفى الشفاء في غزة و شملت الدراسة 200 مريض (96 ذكر و 104 أنثى) وفحصت باستخدام تقنية سلسلة تفاعلات البلمرة (PCR) باستخدام بادئ للبلمرة خاص لكل نوع.

خلال الدراسة حصلنا على 38 عينة موجبة بواسطة فحص PCR ما نسبته 19% من العينات حيث وجدت الكلاميديا في 20 عينة أي بنسبة 10% و الميكوبلازما هومينيس في 6 عينات أي بنسبة 3% و الميكوبلازما جيناتيليوم في عينتين أي بنسبة 1% واليوريابلازما يورياليتكم في 10 عينات أي بنسبة 5%.

وقد أظهرت النتائج عدم وجود فروقات ذات دلالة احصائية بين نسبة اصابة الذكور و اصابة الاناث بالكلاميديا و وجدت الميكوبلازما هومينيس في 5.8 % من الأناث بينما لم توجد في الذكور ووجدت الميكوبلازما جيناتيليوم في 2.1 % من الذكور بينما لم توجد في الأناث و وجدت اليوريابلازما يورياليتكم في 8.7 % من الأناث بينما في 1% من الذكور. نستخلص من هذا البحث أن استخدام تقنية سلسلة تفاعلات البلمرة أثبت أن هناك وجود لهذه الأنواع من البكتيريا في عينات البول سالبة المزرعة البكتيرية وأن هذه التقنية ضرورية ومهمة في تشخيص هذه الأنواع من البكتيريا التي يصعب تشخيصها بطرق الزراعة التقليدية لذلك توصي الدراسة باستخدام هذه التقنية في مراكز الخدمات الصحية لدقة النتائج التي يتم الحصول عليها وسرعتها في التشخيص وذلك يساعد في اختيار العلاج المناسب.

Dedication

To my father's pure soul,

to my mother,

to my wife,

to my son,

Ahmed

and

to my daughters ,

Aya, Shahd, and Lana

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Finally, I want to say that my beloved family especially my mother and brothers, my wife, they always stand beside me and give me encouragement all time and for their never-ending love and support. I am so proud of my family.

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Abbreviations

bp	Base pair
CDC	Center for disease control and prevention
CDs	Constant domains
CF	Complement fixation
DIF	Direct immuno fluorescence
DNA	Deoxy ribonucleic acid
dNTps	Deoxy nucleotide triphosphates
EB	Elementary body
EIA	Enzyme immuno assay
ELISA	Enzyme-linked immunosorbent assay
HPF	High power field
Hsp	Heat shock proteins
Inc	Inclusion membrane protein
IUD	Intrauterine device
LCR	Ligase chain reaction
LGV	Lympho granuloma venereum
LPS	Lipopolysaccharide
$MgCl_2$	Magnesium chloride
MIF	Micro immuno fluorescence
MoPn	Mouse pneumonitis
MOMP	Major outer membrane
NGU	Non-gonococcal urethritis
nm	Nanometer
OD	Optical density
Omp	Outer membrane proteins
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pCT	Chlamydial cryptic plasmid

PID	Pelivic inflammatory disease
Pmps	Polymorphic outer membrane proteins
RB	Reticulate body
rpm	Round per minute
rRNA	Ribosomal RNA
STDs	Sexually transmitted diseases
TE	Tris-EDTA
UTI	Urinary tract infection
VDs	Variable domains
WBC	white blood cells
WHO	World health organization
µg	Microgram
µl	Microliter

Chapter 1

Introduction

Introduction

Sterile pyuria is a condition in which white blood cells (WBCs) are present in the urine (≥10/HPF) without bacteria growing in standard cultures [1].

Sterile pyuria is associated with a number of infective agents including viruses, fungi and atypical or fastidious organisms such as *Chlamydia trachomatis*, Mycoplasmas, and Ureaplasmas [2]. Mycoplasmas, Ureaplasmas and *C. trachomatis* are associated with various diseases of the genitourinary tract, but they are usually not detected by routine microbiological diagnosis [3]. Molecular genetic techniques such as polymerase chain reaction (PCR) are useful for the identification of microorganisms that are difficult to cultivate and for those that grow slowly [4].

C. trachomatis infection of the lower genital tract is one of the most prevalent sexually transmitted diseases (STDs) worldwide [5]. According to the World Health Organization (WHO), approximately 89 million people are newly infected with *C. trachomatis* infections annually worldwide [6]. *C. trachomatis* infection if not treated in an early stage can lead to severe sequelae, such as pelvic inflammatory disease (PID), ectopic pregnancy and tubal infertility [7].
However, 50-80% of infected men and women are asymptomatic [8]. This high number of unrecognized infected individuals provide the reservoir for spreading the infection to other men and women via sexual intercourse .

Several laboratory methods are used for the diagnosis of *C. trachomatis*, these include cytological tests for the detection of intracytoplasmic inclusion bodies, cell culture, Enzyme-linked immunosorbent assay (ELISA), Direct immuno-fluorescence (DIF) and DNA amplification via PCR [9].

PCR is more sensitive than cell culture, it has a high sensitivity and specificity when compared to other tests used for *C. trachomatis* diagnosis, such as direct IF and ELISA which may give false positive results [6].

PCR procedure to detect *C. trachomatis* targets a cryptic chlamydial plasmid (pCT) or the gene of the major outer membrane (MOMP) as templates for PCR amplification [10] .

Mycoplasmas and ureaplasmas, members of the family *Mycoplasmataceae* of the class Mollicutes, are widely distributed in humans, mammals, birds, reptiles, fish and other vertebrates as well as plants [11].

Up to now,13 species of *Mycoplasma* and 2 species of *Ureaplasma* have been isolated in humans [12,13]. Three of these species *Mycoplasma genitalium*, *Ureaplasma parvum* and *Ureaplasma urealyticum*, are thought to be associated with genitourinary infections [12,14].

M. genitalium was first isolated from men with urethritis, but studies that attempted to assess its association with disease were inhibited by the difficulty of growing the organism in culture. Currently the microorganism is detected mainly by PCR [15,16] .

M. hominis has been associated with bacterial vaginosis, PID, post-partum fever and post-abortal fever, as well as a number of gynecological infections [12, 17,18]
Detection of Mycoplasmas employs the 16s rRNA gene for PCR amplification [19].

There is an increasing evidence that *U. urealyticum* is associated with a range of human disorders including non-gonococcal urethritis (NGU) [20]. Previously *U. urealyticum* has been differentiated into biovars 1 and 2. In 1998 *U. urealyticum* biovars 1 and 2 were classified into *U. urealyticum* and *U. parvum,* respectively [21] .

At present, the main method of detecting Ureaplasmas is by culture, but the organism is difficult to isolate and requires special culture media [21] .

Detection of ureaplasmas by PCR employs urease gene as template for amplification [22] .

The goal of the present study was to detect *C. trachomatis*, *M. genitalium*, *M. hominis* and *U. urealyticum* in urine specimens of 200 patients with sterile pyuria by PCR. It is worth mentioning that no previous studies have been conducted on this topic in Gaza strip. This study is important in identifying the etiological agents for many of sterile pyuria cases and would be helpful in choosing the correct treatment.

Objectives of the present study were to :

1. Assess the occurrence of *C. trachomatis,* Mycoplasmas and Ureaplasmas in urine specimens of patients with sterile pyuria.
2. Introduce a rapid and sensitive technique for identifying *C. trachomatis*, Mycoplasmas and ureaplasmas in culture-negative urine specimens.

Chapter 2

Literature Review

2.1. Sterile pyuria

Sterile pyuria is a condition in which WBCs are present in the urine (≥10/HPF) without bacterial growth in a standard culture [1].

The finding of pyuria without bacteriuria can be a diagnostic challenge and warrants further investigation. Sterile pyuria is associated with a number of infectious and non infectious causes as illustrated in **Table 2.1.**

Table 2.1. Causes of sterile pyuria [23].

Infective causes	Non-infective causes
• **Viruses** • **Fungi** • **Atypical or fastidious organisms including:** ▪ *C. trachomatis* ▪ Mycoplasmas ▪ Ureaplasmas ▪ *Mycobacterium* spp ▪ Anaerobic bacteria	• **Systemic and localized diseases:** ▪ Malignant hypertension ▪ Systemic inflammatory diseases • **Structural and physiological abnormalities of the urogenital tract:** ▪ Stones ▪ Polycystic kidneys ▪ Acute interstitial nephritis • **Certain drug treatments**

There are other causes of sterile pyuria including, recent antibiotic treatment, inadequately treated urinary tract infection (UTI), false negative culture due to contamination with antiseptic and contamination of sample with vaginal leucocytes [2].

2.2. *Chlamydia trachomatis*

2.2.1. Taxonomy

C. trachomatis is one of four bacterial species in the genus *Chlamydia*, family *Chlamydiaceae*, class Chlamydiae, phylum Chlamydiae, domain Bacteria. The other three species are *Chlamydia psittaci*, *Chlamydia pneumoniae*, and *Chlamydia pecorum* [24].

The species differ in their host cell tropism, but have similar cell structure and share certain biological properties in the course of their intracellular existence. *C. trachomatis* and *C. pneumoniae* are the two chlamydial species pathogenic to humans, whereas the other species occur mainly in animals and birds [25].

C. trachomatis has been divided into 18 different sub-groups called serotypes or serovars, according to the *Omp1* gene that encode the MOMP. *C. trachomatis* Serovars can be divided into three biovars: trachoma, lympho granuloma venereum (LGV) and mouse pneumonitis (MoPn). The trachoma biovar includes serotypes A, B, Ba, C, D, Da, E, F, G, H, I, Ia, J, and K. The LGV biovar has the serotypes L1, L2, L2a, and L3, but MoPn is a single serovar type [26].

2.2.2. Growth cycle of *Chlamydia*

C. trachomatis is a pleomorphic, non-motile, gram negative bacteria, about 0.2 - 1.5 µm in length. This obligate intracellular bacterium depends on host cell metabolites and shows a unique growth cycle characterized by formation of reticulate body (RB) which is the intracellular replicating form and, elementary body (EB) which is the extracellular infectious form. Infection of a cell is initiated by EBs adhering to the host cell surface and gaining entry into the cell by endocytosis [27].

When inside the cell, the *chlamydia* remain within an enlarging intracellular vacuole, a characteristic inclusion, avoiding lysosomal fusion and subsequent destruction. During the first few hours, EBs differentiate into metabolically active RBs. By using the host cell's energy and nutrient resources, RBs begin to multiply by binary fission. After multiple rounds of division, RBs start to

transform back to EBs. Finally, by exocytosis or host cell lysis, the infectious EBs are released into the cytoplasm, to initiate new cycles in new host cells [28]. The growth cycle of *Chlamydia* is presented in Figure 2.1.

