Detection of *Chlamydia trachomatis* in Endocervical Swabs Using Molecular (PCR) and Enzyme Immunoassay Techniques

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نتيجة الحكم على أطروحة ماجستير

بناءً على موافقة عمادة الدراسات العليا بالجامعة الإسلامية بغزة على تشكيك لجنة الحكم على أطروحة الباحث: إياد أكرم سعيد القوقا لنيل درجة الماجستير في كلية العلوم/قسم العلوم الحياتية-تحاليل طبية

وبموضوعها:

"Detection of Chlamydia Trachomatis in Endocervical Swabs Using Molecular (PCR) and Enzyme Immunoassay Techniques"

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واللجنة إذ تمنح هذه الدرجة فإنها توصي بتقديم الله ولزوم طاعته وأن يسرح علمه في خدمة دينه ووطنه.
والله ولي التوفيق ءآء

عميد الدراسات العليا
د/ مازن إسماعيل هنئة
Declaration

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Abstract

*Chlamydia trachomatis* is an obligate intracellular bacteria characterized by a biphasic developmental cycle of replication. The organism is recognized as one of the major causes of sexually transmissible human bacterial infection throughout the world.

Since there are no previous studies dealing with chlamydial diagnosis in our area, therefore, a comparative study to compare between the diagnostic performance of a PCR and EIA techniques was conducted, to detect *C. trachomatis* infection in 109 endocervical swab specimens. These specimens were collected from female participants, whose ages ranged between 18-52 years with a median age of 29 year, attending gynecology and infertility clinics in Gaza with different complains.

The results obtained showed that the overall confirmed detection rate of *C. trachomatis* was 20%. Infection rate of *C. trachomatis* by plasmid PCR was 21.1%. While, by chromosomal (MOMP) PCR was 17.4% and by EIA was 19.1%.

Diagnostic consistency and diagnostic accuracy were statistically tested. There was no statistical significant difference between PCR and EIA techniques.

The sensitivity of EIA, MOMP- PCR and plasmid- PCR were 73%, 86%, and 100%, respectively. Meanwhile the specificity for EIA, plasmid- and MOMP-PCRs were 94%, 98%, and 100%, respectively.

Efficiency of EIA, MOMP –, and plasmid – based PCR assays were 90%, 97%, and 98%, respectively.

The highest rate of infection was observed in the participants from Northern Gaza Strip and among participants with elementary education level, intrauterine contraceptive device (IUCD) users, and secondary infertility.
Cervical discharge, especially of a mucopurulent character, and contact bleeding were suitable markers for having genital chlamydial infection. These signs were significantly more often expressed in *C. trachomatis* positive women in our study (*P* = 0.0002). There was also a significant statistical relationship between chlamydial infection and infertility (*P*=0.049). The Odds ratio of development of outcomes of cervicitis, contact bleeding, and infertility were 5.9 (95% CI 2.15-15.97), 4.3 (95% CI 1.27-14.45), and 2.6 (95% CI 0.99-6.85), respectively.

PCR proved to be superior and more efficient in the diagnosis of *C. trachomatis* than EIA, therefore it should be used as a technique of choice in diagnosis of *C. trachomatis*.

Although financial constraints may impede the routine use of molecular diagnostic methods in Palestine, it should be borne in our minds that the cost arising from the clinical sequelae of leaving genital *C. trachomatis* infection undiagnosed and untreated may exceed the cost of these diagnostic methods.

**Keywords:**

*C. trachomatis*, Endocervical Swab Specimens, EIA, PCR, Plasmid, MOMP, Cervicitis, Infertility, Gaza.
الكشف عن الكلايميديا التراكوماتس من مسحات من عنق الرحم بتكنولوجيا الهروثة الجزيئية (PCR) الإنجنيسي المناعي

الملخص العربي

تعتبر الكلايميديا التراكوماتس من البكتيريا التي تعيش داخل الخلايا الحية، وهي إجبارية للتمثيل حيث تمتاز بطرق حيوية وهي من أهم مسببات الأمراض البكتيرية المثلثة جنسياً للإنسان في العالم. 

وهي في دراسة سابقة من منطقة تُعَبَّر بتشخيص تلك البكتيريا، فقد أجريت هذه الدراسة المكتوبتينا نتائج كشف عن تلك البكتيريا من مسحات من عنق الرحم. وقد أجريت هذه الدراسة على عدد 109 من السيدات الذين يُعَبَّر بتشخيص عديد النساء والولادة والتعليم في مدينة غزة لأسباب وأعراض مختلفة، وكانت أعمارهن تتراوح ما بين 18 إلى 42 سنة بمتوسط عمر 29 سنة.

أظهرت نتائج هذه الدراسة أن نسبة الامتصاص للحالات المؤكدة بالإصابة كانت 20%. وقد أظهرت نتائج الدراسة أن نسبة الامتصاص للحالات المؤكدة بالإصابة كانت 20%. ونتائج الدراسة أن نسبة الامتصاص للحالات المؤكدة بالإصابة كانت 20%. 

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وهذا وقد تم اختيار صحة وتواجد النتائج الإحصائية وتبين عدم وجود فروقات معنوية ذات دلالة إحصائية بين نتائج تفاعل البوليميريز المتسلسل ونتائج فحص الإنجنيسي المناعي.

وقد أظهرت نتائج هذه الدراسة أن تفاعل البوليميريز المتسلسل هو الأكثر كفاءة ودقة في التشخيص من الفحص الإنجنيسي المناعي حيث كانت الكفاءة للفحص الإنجنيسي المناعي، وفحص تفاعل البوليميريز المتسلسل المعتمد على الكروموسوم، وفحص تفاعل البوليميريز المتسلسل المعتمد على البلازميد هي على التوالي 90%, 97%, 98%, 99%, 100%. بينما كانت النتائج للفحص الإنجنيسي المناعي، ونتائج هذه الدراسة في وجود علاقة معنوية بين علامات الإصابة ومضاعفاتها "والتي تمثلت بشكل جلي في الإجراءات المضادة للالتهابات والنزف الدموي والعملة والإصابة بالكلايميديا.

كما أظهرت النتائج أن أعلى نسبة إصابة تشكلت في منطقة شمال قطاع غزة، وبين السيدات نوات التعليم الابتدائي، والثانية التي تستخدمان البنوك كوسيلة لمنع الحمل، وبين حالات العقم الثانية.

وأشارت هذه الدراسة إلى وجود علاقة معنوية بين علامات الإصابة ومضاعفاتها "والتي تمثلت بشكل جلي في الإجراءات المضادة للالتهابات والنزف الدموي والعملة والإصابة بالكلايميديا.

يمكن استخدام نتائج هذه الدراسة في فحص الإنجنيسي المناعي، وذلك لا بد لأن تكون هي الخيار الأول لتشخيص بكتيريا الكلايميديا. هذا وبالرغم
من القيود المالية التي ربما تشكل عائقاً لاستخدام مثل هذه الطريقة في فلسطين، ينبغي علينا أن ندرك أن تكاليف علاج مضاعفات الكلايميديا قد تنخفض بكثير تكاليف هذه التقنية.

الكلمات المفتاحية: الكلايميديا التراكوماتس، مسحات عنق الرحم، الفحص الإنزيمي المناعي، فاعل البوليميريز المستسلم، البلازميد، MOMP، التهابات الرحم، العقم، غزة.
Dedication

To the person who taught me patience, strife, and pushed me towards success in life and give me all care and happiness.

To my father,

mother,

brothers,

wife and
to my children,

Akram

&

Shahd
Acknowledgement

First of all, I thank God for the beneficent and most merciful.