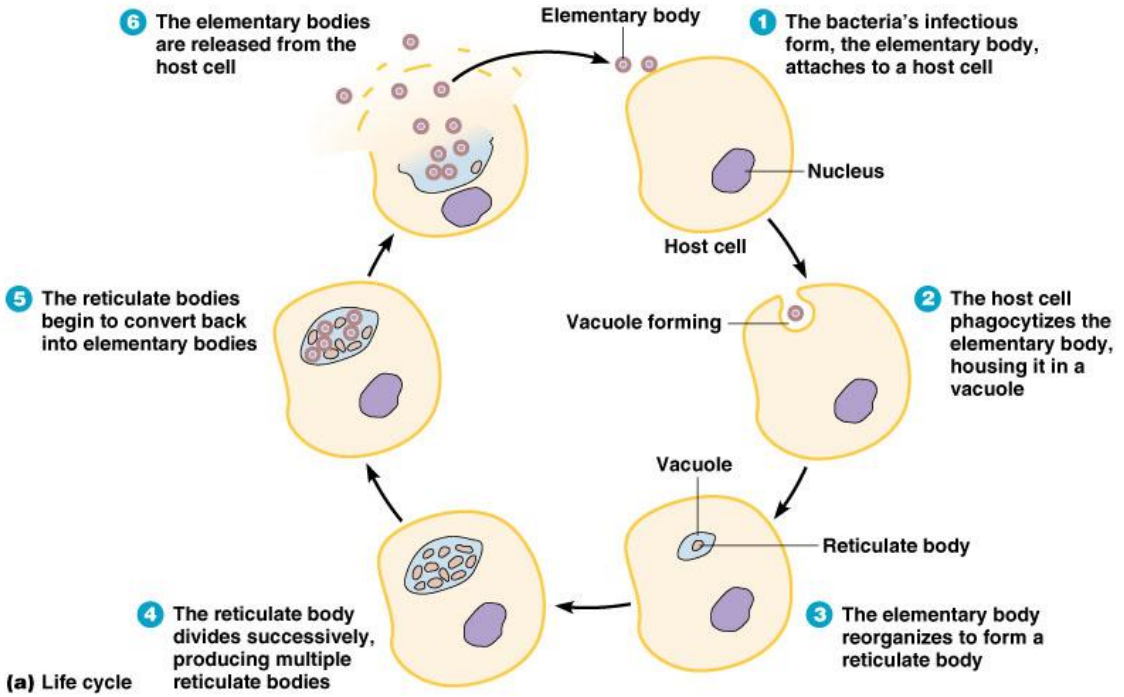

Figure 2.1. Growth cycle of *Chlamydia* [28]

2.2.3. Structural characteristics of *Chlamydia* cell

Like all gram-negative bacteria, chlamydial cells appear to be surrounded by a double membrane. However, unlike other gram-negative bacteria, Chlamydiae do not have a peptidoglycan layer in the space between the two membranes, although the genes for peptidoglycan synthesis are present in the chlamydial genome. The outer cell membrane consists of lipopolysaccharide (LPS) and outer membrane proteins (Omps), the single predominant protein being the MOMP of 38 to 42 KDa, comprising about 60% of Omps. The MOMP of *C. trachomatis* contains serovar, subspecies and species specific epitopes that can be defined by monoclonal antibodies. The MOMP of *C. pneumoniae* is more homogenous and less immunogenic than that of other chlamydiae. Other outer membrane proteins, such as the cysteine-rich 60 KDa protein (Omp2) and the small cysteine-rich 12-15 KDa protein (Omp3), are present in

smaller amounts. In addition, proteins called polymorphic outer membrane proteins (Pmps) have been localized in the outer membrane [24].

Chlamydial LPS, which is present in the outer membrane of both EBs and RBs is an endotoxin generally found in gram-negative bacteria. Chlamydial LPS is structurally similar to the rough form of LPS found in enteric bacteria. It has both chlamydial genus-specific antigens and antigens shared by other members of the *Enterobacteriaceae*. However, the structure of chlamydial LPS is not identical in the different species, and the endotoxin activity of chlamydial LPS is much lower than that of enterobacterial LPS. The proinflammatory cytokine response to *C. trachomatis* at the invasion phase is suggested to be mediated by LPS [24].

Several heat shock proteins (Hsp) have been found in chlamydial cell walls. The genes encoding Hsp10, Hsp60 and Hsp70 have been cloned and sequenced. All three Hsps can be found in the outer membrane complex of both EBs and RBs. These genes are continuously expressed throughout the developmental cycle. The Hsps are highly conserved within chlamydial species, including *C. pneumoniae*. Especially chlamydial Hsp 60, but also Hsp 70 and Hsp 10 have been implicated as important agents in the immunopathology of chlamydial infection. Chlamydiae are obligate intracellular bacteria that occupy a non-acidified vacuole (inclusion) during their entire developmental cycle. These bacteria produce a set of proteins (Inc proteins) that localize to the surface of the inclusion within infected cells. Incs proteins demonstrated and named IncA, IncB to IncG, have been characterized [24].

2.2.4. Genome of *C. trachomatis*

C. trachomatis contains a single chromosome of approximately 1,043,000 bp. The first gene to be analyzed was that coding for MOMP, which was designated *omp1*. *Omp1* was sequenced from *C. trachomatis* L2 strain. Comparison of this gene with that from *C. trachomatis* serovars that were subsequently sequenced revealed extensive *omp1* sequence variation. Most of the polymorphisms were localized to four 40- to 90-bp-long variable domains (VDs), regularly distributed among the relatively conserved constant domains (CDs). Serovar specificity of

C. trachomatis appears to be determined by particular residues within VD1, VD2, and VD4. It was found that for each serovar, the variable domain coding for the most hydrophilic and charged amino acid sequence contained the serovar-specific epitope. Later studies, however, indicated that *omp1* of a given serovar can incorporate multiple distinct serovar-specific epitopes, each of which may be found in a different VD. Collectively, these findings indicate that *omp1* gene product, MOMP, spans the outer membrane of the cell envelop and presents immunologically important epitopes, coded for by one or more VDs, at the cell surface. Heterogeneity in *omp1* constant domains between urogenital and trachoma isolates of the same Ba and C serovars has been identified. The altered nucleotide sequences produce changes in the amino acid sequences of MOMP and could potentially play a role in determining the tissue tropism or virulence of the organism [29].

In addition to the chromosome, chlamydiae commonly possess an extrachromosomal genetic element. The 7.4-kb plasmid pCT was first isolated from a *C. trachomatis* L2 strain. The plasmid is very highly conserved, with less than 1% variation in nucleotide sequence. Because of this sequence conservation, and because of maintenance of this superfluous extrachromosomal DNA, it was suggested that the plasmid might be essential for chlamydial growth or replication. However, several naturally occurring *C. trachomatis* strains lacking the plasmid have since been isolated, including an L2 cultured from a patient with proctitis, a genotype B variant cultured from a male urethral swab, and a serovar E cultured from a male urethral swab. Such strains are thought to be rare, and no plasmid-free ocular isolates have been reported to date. Estimates of the mean number of plasmids per elementary body include, 7 to 10 (*C. trachomatis* L2). This estimate and the possibility of chlamydial infection without the presence of plasmid DNA both have implications for determining the likely sensitivity of some laboratory assays for *C. trachomatis* [29].

2.2.5. Complications of Chlamydial infection

C. trachomatis is frequently implicated in sexually transmitted diseases such as cervicitis, NGU and PID in women and NGU, epididymitis and proctitis in men. If untreated, chlamydial infections can progress to serious reproductive and other health problems with both short-term and long-term consequences. Like the disease itself, the damage that chlamydia causes is often "silent" [30].

In women, untreated infection can spread into the uterus or fallopian tubes and cause PID. This happens in up to 40 percent of women with untreated chlamydia. PID can cause permanent damage to the fallopian tubes, uterus, and surrounding tissues. The damage can lead to chronic pelvic pain, infertility, and fatal ectopic pregnancy. Women infected with chlamydia are up to five times more likely to become infected with HIV, if exposed [31,32].

In pregnant woman, there is some evidence that untreated chlamydial infections can lead to premature delivery. Babies who are born to infected mothers can get chlamydial infections in their eyes and respiratory tracts. Chlamydia is a leading cause of pneumonia and conjunctivitis (pink eye) in newborns [33].

Complications among men are rare. Infection sometimes spreads to the epididymis, causing pain, fever, and, rarely, sterility [30].

2.2.6. Symptoms of Chlamydial infection

Chlamydial infection is known as a "silent" disease because about three quarters of infected women and about half of infected men have no symptoms. If symptoms do occur, they usually appear within 1 to 3 weeks after exposure [30].

In women, the bacteria initially infect the cervix and the urethra. Women who have symptoms might have an abnormal vaginal discharge or a burning sensation when urinating. When the infection spreads from the cervix to the fallopian tubes, some women still have no signs or symptoms; others have lower abdominal pain, low back pain, nausea, fever, pain during intercourse, or bleeding between menstrual periods [8].

Men with signs or symptoms might have a discharge from their penis or a burning sensation when urinating. Men might also have burning and itching

around the opening of the penis. Pain and swelling in the testicles are uncommon [8].

2.2.7. Prevalence of Clamydial infection

C. tracomatis infection is one of the most prevalent STDs in the world. According to WHO, approximately 89 million new *C. trachomatis* infections occur annually worldwide [6].

Studies in different parts of the world had shown high prevalence rates of chlamydial infections e.g., studies made by the Center for Disease Control (CDC)- USA have shown that three to four million new cases of chlamydial infections are detected annually in the United States. They estimated the cost of such infections to be about 1.4 billion dollars/year. Additionally, studies have shown that prevalence rate is higher among sexually active women and young adults [34].

2.2.8. Treatment of Chlamydial infection

Chlamydia can be easily treated and cured with antibiotics. A single dose of azithromycin or a week of doxycycline are the most commonly used treatments. HIV-positive persons with chlamydia should receive the same treatment as those who are HIV negative [35].

2.2.9. Diagnosis of Chlamydial infection

Culture has been the golden standard in chlamydial diagnosis. Since *Chlamydia* is an intracellular organism and requires careful specimen transportation, a high level technical expertise and time-consuming incubation (3 to 7 days) are required. Culture has a specificity that approaches 100%, but it is relatively insensitive being only 50%-80% compared to DNA amplification tests [36]. Since culture detects only viable infectious chlamydial EBs and has minimal potential for contamination, it has still remained the standard for medico-legal purposes [36,37].

The present antigen detection methods are based on the demonstration of genus-specific chlamydial LPS and cannot differentiate between chlamydial

species [36]. For antigen detection, the presence of viable *Chlamydia* is not required and it may therefore be useful in the diagnosis of chronic chlamydial infections, if sufficient amounts of antigens are present [26]. EIA designed for *C. trachomatis* can also be used for detection of the *C. pneumoniae* antigen, since the capture antibody in *Chlamydia* EIA kits is the genus-specific LPS. Antigen detection by EIA, however, is considered more sensitive than culture in chronic *C. trachomatis* infections [26]. EIA also known as (ELISA) assays, are designed to detect antigens or antibodies by producing an enzyme-triggered color change.

The direct fluorescence antibody technique (DFA) adds the advantage of Chlamydia specific antibody staining to the direct examination of clinical specimens. Although the DFA staining method is rapid, the microscopic evaluation of each specimen is laborious and requires highly trained and experienced personnel [36].

Serology methods for diagnosis of chlamydial infections including complement fixation (CF) and Micro-immuno fluorescence test (MIF) are also in use. In the CF test, the target of the antibodies is the genus specific LPS; thus it is not possible to determine the species-specific antibody response with this test. Although the CF test lacks specificity, it is technically much easier than the MIF test and has objective end-point. The treatment with antibiotics may delay or diminish the production or CF antibodies decreasing the sensitivity of the test [36].

The MIF test measures antibodies against chlamydial EB antigen, and the test is able to differentiate both species- and serotype-specific antibodies. It is able to measure separately antibodies in the IgA, IgM and IgG classes, and it is therefore suitable for distinguishing recent from past infections as well as primary from reinfections. If performed and read properly, this test provides a sensitive and specific method for the laboratory diagnosis of chlamydial infections. In the case of acute chlamydial infection, the criterion for serological diagnosis is a fourfold rise in the IgG or IgA titer or a single IgM titer of ≥16 for both *C. pneumoniae* and *C. trachomatis* [38].

Nucleic acid hybridization techniques were successfully used to detect infected cells from tissue culture, ocular swabs, and cervical smears. Unfortunately, the sensitivity of such techniques was thought to be lower than that of culture. Commercial applications of the technique, however, incorporated significant improvements [29].

Two nucleic acid hybridization assays are FDA-cleared to detect *C. trachomatis*: the Gen-probe PACE®2 and the Digene Hybrid Capture® 2 assays. In the Gen–probe hybridization assays, a DNA probe that is complementary to a specific sequence of *C. trachomatis* rRNA hybridizes with any complementary rRNA that is present in the specimen. A competitive probe version of PACE 2 assay is commercially available. In this version, the test is repeated on initially positive specimens with and without adding an unlabeled probe. The unlabeled probe competitively inhibits binding of the labeled probe; a reduction in signal when the assay is performed with the unlabeled probe is interpreted as verification of the initial positive test result [38].

RNA hybridization probes in the Digene assay are specific for DNA sequences *of C. trachomatis* including both genomic DNA and cryptic plasmid DNA. Technical requirements and expertise are necessary for performing nucleic acid hybridization tests. One of the advantages of the nucleic acid hybridization tests is the ability to store and transport specimens for few days without refrigeration before receipt and testing by laboratory [38].

The development of tests based on the nucleic acid amplification technology has been the most important advances in the field of chlamydial diagnosis. Since all nucleic acid amplification technologies detect nucleic acid targets, they do not depend on the viability of the organism. The fact that nucleic acid amplification is exquisitely sensitive and highly specific offers the opportunity to use also non-invasive sampling techniques. Nucleic acid amplification tests have been used to detect *C, trachomatis* in first-void urine specimens and vaginal swabs and *C. pneumoniae* in sputum, circulating WBCs and tissues. The most widely known method of DNA amplification technology is PCR. The specificity of the PCR method compared to culture is 95-100% for both *C. pneumoniae* and *C. trachomatis* [24]. Due to the inhibitory factors present in

specimens, the sensitivity of PCR has been variable. However, it has been estimated that PCR, in general, is at least 25% more sensitive than culture. LCR is another commercially available nucleic acid amplification technology used for the diagnosis of *C. trachomatis*. Recently, a quantitative real-time PCR technique has also been developed [24].

A number of different nucleic acid sequences have been used as targets in PCRs for detection of *C. trachomatis*. These include the chlamydial plasmid pCT, *omp1*, coding for MOMP, the gene coding for 16s rRNA, and *omp2,* coding for OmcB. With the exception of pCT, all of these targets are sequences found on the *C. trachomatis* chromosome, which include two complete rRNA operons and single copies of *omp1* and *omp2* [29].

A commercial PCR kit, (Maxim Biotech Inc, USA) targets a 364-bp sequence within pCT was used in the present study. Roche Diagnostic produces semi-and full automated Amplicor system known as the cobas Aamplicor, targeting a pCT specific sequence. For urogenital swabs and urine, the two formats appeared to have comparable sensitivity and specificity [39].

2.2.10. Detection of *C. trachomatis* by PCR

Mahoney *et al.* (1993) in Canada, have shown that PCR was more sensitive than culture or EIA for detecting *C. trachomatis* in genitourinary tract specimens when various DNA targets were used for amplification, including the cryptic plasmid, (MOMP), or rRNA genes. Results obtained with both purified chlamydial and plasmid DNA have demonstrated that plasmid-based PCR are 10 to 1,000 times more sensitive than chromosome based assays. Plasmid-based assays detected 38 of 38 confirmed Chlamydiazyme positive specimens, whereas the assays with the MOMP and rRNA primers detected 36 of 38 and 29 of 38 confirmed Chlamydiazyme positive specimens, respectively [10].

Jaschek *et al.* (1993) in USA, have used MOMP-based PCR to detect *C. trachomatis* in male urine specimens, in comparison with uretheral culture; a total of 530 male urine specimens were collected from 322 symptomatic and

208 asymptomatic men attending sexually transmitted diseases (STDs) clinics in Baltimore. Fifty two specimens were PCR positive and culture positive, 449 specimens were negative by both tests, 25 specimens were positive by PCR and negative by culture, and 4 specimens were positive by culture and negative by PCR. As compared to culture, the sensitivity of the PCR was 92.8% and its specificity was 94.7**%** [40].

Gaydos *et al.* (1998) in the USA, have conducted a large survey of women in U.S. military recruits. Urine specimens from 13,204 new female U.S. army recruits from 50 states were screened by LCR for *C. trachomatis* infection, information on potential risk factors was obtained by a questionnaire.
Results of their study demonstrated that the overall prevalence of chlamydial infection was 9.2%, with a peak of 12.2% among the 17-year-old recruits. A screening program for subjects 25 years of age or less would have identified 95.3% of infected women. The authors concluded that the prevalence of chlamydial infection is high among female military recruits. A control program that screens female recruits who are 25 years old or younger with urine DNA-amplification assays has the potential to reduce infection, transmission, and the sequelae of chlamydial infection [41].

Jayanti *et al.* (2001) in India, have employed a primer pair specific to MOMP gene common to all serotypes of *C. trachomatis*, and the study concluded that PCR technique could be used for better diagnosis of *C. trachomatis* infection in comparison to the other techniques [42].

Basarab *et al.* (2002) in the UK, have tested 71 sterile pyuria samples from males aged 18-30 years by (EIA) and if reactive, by IF. Subsequently samples were studied by PCR to assess the presence of *C. trachomatis* in these samples. Nine samples were EIA/IF-positive and PCR-positive, three samples positive by EIA/IF were PCR- negative, and 15 samples negative by EIA were PCR- positive. The authors concluded that EIA/IF are less sensitive than PCR [43]

Awwad *et al.* (2003) in Jordan, have investigated the prevalence of *C. trachomatis* infection among 230 patients, 130 with signs or symptoms associated with urethritis, and 100 asymptomatic patients, attending the Jordan University Hospital, Urology clinic. Routine urine examination and the leukocyte esterase test were done for each patient. *C. trachomatis* infection was detected using first-void urine specimens and a cryptic plasmid-based PCR technique specific for *C. trachomatis*. The prevalence of chlamydial infection was 4.6% among symptomatic patients with urethritis. The difference in prevalence was statistically insignificant between males and females, as well as in relation to their marital status. Two-thirds of the Chlamydia-positive patients also had urine positive for leukocyte esterase. The low prevalence of chlamydial infection in association with urethritis among Jordanian patients might be due to the conservative behavior of the Jordanian society towards free sexuality [44]**.**

George *et al.* (2003) in India, have conducted a study to evaluate the diagnostic efficiency and feasibility of PCR assays using genital and urine specimens from men and women in India. Genital swabs and urine specimens collected from men and women attending STDs clinic, were tested by culture and a plasmid based PCR. Culture was positive in 27 (18.9%) patients. PCR gave positive results for 46 (32.2%) cases using genital specimens, and the positivity rate in urine was 25.2%. After the discordant results between culture and PCR were excluded by using MOMP PCR, the overall sensitivity, specificity, and positive and negative predictive values for plasmid PCR in genital specimens were 100%, 98%, 95.7%, and 100%, respectively, corresponding values for urine PCR were 81.8%, 100%, 100%, and 92.5%, respectively. The prevalence of confirmed *C. trachomatis* infection was 30. 8% (44 out of 143) in that STD population [45].

Bamberger *et al.* (2003) in "Israel", have examined the prevalence of *C. trachomatis* infection in urine specimens of 708 Israeli female soldiers by PCR (Roche Amplicor, Branching, NJ), and the prevalence was 3.2% (23 of 708 were positive). An important limitation of this study was that the sample

population might not be entirely representative of the entire population of female soldiers in Israel Defense Forces. In addition over-sampling those most concerned with their health (i.e., "the worried well") might partially explain the lower than expected prevalence rate [46].

Klavs *et al.* (2004) in Slovenia, have estimated the prevalence and risk factors for genital *C. trachomatis* infection in Slovenian adults aged 18-49 years. Respondents were invited to provide urine specimens for testing for *C. trachomatis*. Urine specimens were frozen at -20° C. The Amplicor PCR test (Roche Diagnostic) for *C. trachomatis* was performed on pool sizes of 5 urine samples. Internal control was used to identify inhibitory specimens, and specimens from reactive pools were retested individually. The study demonstrated a relatively high prevalence of genital *C. trachomatis* infection among the 18-24 year old Slovenians. The presence of relatively low risk sexual behavior and low reported incidence rates of Chlamydia infection, suggest serious gaps in the diagnosis and treatment of the condition. The results recommended the introduction of Chlamydia screening in Slovenia [47].

Fallah *et al.* (2005) in Iran, have conducted a study for detection of *C. trachomatis* from urine specimens by PCR in Iranian women with cervicitis. The result of this study indicated that PCR technique is a useful method for detecting *C. trachomatis* in urine. A total of 122 consecutive women with cervicitis who attended Obstetric & Gynecology Clinic of Shoosh, Tehran-Iran were involved in the study. Results of the study showed that DNA specimens were extracted only in 94 specimens from 122 collected urine specimens, and 28 specimens were lost. Fourteen of the 94 specimens (15%) were positive by PCR using specific primers for MOMP and cryptic plasmid. The result of PCR by MOMP primers and cryptic plasmid were the same. The highest *C. trachomatis* cervical infection frequency was found in women with 28 to 38 years old group, elementary education level group, and users of intrauterine device (IUD) for contraception [48].

2.3. Mycoplasmas

2.3.1. Overview

Mycoplasmas (members of the class Mollicutes) are specific and unique species of bacteria and are considered the smallest free living organisms known on the planet [49].

The primary differences between Mycoplasmas and other bacteria is that bacteria have a solid cell-wall structure and they can usually grow in simple culture media. Mycoplasmas however do not have a cell wall and like a tiny jelly fish with a pliable membrane, can take on many different shapes which make them difficult to identify. Mycoplasmas can be very hard to culture in the laboratory [49].

Mycoplasmas have very small amount of DNA, usually require cholesterol for growth and membrane function and are filterable through the usual bacteriological filters [50].