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## Abbreviations

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<td>bp</td>
<td>Base pair</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>Center for disease control and prevention</td>
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<tr>
<td>LGTis</td>
<td>Lower genital tract infections</td>
</tr>
<tr>
<td>LGV</td>
<td>Lymphogranuloma venereum</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MIF</td>
<td>Microimmunofluorescence</td>
</tr>
<tr>
<td>MOH</td>
<td>Ministry of health</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MOMP</td>
<td>Major outer membrane protein</td>
</tr>
<tr>
<td>MoPn</td>
<td>Mouse pneumonitis</td>
</tr>
<tr>
<td>NAATs</td>
<td>Nucleic acid amplification tests</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative predictive value</td>
</tr>
<tr>
<td>OCP</td>
<td>Oral contraceptive pill</td>
</tr>
<tr>
<td>OME</td>
<td>Otitis media with effusion</td>
</tr>
<tr>
<td>Omp</td>
<td>Outer membrane protein</td>
</tr>
<tr>
<td>Omp2</td>
<td>60 kDa cysteine-rich outer membrane protein</td>
</tr>
<tr>
<td>Omp3</td>
<td>12 kDa outer membrane protein</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCE</td>
<td>Plasma cell endometritis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pCT</td>
<td>Chlamydia cryptic plasmid</td>
</tr>
<tr>
<td>PID</td>
<td>Pelvic inflammatory disease</td>
</tr>
<tr>
<td>Pmp</td>
<td>Polymorphic outer membrane protein</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive predictive value</td>
</tr>
<tr>
<td>PROM</td>
<td>Premature rupture of membranes</td>
</tr>
<tr>
<td>RB</td>
<td>Reticulate body</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RTIs</td>
<td>Reproductive tract infections</td>
</tr>
<tr>
<td>SDA</td>
<td>Strand displacement amplification assay</td>
</tr>
<tr>
<td>STDs</td>
<td>Sexually transmitted diseases</td>
</tr>
<tr>
<td>STIs</td>
<td>Sexually transmitted infections</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris- Acetate- EDTA</td>
</tr>
<tr>
<td>TMA</td>
<td>Transcription- mediated amplification assay</td>
</tr>
<tr>
<td>UGTIs</td>
<td>Upper genital tract infections</td>
</tr>
<tr>
<td>VDs</td>
<td>Variable domains</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
</tbody>
</table>
Chapter One

Introduction
1.1. Background of the problem

*Chlamydia trachomatis* is ubiquitous pathogen world-wide and causes ocular, urogenital, and respiratory infections in humans [1]. *C. trachomatis* infection of the lower genital tract is one of the most prevalent sexually transmitted diseases (STDs) in the world [2]. According to the World Health Organization (WHO), approximately 89 million new *C. trachomatis* infections occur annually worldwide [3]. It has been estimated that there are approximately 4 millions new *C. trachomatis* infections each year in the United States [4]. *C. trachomatis* is the leading cause of bacterial STDs in both developed and developing countries, producing lower and upper genital tract infections in males and females, including nongonococcal urethritis, epididymitis and proctitis in males; endocervicitis and pelvic inflammatory disease (PID) in females; and proctitis and reactive arthritis in both. PID and its sequelae such as chronic pelvic pain, ectopic pregnancy, and infertility, account for approximately 80% of the estimated $2.5 billion annual cost of these infections in the United States [5].

Symptoms of chlamydial infection in women include: abnormal genital discharge, painful or frequent urination, burning or itching in vaginal area, redness, swelling or soreness of the vulva or pain in the pelvis during sexual contact. However, up to 80 percent of infected women and 25 percent of infected men may have no symptoms. The infection can be passed from mother to the baby and may cause premature rupture of membrane, preterm birth, pneumonia and conjunctivitis of the baby’s eyes [6]. Additionally, recent studies have suggested that chlamydial genital infections may be a risk factor facilitating sexual transmission of HIV and human papilloma virus (HPV)—induced cervical neoplasia, therefore improved means for prevention and control of early diagnosed cases are necessary [7,8].
Over 18 serotypes of the organism have been identified. Serotypes A, B, Ba, and C are the primary causes of trachoma, a chronic ocular disease found predominantly in developing countries. Serotypes D, Da, E, F, G, H, I, Ia, J, Ja, and K cause lower genital tract infections (LGTIs) and upper genital tract infections (UGTIs). The lymphogranuloma venereum (LGV) serotypes, which include L1, L2, L2a, and L3, also cause STDs, but these infections tend to be associated with more severe invasive disease, such as suppurative proctitis and lymphadenitis [5].

Several laboratory methods are used for the diagnosis of *C. trachomatis*, these include cytological tests for the detection of intracytoplasmic inclusions, cell culture, enzyme immunoassay (EIA), direct immunofluorescence, DNA hybridization techniques and DNA amplification such as polymerase chain reaction (PCR) [37].

Serology has limited value in testing for uncomplicated genital *C. trachomatis* infection and should not be used for screening because previous chlamydial infection frequently elicits long-lasting antibodies that cannot be easily distinguished from the antibodies produced in a current infection [9].