The accepted name was chosen because Mycoplasmas were observed to have a fungi like structure, hence "Myco" and it also had a flowing plasma-like structure without cell wall-hence 'plasma' [49,50].

The first human strain was isolated in 1932 from an abscessed wound. The first connection between Mycoplasmas and rheumatoid diseases was made in 1939 by Drs. Swift and Brown. Unfortunately Mycoplasmas did not become part of medical school curriculum until late 1950 when one specific strain was identified and proven to be a cause of atypical pneumonia and named *Mycoplasma pneumoniae* [51,52]. The association between immunodeficiency and autoimmune disorders with mycoplasmas was first reported in the mid 1970s in patients with primary hypogammaglobulinemia (an autoimmune disease) and infection with four species of mycoplasma that had localized in joint tissue. Since that time, scientific testing methodologies have made critical technological progress and along with it, more mycoplasma species have been identified and recorded in animals, humans and even plants [51,52].

2.3.2. Common human Mycoplasma pathogens

Mycoplasma pathogens have been discovered in the urogenital tract of patients suffering from PID, urethritis, and other urinary tract diseases [53]. They have been discovered in the heart tissues and fluid of patients suffering from carditis, pericarditis, tachycardia, hemolytic anemia, and other coronary heart diseases [51,54,55]. They have been found in the cerebrospinal fluid of patients with meningitis and encephalitis, seizures, Alzheimer's and other central nervous system infections, diseases and disorders [56-58]. They have even been found regularly in the bone marrow of children with leukemia [59-61]. It is amazing that one single tiny bacterium can be the cause of so many seemingly unrelated diseases in humans. But as with all mycoplasma species, the disease is directly related to where the mycoplasma resides in the body and which cells in the body it attaches to or invades. Up to now, 13 species of Mycoplasmas have been isolated in human, the main ones are shown in **Table 2.2 .** [62-66]

Table 2.2. Common human Mycoplasma pathogens and associated diseases.

Pathogen	Implicated Diseases
Mycoplasma genitalium	Arthritis, chronic NGU, PID, other urogenital infections, infertility.
Mycoplasma fermentans	Arthritis, Gulf War Syndrome, Fibromyalgia, Chronic Fatigue Syndrome, Lupus.
Mycoplasma salivarium	Arthritis, Eye and ear infections, gingivitis.
Mycoplasma hominis	PID, infertility, NGU, vaginitis, cervicitis,amnionitis.
Mycoplasma pneumoniae	Pneumonia, asthma, upper and lower respiratory diseases, heart diseases.
M. incognitos and M. penetrans	urogenital infections, Autoimmune disorders.
Mycoplasma pirum	Urogenital infections and diseases .
Mycoplasma faucium, M. lipophilum and M. buccale	Diseases of the gingival crevices and respiratory tract.

2.3.3. Interaction of Mycoplasmas in the body

Mycoplasmas are able to hide inside the cells of the host (patient) or to attach to the outside of host cells. Whether they live inside or outside the host cell, they depend on host cells for nutrients such as cholesterol, amino acids, etc. They compete with the host cells for these nutrients which can interfere with host cell function without killing the host cell [50]. Mycoplasmas have very little DNA of its own, but is capable of using DNA from a host cell. When a mycoplasma takes over the DNA of the host cell, anything can happen - including causing that cell to malfunction in many different ways and/or die, or can cause DNA mutation of the host cell [50]. Mycoplasmas attach to host cells with a tiny arm coated in protein which attaches to the protein coating of host cells. For this reason, antibiotics like tetracycline, which are classified as "protein synthesis inhibitors" are often used against mycoplasma infections. While these antibiotics may block this protein attachment and very slowly starve it from the nutrients it needs from host cells to thrive and replicate, it still takes a healthy immune system to actually kill the mycoplasma for good [67]. Mycoplasmas are highly adaptable to changing environments and can move anywhere in the body, attaching to or invading virtually any type of cell in the body. The mycoplasma adhesion proteins are very similar to human proteins. Once adhered to the host cell, the mycoplasma can completely mimic or copy the protein of the host cell. This can cause the immune system to begin attacking the body's own cells. Mycoplasma can also attach to or invade immune system cells, like the very phagocytes (natural killer cells) that are supposed to kill them. Inside these phagocytes, they can be carried to new locations of inflammation or disease-hidden away like a spy who has infiltrated the defending army [67].

When a mycoplasma attaches to a host cell, it generates and releases hydrogen peroxide and superoxide radicals which cause oxidative stress and damage to the surrounding tissues [50].

2.3.4. Treatment of Mycoplasmal infection

In immunodeficient patients it can be very difficult to treat these mycoplasma infections with appropriate broad spectrum antibiotics which are

immunosuppressive themselves. Although the tetracycline and erythromycin types of antibiotics are effective for some mycoplasmal infections, *M. fermentans*, *M. hominis* and *M. pirum* strains are usually resistant to erythromycin, and tetracycline-resistant strains of *M. hominis* and *U. urealyticum* have been reported. However, these antibiotics have a very limited ability to directly kill these mycoplasmas, and their efficacy eventually depends on an intact host immune system to eliminate the mycoplasmas. These types of protein inhibiting antibiotics will stop the protein adhesion of the mycoplasma to host cells but won't directly kill the mycoplasma itself [68,69].

With an already weakened immune system, many patients lack the ability to mount a strong antibody response against these pathogens to kill them effectively. Oflavoids and biotin are necessary and helpful when recovering from a mycoplasmal infection. Fibromyalgia and other mycoplasma infections need long term antibiotic therapy. Regardless of how many physicians and rheumatologists are treating their arthritis, the more popular conventional protocols involve rotating multiple 6 week cycles of Minocycline or Doxycycline (200-300 mg/day), Ciprofloxacin (1,500 mg/day), Azithromycin (250-500 mg/day, and/or Clarithromycin (750-1,000 mg/day) among others [69,70]. Sometimes the side effects of these strong antibiotics can be as bad as the symptoms of the diseases they are treating since a minimum of 6 months and up to two years of antibiotic therapy may be required. Many doctors now believe that antibiotics should not be used solely or exclusively to treat mycoplasmal infections, without addressing rebuilding the immune system which is imperative for a complete recovery and eradication of infection. Others are using more natural antibiotics found in plants which can be as effective or more effective with fewer side effects or negative impact on the body. These include olive leaf extract products, urva ursi, and Neem leaf or seed extracts [68,69].

2.3.5. Diagnosis of Mycoplasmas

Testing for mycoplasmas is much harder and more complicated than testing for all other bacteria, which is one of the main reasons conventional medical practitioners misdiagnose or miss these types of infections [68,69]. The most

reliable testing method is PCR. Even performing a PCR lab test on a standard whole blood sample may not find the mycoplasma, simply because the mycoplasma may be residing in other fluids and tissues in the body and not the blood (i.e.; the fluid in the joints, in the spinal fluid, or in any tissue cell like heart, liver, pancreas, endocrine organs, etc.) [68,69]. PCR test is generally performed by specific *Mycoplasma* species primers and employs the 16s rRNA gene for amplification.

2.4. Ureaplasmas

2.4.1. Overview

Important mollicutes known to cause disease in human also include members of the genus, *Ureaplasma*. The *Ureaplasma* species are distinguished by their ability to hydrolyze urea to carbon dioxide and ammonia by the action of the enzyme urease [70].

In ureaplasma 14 serotypes have been established by various serological methods [71,72]. The serotype standard strains can be divided into two groups or biovars, one biovar includes serotype standard strains 1,3,6 and 14 and is designated biovar 1. The other biovar 2 or T960, is the name of the type strain of *U. urealyticum*. Biovar 1 has been called "parvo" because the genomes of these four serotype standard strains are much smaller than these of the serotype standard strains of biovar 2 [73].

The importance of ureaplasmas is obscured by the many asymptomatic persons from whom ureaplasmas can be isolated from urogenital specimens. However, male NGU [74,75] and chorioamnionitis may be caused by *U. urealyticum* [76,77]. Patients suffering from clinical chorionamnionitis may deliver prematurely. *U. urealyticum* is isolated more often from preterm infants, stillborn fetuses, and spontaneously aborted fetuses than from fully developed neonates and fetuses from induced abortions.

U. urealyticum may cause respiratory distress syndrome, pneumonia, and meningitis [78].

2.4.2. Diagnosis of Ureaplasmas

The prevalence of clinical disease associated with ureaplasmas is probably underestimated due to the limitation of laboratory diagnoses. Ureaplasmas are fastidious organisms requiring vigorously quality-controlled medium for cultivation and several days of incubation. These procedures are costly and laborious and typically require special facilities [21,79,80].

The treatment of ureaplasmas is predicted upon rapid detection of infection. Using PCR, ureaplasmas can be detected rapidly and accurately such that treatment may be established in the early stages of infection. This method also avoids the problems associated with culturing since the bacteria do not need to be grown in order to confirm their presence in a sample [79,80] .

The PCR specifically detects the genetic material of ureaplasmas, thus providing a sensitive and definitive method of diagnosis. Detection of ureaplasma employs the urease gene for PCR amplification [22].

2.4.3. Treatment of ureaplasmal infection

Ureaplasma spp. are generally susceptible to the macrolides, tetracycline, chloramphenicol, spiramycin, lincomycin, clindamycin, nitrofurantoin, and aminoglycosides in vitro. Because tetracycline and chloramphenicol should not be used in pregnant women, erythromycin and lincomycin are safer, although less effective [68,69].

2.5. Detection of Mycoplasmas and Ureaplasmas by PCR

Many epidemiological research indicates that Mycoplasmas and Ureaplasmas have a role in the etiology of UTI and that this role has not been established because of the difficulty of isolating them from clinical samples. PCR-based assays have facilitated the detection of Mycoplasmas in clinical samples.

Teng *et al.* (1994) in China, have detected *U. urealyticum* in 50 specimens, including sperm, urine, and prostate secretions from hospital patients with urogenital infections by PCR and culture. Five positive and a further 4 doubtful

diagnoses were made by culture, whereas PCR detected *U. urealyticum* in 12 samples [81].

Povlesn *et al.* (1998) in Denmark, have investigated the prevalence of *U. urealyticum* by PCR and culture in 453 urogenital specimens from women and 105 urethral specimens from men. Among the specimens from women, 63% were PCR positive as well as culture positive, 0.9% were positive only by PCR, and 4% were positive only by culture. Among the specimens from men, 15% were PCR positive as well as culture positive, 1% were positive only by PCR, and 9% were positive only by culture. By using culture as the reference method, the PCR had a sensitivity of 94% and a specificity of 98% when applied to specimens from women and a sensitivity of 64% and a specificity of 99% when applied to specimens from men [22].

Yoshida *et al.* (2001) in Japan, have used PCR and phylogenetic analysis of a partial 16s rRNA gene to detect mycoplasmas and ureaplasmas in urine specimens from urethritis patients in the department of urology, Toyota hospital, Japan. The PCR amplified 42 of 148 urine specimens from urethritis patients (15 were *M. genitalium*, 2 were *M. hominis*, 19 were *U. urealyticum*, 5 were *U. parvum* and one sample contained both *M. genitalium* and *U. urealyticum*) and 15 of 42 urine specimens from asymptomatic patients (one sample was *M. genitalium*, one sample was *M. faucium*, 9 were *U. parvum* and 4 were *U. urealyticum*) [19].

Leadro *et al.* (2002) in the USA, have examined the prevalence of *M. genitalium* in men with urethritis at (STDs) clinic in New Orleans (97 men with urethritis and 184 asymptomatic men) by PCR, the results of their study showed that *M. genitalium* infection rates in symptomatic and asymptomatic men who were negative for *C. trachomatis* and *Neisseria gonorrhoeae* were 25% and 7%, respectively, *M. genitalium* coinfection rates among men with chlamydial and gonococcal urethritis were 35% and 14%, respectively. Among men with urethritis, the sensitivities of PCR of urine and swab specimens for the

detection of *M. genitalium* were 87% and 91%, respectively. The authors concluded that *M. genitalium* is associated with (NGU) in that population [82].

Maedo *et al.* (2004) in Japan, have used PCR assay to detect *M. genitalium*, *M. hominis*, *U. parvum* and *U. urealyticum* in first-voided urine specimens from patients with (NGU). A total of 153 male patients with NGU, who visited one of 24 clinics in Japan, were recruited for this study. All were examined using PCR for the presence of *M. genitalium*, *M. hominis*, *U. parvum* and *U. urealyticum* (b in first-voided urine specimens. They were also examined for the presence of *C. trachomatis*. Of these 153 patients, 73 (47.7%) were positive for *C. trachomatis*. Overall, the prevalence was 17.0% for *M. genitalium,* 16.3% for *U. urealyticum* , 7.8% for *U. parvum* and 2.6% for *M. hominis*. In the 80 patients with non-chlamydial NGU, the prevalence of *M. genitalium, U. urealyticum* , *U. parvum* and *M. hominis* was 23.8%, 18.8%, 8.8% and 2.6%, respectively. This study showed that *M. genitalium* and *U. urealyticum* might be pathogens of NGU and could be associated with persistent and recurrent urethritis. When patients with NGU are treated, such pathogens should be taken into account. The study aiso indicated that PCR assay provides a useful method for diagnosing NGU caused by *M. genitalium* and *U. urealyticum* [83].

Daxboeck *et al.* (2005) In Austria, have examined 30 urine samples from patients with sterile pyuria (21 females, 9 males) by PCR for detection of *M. hominis* and *U. urealyticum. M. hominis* was detected in 2 samples (7%) and *U. urealyticum* was detected in 6 samples (20%) [4].

Dhawan *et al.* (2005) in India, have determined the prevalence of *U. urealyticum* in Indian adults with symptoms of genital discharge. Urine specimens from 100 patients attending an STD clinic at a tertiary care hospital in Delhi were screened prospectively for infection with *U. urealyticum*. The prevalence of *U. urealyticum* was found to be 32% by culture and 45% by PCR. *U. urealyticum* was recovered from 8 (47%) and 37 (45%) symptomatic men and women, respectively. The agreement between PCR and culture was 93.75%. PCR improved the test sensitivity by 13% compared to culture. The

results confirmed the need to use a sensitive and reliable molecular method to prevent the under-diagnosis of ureaplasma infection and to facilitate better clinical management of this infection in India [84].

Yoshida *et al.* (2005) in Japan, have determined which subtypes of *U. urealyticum* are associated with NGU by PCR-based assay. The prevalence of *U. urealyticum* subtypes in 106 ureaplasma-positive men with urethritis was compared with that in 30 ureaplasma-positive men without urethritis. In men with nonchlamydial NGU and men with *M. genitalium*-negative nonchlamydial NGU, only *U. urealyticum* subtype 1 (serovars 2, 5, 8, and 9) was detected significantly more often than in men without urethritis. This study suggests that subtype 1 of *U. urealyticum* (serovars 2, 5, 8, and 9) is associated with NGU independently of *C. trachomatis* or *M. genitalium* [85].

Takahashi *et al.* (2006) in Japan, have investigated the occurrence of *Mycoplasma* and *Ureaplasma* in young men in Sapporo, Japan. In addition, they examined the associations among *C. trachomatis*, *Mycoplasma*, and *Ureaplasma*. A survey of 100 asymptomatic healthy male volunteers was carried out. *C. trachomatis*, *M. genitalium*, *M. hominis*, *U. urealyticum*, and *U. parvum* in first-voided urine specimens were diagnosed by PCR. Detection rates were 1% for *M. genitalium*, 4% for *M. hominis*, 12% for *U. urealyticum*, and 23% for *U. parvum*. *C. trachomatis* was detected in 6% of samples. No *M. hominis*, *U. urealyticum*, or *U. parvum* were detected simultaneously in any sample positive for *C. trachomatis*. The detection rate of urinary *M. genitalium* was extremely low, which is similar to previous reports from Japan. The detection rates of urethral *U. urealyticum* and *U. parvum* were significantly related to sexual activity [86].

Cohen *et al.* (2006) in Kenya, have assessed the risk factors for persistence of *M. genitalium* in a highly exposed female population in Kenya. Two hundred fifty-eight sex workers in Nairobi, Kenya, 18 to 35 years of age, were enrolled. Every 2 months, urine samples were collected for *M. genitalium*, *C. trachomatis*, and *N. gonorrhoeae* testing by PCR. At enrollment, 16% were

infected with *M. genitalium*. The high incidence of *M. genitalium*, greater than that for both *C. trachomatis* (14.0%) and *N. gonorrhoeae* (8%), association with common sexually transmitted infection risk factors, and persistence in the female genital tract supports its role as a common sexually transmitted infection in Kenyan women [87].

Chapter 3

Materials and Methods

3.1. Materials

3.1.1 PCR primers

Oligonucleotide primers for the PCR were adapted from the published nucleotide sequences, and are shown in **Table 3.1.**

Table 3.1. Sequences of the primers used in PCR

Organism	Primer name	Sequence 5' to 3'	Reference
M. genitalium	MgPaW1*	5-AAG TGG AGC GAT CAT TAC TAA C-3	[82]
	MgPaR1*	5-CCG TTG TTA TCA TAC CTT CTG A-3	
M. hominis	MHF*	5-ATA CAT CGA TGT CGA GCG AG-3	[88]
	MHR*	5-CAT CTT TTA GTG GCG CCT TAC-3	
C. trachomatis	CTR**	5-GCA AGA TAT CGA GTA TGC GTT GTT AGG-3	
	CTF**	5-TTC ATT GTA CTC ATT AAA CGA GGC GG-3	
U. urealyticum	U5*	5-CAA TCT GCT CGT GAA GTA TTA C-3	[22]
	U4*	5-ACG ACG TCC ATA AGC AAC T-3	

***(All primers were synthesized by operon Biotechnologies, Germany).**

****(These primers were included in *C. trachomatis* cryptic plasmid, primer set kit)**

3.1.2. Bacterial culture media

- MacConkey agar (Himedia, India)
- Blood agar (Himedia, India**)**
- Sabouraud agar (Himedia, India**)**

3.1.3. Reagents and materials

- Agarose Molecular Biology grade (Promega, USA)
- PCR Master mix (Promega, USA)
- DNA Marker, 100bp ladder **(**Promega, USA)
- EDTA disodium salt (Promega, USA)
- Ethidium bromide (Promega, USA)

- Ethanol 70% (Sigma, USA)
- Absolute isopropanol (Sigma, USA)
- Tris base (Promega, USA)
- DNAse, RNAse free water (Promega, USA)
- Phosphate buffer saline
- Microfuge tubes-1.5 ml capacity
- Microfuge tubes for PCR-thin wall 0.2 ml and 0.5 ml capacity
- Pipettes (Eppendrof)
- Sterile pipette tips
- Bacterial positive control

3.1.4. Enzymes

- Taq DNA polymerase
- Proteinase K
- RNAse

3.1.5. Commercial Kits

- Ez-PCR Mycoplasma test kit (Biological industries, Israel)
- Bacteria, *Chlamydia trachomatis* cryptic plasmid, primer set kit (Maxim Biotech. Inc., USA)
- Master pure™ DNA purification Kit (EPICENTR Technologies, USA)

3.1.6. Apparatus and Equipments

- Thermal cycler (Eppendrof Master cycler Personal, Germany)
- L.G. Microwave oven (LG, Korea)
- Gel documentation system (SciE-plAs, Japan)
- Vortex mixer (GEMMY industrial CORP, Taiwan)
- Power supply (BIO-RAD, USA)
- Micro centrifuge (Centurion Scientific Ltd., UK)
- Freezer, Refrigerator

- Electrophoresis Tank (Cleaver scientific Ltd., UK)
- Incubator
- Water bath (GEMMY industrial CORP, Taiwan)
- Weight mod
- Microscope
- Computer
- Spectrophotometer (Nano Drop Technologies, USA)

3.2. Clinical specimens

Urine samples from patients aged >18 year with symptoms (discharge, dysuria, pain, frequent urination) attending the genitourinary clinic at Al-shifa hospital-Gaza were collected in sterile containers.

3.2.1. Analysis of sterile pyuria samples

A total of 200 samples of sterile pyuria (**96 males , 104 females**) were Collected during the period **February 2006** to **April 2007** and were analyzed as follows:

- Routine urine examination.
- Any sample containing more than 10 leukocytes / HPF was cultured on MacConkey agar, blood agar, and sabouraud agar to detect the presence of bacteria and *Candida* .
- Samples negative for culture (showing no significant growth after 24 hr) were stored frozen at (-70° C) for 4-6 days until processing by PCR assay .

3.3. Polymerase Chain Reaction (PCR)

3.3.1 . DNA extraction from urine specimens

DNA extraction was done by using Master pure™ DNA purification kit

Components of the kit

- Red cell lysis solution (120ml)
- Tissue and cell lysis solution (60ml)

- 2X T and L lysis solution (50ml)
- MPC protein precipitation reagent (50ml)
- Rnase A 5 µg/µl (200 µl)
- Proteinase K 50 µg/µl (200 µl)
- TE buffer (7ml)

Procedure of extracting DNA from urine

The urine specimens were thawed at room temperature, 1 ml urine was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for 30 minutes at 4° C. Supernatant was discarded and the pellet was washed with 1ml of phosphate buffer saline (pH 7.4) and centrifuged again at 13,000 rpm for 15 minutes at 4° C. Supernatant was discarded and DNA was extracted from pellet using the Master pure™ DNA purification kit following the manufacturer's instructions:

- Three hundred µl of tissue and cell lysis solution containing 1µl of 50 µg/µl proteinase K were added to each sample.
- The samples were incubated at 65°C for 15 minutes and mixed every 5 minutes.
- The samples were cooled to 37°C and 1µl of 5µg/µl Rnase A was added to each sample and mixed thoroughly.
- The samples were incubated at 37°C for 50 minutes.
- The samples were placed on ice for 3-5 minutes.
- Hundred seventy five µl of MPC protein precipitation reagent was added to each sample and vortex mix vigorously for 10 seconds.
- The samples were centrifuged at 13,000 rpm for 10 minutes.
- The supernatant was transferred to microcentrifuge tube end pellet was discarded.
- Five hundred µl of isopropanol were added to the recovered supernatant and the tubes were inverted several (30-40) times.
- The samples were centrifuged at 4°C for 10 minutes.
- The isopropanol was poured off carefully without dislodging the DNA pellet.