Molecular genetics techniques are useful for the identification of microorganisms that are difficult to cultivate, such as *C. trachomatis* and for those that grow slowly [10]. Techniques to detect *C. trachomatis* in the clinical specimens such as nucleic acid amplification techniques like PCR and ligase chain reaction (LCR) have been developed. These DNA amplification methods are more sensitive than cultural recovery or antigen detection tests [11]. The enzyme immunoassay test is still widely used for the diagnosis of *C. trachomatis*. Although molecular amplification analysis is increasingly used for confirmation testing, its use as a routine screening test for *C. trachomatis* is limited by the high cost for each test compared with current routine methods [12]. The PCR procedure may target chlamydial plasmid as a template for PCR amplification, or the gene of the major outer membrane protein “MOMP”, or ribosomal DNA "rDNA" [13].
1.2. Justification of the study

♦ No previous studies dealing with diagnosis of \textit{C. trachomatis} have been conducted in our area.
♦ \textit{C. trachomatis} infection is considered a worldwide problem, therefore we expect to find the infection in our area.
♦ Infected cases may undergo serious sequelae and need early and prompt treatment.
♦ No published data concerning \textit{C. trachomatis} diagnosis is available and we intend to introduce a technique for routine diagnosis.
♦ Our study may lead to developing a comprehensive screening program aiming at treating and reducing the infection rate of \textit{C. trachomatis}.

In this study, we intend to determine the occurrence of \textit{C. trachomatis}, evaluate the performance of an improved EIA kit (IDEIA PCE Chlamydia) in the detection of \textit{C. trachomatis} in endocervical swab specimens from females attending health care clinics (especially gynecology clinic in AL-Shifa hospital, private hospitals and clinics) and compare the results with those of plasmid- & MOMP-based PCR assays.

1.3. Aims of the study

To determine the occurrence of \textit{C. trachomatis} infection in females attending gynecology and infertility health centers in Gaza strip, and to evaluate the diagnostic utility of PCR, and EIA techniques.

1.4. Objectives of the study

♦ To detect the presence of \textit{C. trachomatis} by EIA (IDEIA™ PCE Chlamydia)
♦ To detect the presence of \textit{C. trachomatis} by molecular DNA amplification (PCR), and to emphasize the diagnostic value of PCR in detection of truly infected female patients.
♦ To compare the performance of the two techniques in detecting *C. trachomatis* among symptomatic cases.

1.5. **Statement of the problem**

♦ What is the occurrence rate of *C. trachomatis* in cervicitis cases in Gaza?
♦ What percentage of infertility cases can be attributed to *C. trachomatis*?
♦ What are the risk factors of *Chlamydia* infection?
♦ What are the advantages and disadvantages of PCR and EIA techniques in diagnosis of *C. trachomatis*?
♦ Is there any difference in the performance of PCR and EIA techniques in detection of *Chlamydia*?

1.6. **Limitation of the study**

♦ Difficulties in endocervical specimens collection, since some women denied participation.
♦ Difficulties in obtaining material and kits for both EIA and PCR, in addition to their high cost, made it impossible to include the asymptomatic cases in our study.
Chapter Two

Review of the literature
2.1. Historical perspective

2.1.1. Taxonomy

*Chlamydiae* are obligate intracellular gram-negative eubacteria with a unique biphasic life cycle. Originally, they were taxonomically categorized as a separate order, *Chlamydiales*, which contained the single family, *Chlamydiaceae*, and only one genus, *Chlamydia*. The genus included four species, which 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*, *C. psittaci*, *C. pneumoniae* and *C. pecorum* [14].

In 1999, a radical change of the chlamydial taxonomy was proposed on the basis of molecular markers. According to the proposed taxonomy, the family *Chlamydiaceae* was divided into two genera, *Chlamydia* and *Chlamydophila*, with nine species [15]. However, the proposal to change the taxonomic nomenclature for the *Chlamydiaceae* family has not been generally accepted in the field [16]. *C. trachomatis* and *C. pneumoniae* are the two chlamydial species pathogenic to humans, whereas the other species occur mainly in animals and birds.