- The DNA pellet was rinsed twice with 75% ethanol then all of residual ethanol were removed with a pipette.
- The eluted DNA was resuspended in 35 µl of TE buffer. The DNA solution can directly be used or stored at 4°C for longer storage periods.

3.3.2. Detection and measurement of extracted DNA

3.3.2.1. Agarose gel electrophoresis

The quality of the isolated DNA was determined by running 5 µl of each sample on ethidium bromide-stained 2.0% agarose gels and the DNA was visualized on a short wave U.V. transilluminator.

3.3.2.2. Spectrophotometery

The optical density (O.D.) at 260 nm of diluted fractions of isolated DNA samples was measured by a spectrophotometer and the DNA concentration was calculated by considering 1 $O.D._{(260\ nm)}$ = 50µg/ml DNA and taking into account the dilution factor.

3.3.3. Detection of *C. trachomatis* by PCR

The extracted DNAs were subjected to PCR with primers specific for *C. trachomatis* cryptic plasmid. These primers were included in *C. trachomatis* cryptic plasmid, primer set kit.

Components of the kit

- Pre-mixed primers (1000 µl**)**
- Optimized PCR buffer (750 µl x 4 tubes)
- Positive control cDNA (100 µl**)**
- Size marker, 100bp ladder (100 µl)
- Double distilled water (1000 µl)

Master mixture preparation

We added 250 µl each of pre-mixed primers to each tube of optimized PCR buffer. The master mixture was aliquoted and stored at -20°C.

Reaction mixture preparation

Reaction mixture was prepared in a PCR tube by combining the reagents as shown in **Table 3.2**.

Table 3.2. *C. trachomatis* PCR reaction mixture

Reagents	**Volume**
Master mixture (dNTPs,$MgCl_2$,primers)	20µl
DNA template	5µl
Taq polymerase	0.1µl
Total volume	25.1µl

PCR tubes were then placed in a thermal cycler and PCR amplification was done according the program described below. Upon completion of PCR, the products were analyzed on ethidium bromide-stained 2% agarose gel electrophoresis.

Temperature cycling program

The thermal cycling program was set as follows :

Step1 : initial denaturation for 1 minute at 96° C

Step2 : 35 cycles of

Step2.1 : denaturation for 1 minute at 95° C

Step2.2 : annealing for 1 minute at 58° C

Step2.3 : elongation for 1 minute at 72° C

Step3 : final extension for 10 minutes at 72° C

Expected *C. trachomatis* PCR results

PCR products were analyzed by electrophoresis on 2 % agarose gel containing ethidium bromide. The gel was visualized by U V illumination and photographed. DNA molecular size standard (100 bp ladder) was included in each agarose gel. A band of 364 bp in size was taken as an indication of a positive result .

3.3.4. Detection of *M. genitalium* by PCR

Reaction mixture was prepared in a PCR tube by combining the reagents shown in Table 3.3

Table 3.3. *M. genitalium* PCR reaction mixture

Reagents	volume
PCR master mix	12.5µl
DNA template	2.5µl
Primer1 MgpaW1	2µl
Primer2 MgpaR	2µl
H20	6µl
Total volume	25µl

PCR tubes were then placed in a thermal cycler and PCR amplification was done according to the program described below.

Temperature cycling program

Step 1: initial denaturation at 95° C for 10 minutes

Step 2: 35 cycles of:

Step 2.1: denaturation at 94° C for 40 seconds

Step 2.2: annealing at 56° C for 40 seconds

Step 2.3: elongation at 72° C for 40 seconds

Step 3: final extension at 72° C for 15 minutes

Expected *M. genitalium* PCR results

PCR products were visualized as described before. A band of 495 bp in size indicates a positive result for *M. genitalium .*

3.3.5. Detection of *M. hominis* by PCR

Reaction mixture was prepared in a PCR tube by combining the reagents as described for *M. genitalium* except that primers MHR and MHF were used instead. PCR tubes were then placed in a thermal cycler and PCR amplification was done as described for *M. genitalium* except that the annealing temperature was 58° C.

Expected *M. hominis* PCR result

A positive PCR test should yield a 270 bp DNA fragment which would appear as an intense band on an ethidium bromide – stained 2.0% agarose gel .

3.3.6. Detection of *U. urealyticum* by PCR

Reaction mixture was prepared in a PCR tube by combining the reagents as described for *M. genitalium* but using primers u4 and u5. PCR tubes were then placed in a thermal cycler and PCR amplification was done according to the program described below .

Temperature cycling program

The thermal cycler program was set as follows :

Step 1 : initial denaturation at 94° C for 2 minutes

Step 2 : 50 cycles of

Step 2.1 : denaturation at 94° C for 30 seconds

Step 2.2 : annealing at 56° C for 1 minute

Step 2.3 : elongation at 72° C for 45 seconds

Step 3 : final extension at 72° C for 5 minutes

Expected *U. urealyticum* PCR results

A positive PCR test should yield a 429 bp DNA fragment that would appear as an intense band on ethidium bromide – stained 2.0% agarose gel.

3.4. Data analysis

The data were analyzed by a personal computer using SPSS 8.0. Differences in proportions were assessed by Chi–square test and p values < 0.05 were considered statistically significant.

Chapter 4

Results

Results

In the present study 200 urine samples from patients with sterile pyuria (96 male , 104 female) aged > 18 years in Gaza, Palestine were subjected to PCR for detection of *C. trachomatis, U. urealyticum*, *M. hominis* and *M. genitalium*. The results can be summarized as follows:

4.1. Occurrence of *C. trachomatis, U. urealyticum, M. hominis and M. genitalium*

The PCR amplified DNA from 38 of the 200 urine samples (19%) from patients with sterile pyuria. *C. trachomatis* was detected in 20 samples (10%), *U. urealyticum* in 10 samples (5%), *M. hominis* in 6 samples (3%) and *M. genitalium* in 2 samples (1%), **Table 4.1.**

Table 4.1. Microorganisms identified in the 200 studied specimens

Microorganisms				
C. trachomatis n (%)	*U. urealyticum* n (%)	*M. hominis* n (%)	*M. genitalium* n (%)	**Total n (%)**
20 (10)	10 (5)	6 (3)	2 (1)	**38 (19)**

4.2. Occurrence of microorganisms according to the sex of patients

Ninety six samples (48%) were from male patients and 104 (52%) from female patients. The occurrence of the investigated microorganisms with regard to gender of patients is presented in **Table 4.2.** As can be seen in the table, the difference in occurrence of *C. trachomatis* was statistically insignificant between males and females but, that of *U. urealyticum* was significant. *M. hominis* was detected only in samples collected from female patients. On the contrary, *M. genitalium* was detected only in men

Table 4.2. Occurrence of microorganisms according to the sex of patients (N=200)

Microorganisms	Sex of patients						
	male		female		total		P value
	n	(%)	n	(%)	n	(%)	
C. trachomatis	11	(5.5)	9	(4.5)	20	(10.0)	0.509
U. urealyticum	1	(0.5)	9	(4.5)	10	(5.0)	0.008*
M. hominis	0	(0.0)	6	(3.0)	6	(3.0)	0.005*
M. genitalium	2	(1.0)	0	(0.0)	2	(1.0)	0.085

4. 3. Distribution of the samples according to the number of white blood cells per high power field :

Most of the samples (68%) contained 10-20 WBCs / HPF. The distribution of the samples according to the WBC/HPF is provided in **Table 4.3.**

Table 4.3. Distribution of the samples according to the WBC/HPF

WBC/HPF	Number and percentage of all tested samples			
	Number of subjects	Percentage	Number of positive samples	Percentage
10-20	136	68%	19	9.5%
21-30	32	16%	7	3.5%
31-40	9	4.5%	4	2.0%
41-50	1	0.5%	0	0%
Above 50	22	11%	8	4%
Total	**200**	**100%**	**38**	**19%**

4.4. Occurrence of microorganisms according to the WBC/HPF in tested samples:

The occurrence of microorganisms according to the number of WBC/HPF is provided in **Table 4.4.** As can be seen in the table, the relation between the number of WBC/HPF and *U. urealyticum* infection was statistically significant.

Table 4.4. Occurrence of microorganisms according to the WBC/HPF

Microorganisms	WBC/HPF						
	10 - 20	21 - 30	31 - 40	41 - 50	Above 50	Total	P value
C. trachomatis	16	2	1	0	1	20	0.711
U. urealyticum	0	4	3	0	3	10	0.00*
M. hominis	3	0	0	0	3	6	0.109
M. genitalium	0	1	0	0	1	2	0.252
Negative samples	117	25	5	1	14	162	
Total	**136**	**32**	**9**	**1**	**22**	**200**	

4.5. PCR results :

The following figures (Figures 4.1. to 4.4.) represent the PCR results for *C. trachomatis, M. hominis, M. genitalium*, and *U. urealyticum,* respectively.

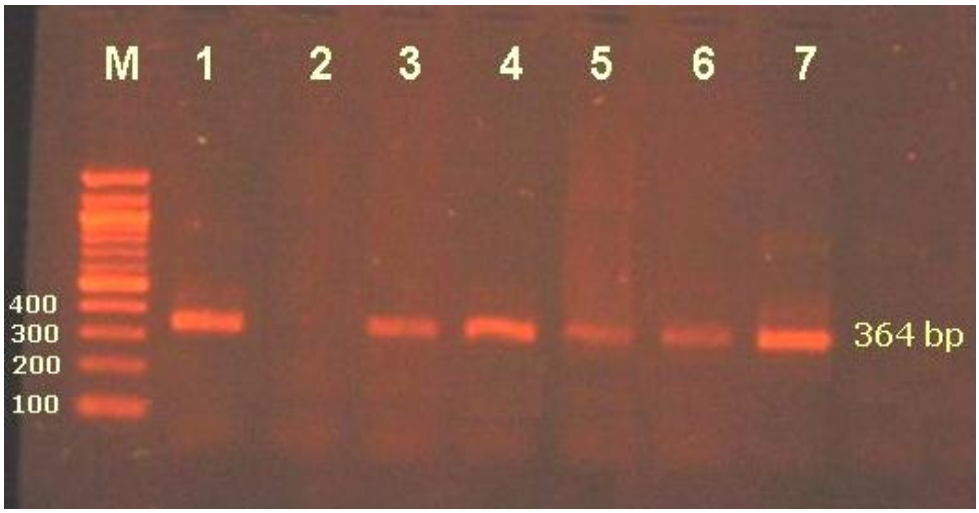

Figure 4.1. A photograph of Ethidium bromide stained 2% agarose gel showing the PCR result for identification of *C. trachomatis* targeting cryptic plasmid. All positive samples yielded an amplicon of 364 bp. M:100bp DNA marker, lane1: positive control, lane2: negative (no DNA) control, lane3 to lane7: positive samples with 364bp product.

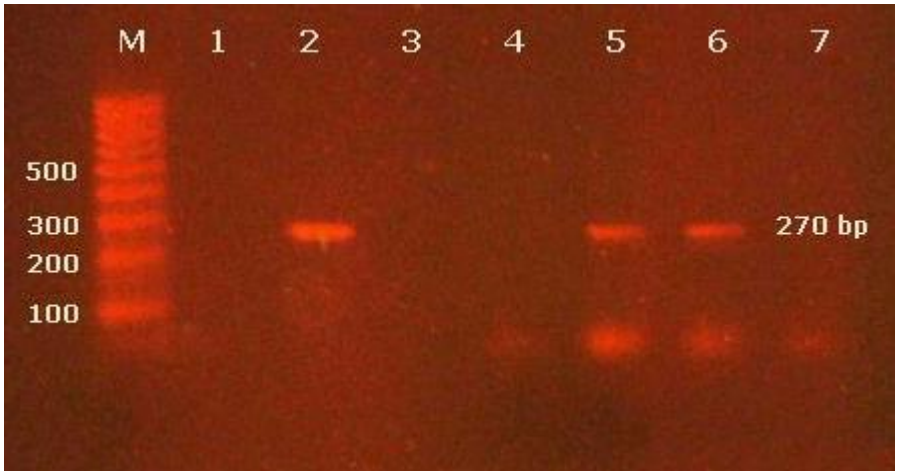

Figure 4.2. A photograph of ethidium bromide stained 2% agarose gel showing the PCR result for identification of *M. hominis* targeting 16s rRNA gene. PCR assay yields an amplicon of 270bp, M: 100bp DNA marker, lane1: negative control, Lanes 3, 4, 7: negative samples, lanes2, 5, 6: positive samples with 270bp product .

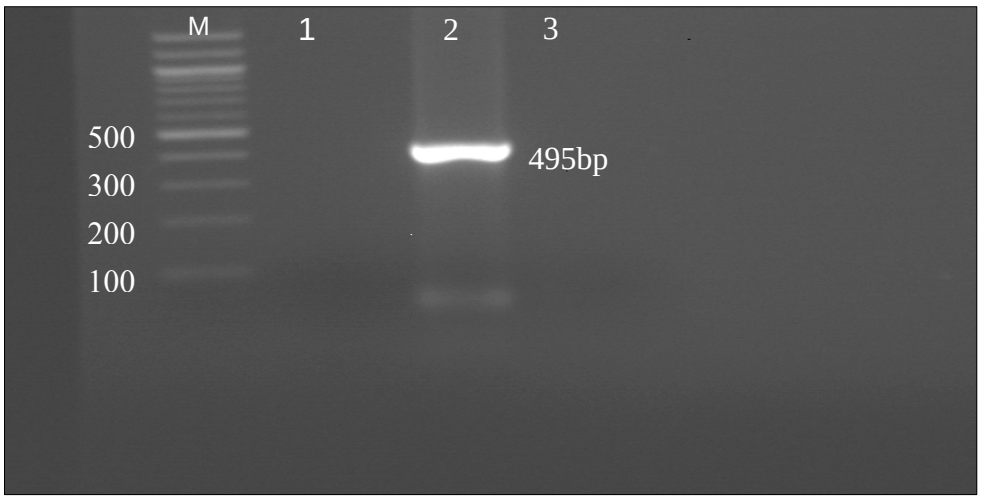

Figure 4.3. A photograph of ethidium bromide stained 2% agarose gel showing the PCR result for identification of *M. genitalium*. The PCR target was a 495bp beginning 85bp upstream from the *M. genitalium* adhesion gene start codon. M : 100bp DNA marker, lane1: negative control, lane2 : positive sample with 495bp product, lane3 : negative sample

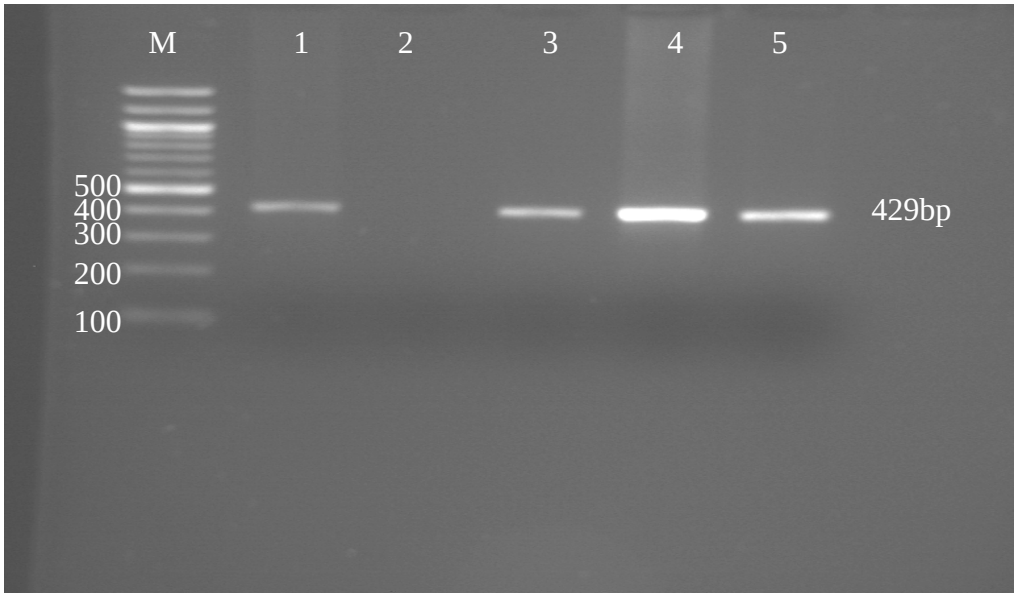

Figure 4.4. A photograph of ethidium bromide stained 2% agarose gel showing the PCR result for identification of *U. urealyticum* targeting the urease gene. All positive samples yielded an amplicon of 429bp. M : 100 bp DNA marker, lan2: negative control, lanes1, 3, 4, 5 : positive samples with 429bp product.

Chapter 5

Discussion

Discussion

Sterile pyuria in patients with clinical symptoms consistent with UTI can be a diagnostic challenge and warrants further investigation for detection of fastidious and atypical microorganisms such as *C. trachomatis*, *M. hominis*, *M. genitalium*, and *U. urealyticum*. These microorganisms are associated with various diseases of the genitourinary tract, but they are usually not detected by routine microbiological diagnosis.

This study was performed in order to determine the occurrence of *C. trachomatis*, *M. hominis*, *M. genitalium*, and *U. urealyticum* in urine specimens collected from 200 patients (104 female, 96 male) with sterile pyuria by PCR using specific primers for each species.

These organisms are not screened by routine examination of urine samples in health laboratories in Gaza strip, and this is the first study that employed PCR assay for detection of these organisms in sterile pyuria specimens in Gaza strip.

However, we acknowledge that this study has various limitations such as difficulties in collecting data pertinent to the patients, and obstacles and delays in obtaining materials and kits, not to mention the high cost of those reagents that made it impossible to include asymptomatic cases in our study. Therefore, further studies should be conducted on this topic for investigating the presence of such microorganisms in asymptomatic persons from the general population.

The results of the study showed that 38 of the 200 urine specimens (19%) were PCR-positive, with microorganisms detected as follows: *C. trachomatis* in 20 samples (10%), *M. hominis* in 6 samples (3%), *M. genitalium* in 2 samples (1%), and *U. urealyticum* in 10 samples (5%) as indicated in Table 4.1.

Findings of this study showed that PCR testing of sterile pyuria could identify a significant number of causative microorganisms and should demonstrate to the

clinicians the advantage of detection of the fastidious microorganisms in urine from the patients with UTI symptoms, when standard cultures fail to detect microbial infection. Since the detection of those microorganisms should constitute an essential part of the diagnosis and management of patients. The association of sterile pyuria with these organisms infections, however, ought to be discussed.

C. trachomatis infections are the most prevalent sexually transmitted bacterial infections among women and men worldwide [39]. Screening for these infections is important not only to identify infected symptomatic individuals for the diagnosis and management of their infections but also to identify asymptomatic individuals who serve as reservoirs for *C. trachomatis* infections [39].

Traditionally, the gold standard for the identification of *C. trachomatis* is culture, however, culture is time-consuming and labor-intensive. It takes 3 to 6 days to complete, and it requires access to specialized facilities and trained personnel [40].

DFA tests utilize fluorescein-conjugated monoclonal antibodies to detect EBs of *C. trachomatis* through microscopic analysis. EBs are hard to detect in urine specimens because they are usually present in very low number and may be masked by a large amount of debris in the urine. Moreover, DFA tests require detection by highly competent personnel and are time-consuming [36].

ELISAs have also been evaluated as screening tests for the rapid identification of infected individuals by using first-catch urine [89,90]. ELISAs are relatively fast and easy to complete, but sensitivities of the tests for urine specimens remain relatively low [91,92]

Wu *et al.* (1992) found that the sensitivities of both the PCR and ELISA were 90.9% for male urine compared with urethral culture. Overall, for both males and females, the PCR was more sensitive (95.6%) than the ELISA (87.0%). Specificities were 97.7% (99.4% for men and 95.7% for women) for the ELISA and 98.0% (99.4% for men and 96.5% for women) for the PCR [93].

Two main DNA targets have been used for detection of *C. trachomatis* by PCR namely the cryptic plasmid and chromosomal MOMP gene.
Mahoney *et al.* (1993) found that the plasmid-based PCRs are 10 to 1,000 times more sensitive than chromosome based assays for detection of *C. trachomatis* [10]. Therefore, we used plasmid-based PCR to detect *C. trachomatis* in first-void urine specimens from patients with sterile pyuria.

In the current study, the occurrence of chlamydial infection was 10% in the 200 urine specimens collected from symptomatic patients with sterile pyuria. In samples from women (104 samples) *C. trachomatis* was detected in 9 samples (8.7%) and in 11 of the 96 male specimens (11.5%). The difference in occurrence was statistically insignificant (P=0.509) between males and females (as shown in Table 4.2.).

When compared to other studies from other countries (as shown in Table 5.1.), our results are still lower than that reported in Egypt, Iran, Senegal, India, Bangladesh, and the UK. However, our results is higher than that reported in Jordan, and Ghana.

Table 5.1. Prevalence of *C. trachomatis* in different countries by PCR

Country (year)	Prevalence of C. trachomatis	Study population	Reference
Egypt (2001)	31%	symptomatic women	[33]
Iran (1991)	22%	Patients with urethritis	[94]
Iran (2005)	15%	Women with cervicitis	[48]
Senegal (2000)	28.5%	Sex workers women	[95]
India (2003)	30.8%	Symptomatic men and women	[96]
Bangladesh (2004)	43.5%	Hotel-based sex workers	[97]
The UK (2002)	21%	Males with sterile pyuria	[43]
Jordan (2003)	4.6%	Symptomatic patients	[44]
Ghana (2004)	3%	Gynecological patients	[98]
Eastern Europe countries	6-25%	Symptomatic women	[99]

Variations of *Chlamydia* prevalence between countries and studies could be due to several factors such as, study population (selection of high risk group, symptoms, education level, sample size, etc.), rate of infection in the study area, hygiene level and socioeconomic status of the study area, culture of the society whether it is open or conservative, and the technique and the DNA target of PCR used.

Association of sterile pyuria with chlamydial infection

Our results revealed that the occurrence of *C. trachomatis* was higher than the occurrence of the other microorganisms detected in this study. These results suggest that *C. trachomatis* infection should be strongly considered in urine samples that show sterile pyuria.

This is congruent with the conclusion of Basarab *et al.* (2002) who showed that a significant number of *C. trachomatis* could be detected in urine specimens from sterile pyuria (as shown in Table 5.1.).

Mycoplasma hominis is a heterogeneous genital mycoplasma [100] found in at least two-thirds of women with bacterial vaginosis (BV), compared to 10% of healthy women [101,102]. *M. hominis* has also been isolated from the endometrium and fallopian tubes of 10% women with salpingitis. However, its role as a primary pathogen is doubtful since it co-exists with many other bacteria in BV [103]. Isolation from other sites than the genitourinary tract has also been reported [104].

Culture is the most commonly employed method for detection of genital *Mycoplasma,* but it requires special handling, complex media, and cultivation. Furthermore, positive samples need further testing to determine the species [105].

Comparison between culture method and PCR has been performed and showed that a PCR assay was as sensitive as culture for detection of *M. hominis* from clinical samples. In addition it was very specific [106].

In the present study we employed the 16s rRNA gene as a target for PCR amplification to detect the occurrence of *M. hominis* in the urine samples from patients with sterile pyuria. The results showed that *M. hominis* was present in 6 samples (3%). The occurrence of *M. hominis* among women was 5.8% and it was not detected in men (Table 4.2.). This finding deserves further investigation to determine whether *M. hominis* has a role in disease or just in colonization and to improve the association of sterile pyuria with urogenital *M. hominis* infection or colonization.

Our finding is nearly congruent with that of other studies such as that of Doxboeck *et al.* (2005) In Austria, who examined 30 urine samples from patients with sterile pyuria (21 females, 9 males) by PCR for detection of *M. hominis* and the microorganism was detected in 2 samples (7%). The authors, however, concluded that this finding does not indicate an association of sterile pyuria with urogenital mycoplasma infection or colinization [4]

Takahashi *et al.* (2006) in Japan**,** have investigated the occurrence of *M. hominis* in first-voided urine specimens from young men. They found that the occurrence of *M. hominis* was 4%. They concluded that there is a need to determine whether this pathogen has a role in STDs or just in colonization [86].

Maeda *et al.* (2004) in Japan detected *M. hominis* in first-voided urine specimens from patients with NGU, they found a prevalence of 2.6% and they concluded that *M. hominis* might be non-pathogenic of NGU and could not be associated with urethritis [83].

Baczynska *et al.* (2004) in Denmark where they reported that the prevalence of *M. hominis* among 83 infertile women was 2.4% [107].

Yoshida *et al.* (2001) In Japan, have used PCR and phylogenetic analysis of a partial 16s rRNA gene to detect *M. hominis* in 148 urine specimens from urethritis patients. The prevalence of *M. hominis* was 1.4% [19].

The low prevalences recorded in the aforementioned studies may suggest that *M. hominis* is not the primary cause of UTIs. However, we recommend to execute further investigation and increasing sample size. In addition, carful study of personal behavior-related to sexual activity should be considerd.

M. genitalium was first isolated in urethral cultures from two men with NGU in 1980 [108]. Although *M. genitalium* has been proposed as a cause of human NGU, the precise role of this mycoplasma in the etiology of NGU has not been established because of the immense difficulty of isolating it from clinical samples. However, PCR-based assays have facilitated the detection of *M. genitalium* in clinical samples [16], and a significant association has been demonstrated between *M. genitalium* and NGU [109]. In experimentally infected chimpanzees, *M. genitalium* has been shown to induce symptomatic genital infections with inflammatory and antibody responses, suggesting that, *M. genitalium* may be a pathogen of NGU [110].

The PCR target for detection of *M. genitalium* employed in this study was a 495-bp fragment beginning 85-bp upstream from *M. genitalium* adhesion gene start codon. The results showed that the occurrence of *M. genitalium* was 1% and it was detected in men only. The detection of *M. genitalium* in urine samples of 2 of 96 men (2.1%) with UTI symptoms and sterile pyuria by PCR in this study and previous studies that used these assays should be considered an evidence for the association between urethritis in men and *M. genitalium* infection.

When compared to the results of other studies, our findings are similar to those reported by Takahashi *et al.* (2006) in Japan, where they recorded a 1% prevalence of *M. genitalium* among men [86].

Our results, however, are considerably lower than reported by Leandro *et al.* (2002) in the USA, where they examined the prevalence of *M. genitalium* in men with urethritis at (STDs) clinic in New Orleans (97 men with urethritis and

184 asymptomatic men) by PCR, the results of their study showed that *M. genitalium* infection rates in symptomatic and asymptomatic men were 25% and 7%, respectively. The authors concluded that *M. genitalium* is associated with (NGU) in that population [82].

In Kenya**,** the prevalence of *M. genitalium* infection among female sex workers was 16% as reported by Cohen *et al.* (2006). The high incidence of *M. genatilum* might be due to selection of highly symptomatic females [87].

In Japan, Yoshida *et al.* (2001) have used PCR to detect *M. genitalium* in urine specimens from urethritis patients. Fifteen of the 148 (10.1%) urine specimens were positive for *M. genitalium* [19]. In another study in Japan Maedo *et al.* (2004) reported that the prevalence of *M. genitalium* was 17.0% in first-voided urine specimens from153 male patients with NGU [83].

The difference between our results and those reported by previous studies may be due to the difference in the populations studied and the prevalence of the microorganism in the study area.

Another issue that needs further investigation is the role of *M. genitalium* in genital tract inflammatory disease in women.

U. urealyticum has been shown to be associated with a range of human disorders including respiratory infection [111], intrauterine infection [112] and NGU[20].

At present, the main method of detecting *U. urealyticum* is by culture, but the organism is difficult to isolate and requires special culture media [80]. *U. urealyticum* can also be detected rapidly and accurately by PCR such that treatment may be established in the early stages of infection, also this method avoids the problems associated with culturing.

In the present study we employed the urease gene for PCR amplification to detect the occurrence of *U. urealyticum* in the urine samples from patients with sterile pyuria. The occurrence of *U. urealyticum* was 5% (Table 4.1).

U. urealyticum was detected in 9 (8.7%) of female samples and in only one (1%) of the male sample. The difference in occurrence was statistically significant (P=0.008) between males and females (as shown in Table 4.2).

Detection of the microorganism in urine specimens leaves the clinical significance of *U. urealyticum* open to question. It should be noted that 60% of healthy women carry *U. urealyticum* in their urogenital tract. Unlikely, in male urogenital tract this microorganism should not appear. If present in male urethra, it should always be treated as a pathogen [113].

It is known that other organisms can cause the clinical disorders observed and also that not all serotypes of *U. urealyticum* are pathogenic. The PCR primers used for this study amplify a DNA sequence from all *Ureaplasma* strains that have been tested, but do not distinguish between biovars [114].
Robertson *et al.* reported that an association between *U. urealyticum* and infection should be understood in terms of biovars rather than serotypes [115].

Further investigation using PCR primers that distinguish between biovars should provide additional information regarding the pathogenicity of *U. urealyticum,* especially in women.

In a study from Japan, Yoshida *et al.* (2005) have determined which subtypes of *U. urealyticum* are associated with NGU by a PCR-based assay. The prevalence of *U. urealyticum* subtypes in 106 ureaplasma-positive men with urethritis was compared with that in 30 ureaplasma-positive men without urethritis. Only *U. urealyticum* subtype 1 (serovars 2, 5, 8, and 9) was detected significantly more often than in men without urethritis. This study suggested that subtype 1 of *U. urealyticum* (serovars 2, 5, 8, and 9) is associated with NGU [85].

When we compared our results to other studies, our results are still lower than those reported in Japan, China, Austria, and India (as shown in Table 5.2)

Table 5.2. Prevalence of U. urealyticum in different countries by PCR

Country (year)	Prevalence of U. urealyticum	Study population	Reference
Japan (2004)	15.8%	Men with urethritis	[116]
Japan (2004)	16.3%	Male patients with NGU	[83]
Japan (2005)	12.8%	Patients with NGU	[19]
China (1994)	24%	Patients with urogenital infection	[81]
Austria (2005)	20%	Patients with sterile pyuria	[4]
India (2005)	47% and 45%	Symptomatic men & women respectively	[97]

These variations could be explained by differences in the study population and rate of infection in the study area. Previous studies have included populations that have much higher incidences and prevalences of STD than does the population of our study.

Finally, PCR assay provide a rapid and effective measure to detect fastidious and atypical microorganisms in sterile pyuria cases which is useful for identification of etiological agents and the consequent management and treatment of patients.

Chapter 6

Conclusion & Recommendations

Conclusion and Recommendations

Findings from this study demonstrated that:

- Significant numbers of *C. trachomatis*, *Mycoplasma* spp. and *ureaplasma* spp., which are not screened for during routine examinations of urine samples in Palestinian health laboratories in Gaza strip, were present in sterile pyuria cases.
- Association of these organisms with urogenital tract infections should be considered and Identification of these microorganisms should not be neglected for effective treatment and control strategies.
- PCR assay is a rapid technique for the detection of these bacteria.

Therefore we strongly recommend clinicians not to neglect *Chlamydia trachomatis*, *Mycoplasma* spp and *Ureaplasma* spp in sterile pyuria samples and determine the etiology of UTI before prescribing antibiotics, to prevent the unnecessary use of the valuable broad spectrum antibiotics. The cost, medically and financially, of screening for these pathogens by PCR and treating infected patients is likely to be less than ignoring it.

Recommendations

- It is recommended to use the PCR technique as a routine test for diagnosis of these microorganisms in our clinical laboratories.
- A heightened educational initiative for clinicians and lab technicians to start screening for these microorganisms, especially in cases of sterile pyuria.
- Further investigations should be done including asymptomatic cases and symptomatic without sterile pyuria to compare the prevalence of these microorganisms among these groups.
- Further investigations should be done to provide further information about the pathogenicity of *U. urealyticum* and *M. hominis* in both men and women in conjunction with sufficient information of the study population.

- STDs clinics are not available, it recommended to establish such clinics to minimize the danger of such diseases. We also recommend the establishment of a database for STDs in Gaza strip.

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Appendices

Appendices

Appendix A

Composition of buffer and reagents

50x TAE buffer

Composition:

Tris base …… 242 g

glacial acetic acid ……. 57.1 ml

EDTA ……..…18.6 g

H2O to …….. 1000 ml

pH 8.0

Ethidium bromide (stock solution)

Ethidium bromide 10 mg/ml in water.

DNA loading buffer

bromphenol blue	0.25 g
xylene cyanol	0.25 g
glycerine	30 ml
H2O	70 ml

Composition of PBS:

Potassium phosphate	50 mM
NaCl; pH= 7	150 mM.