*C. trachomatis* species have been divided into 18 serotypes or serovars, according to the *Omp1* gene that encodes the MOMP. Typing of the *Omp1* gene is used to classify *C. trachomatis* strains. Further, *C. trachomatis* serotypes can be divided into three biovars: trachoma, lymphogranuloma venereum (LGV) and mouse pneumonitis (MoPn). The trachoma biovar includes the 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. The number of serotypes within the *C. psittaci* species is unknown, but it is genetically more heterogeneous than *C. trachomatis*. *C. pecorum*, which causes infections in ruminants, was established as a species distinct from *C. psittaci*. Recently, studies on the possible specific relationships between the
*Chlamydiae* have highlighted the need for assessment of the taxonomy of chlamydia-related organisms. This is currently under discussion [17].

So far, it seems that *C. pneumoniae* has only one serovar, with almost 100% DNA homology between the strains but less than 10% homology with the other *Chlamydiae* [17].

The essential features of the three chlamydial species are presented in Table 2.1. below.

### Table 2.1: Essential features of chlamydial species [14].

<table>
<thead>
<tr>
<th>Feature</th>
<th><em>C. pneumoniae</em></th>
<th><em>C. trachomatis</em></th>
<th><em>C. psittaci</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural hosts</td>
<td>human, (koala, horse, frog)</td>
<td>human</td>
<td>birds and mammals</td>
</tr>
<tr>
<td>Host cell tropism</td>
<td>epithelial cells, mononuclear lymphocytes, endothelial cells</td>
<td>epithelial cells, mononuclear lymphocytes (LGV)</td>
<td>mononuclear lymphocytes, epithelial cells other cell types</td>
</tr>
<tr>
<td>Major human disease</td>
<td>pneumonia, respiratory tract infection</td>
<td>trachoma, STD</td>
<td>pneumonia abortion</td>
</tr>
<tr>
<td>Transmission</td>
<td>aerosol</td>
<td>sexual, neonatal; hand to eye, flies (trachoma)</td>
<td>aerosol, excretions</td>
</tr>
<tr>
<td>Number of serovars</td>
<td>1(?)</td>
<td>18</td>
<td>unknown</td>
</tr>
<tr>
<td>DNA homology with <em>C. pneumoniae</em> (%)</td>
<td>94–100</td>
<td>&lt;5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>MOMP-containing species-specific antigens</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Sensitivity to macrolides and tetracycline</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Sensitivity to sulpha</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Glycogen in inclusions</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Morphology of elementary body</td>
<td>Pear-shaped or round</td>
<td>round</td>
<td>round</td>
</tr>
</tbody>
</table>
2.1.2. Biology of *Chlamydia*

2.1.2.1. Growth cycle

*Chlamydiae* are intracellular bacteria that have a unique biphasic developmental cycle with two distinct morphological forms. The extracellular, infectious form (0.3 μm) is called elementary body (EB), and the intracellular, replicating form (1.0 μm) is called reticulate body (RB). Infectious EBs start the cycle by attaching to a susceptible host cell membrane. They gain access into the host cell via either parasite-specified phagocytosis or receptor-mediated endocytosis. Tyrosine phosphorylation of host cell proteins and actin cytoskeletal rearrangement may be involved [18]. When inside the cell, the chlamydiae 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 [19]. In cell culture conditions, the duration of the developmental cycle is between 2 and 3 days. In natural infections, the situation is more complicated, and the normal Chlamydia development is easily disturbed. Certain circumstances (nutrient deficiency, interferon gamma, antibiotics) may result in morphological alterations of RBs and the emergence of enlarged, atypical chlamydial forms. These aberrant forms may persist inside the host cell in a viable but non-culturable state for a long time and result in persistent infection despite activation of the immune defence mechanisms [14].

The cycle of both normal and altered development of *Chlamydia* is presented in Figure 2.1.
2.1.2.2. Structural characteristics

Like all gram-negative bacteria, chlamydial cells appear to be surrounded by a double membrane. However, unlike the 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 (Omp), the single predominant protein being the MOMP of 38 to 42 kDa, comprising about 60% of the 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 the 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 [14].

![Developmental cycle of Chlamydia](image)
Chlamydial lipopolysaccharide (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 [14].

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 Hsps 60, but also Hsp 70 and Hsp10 have been implicated as important agents in the immunopathology of chlamydial infections. *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 [14]. A model of chlamydial elementary body cell wall is depicted in Figure 2.2.

![Figure 2.2: A model of chlamydial elementary body cell wall [18]](image_url)
2.1.2.3. Genome of *Chlamydia trachomatis*

2.1.2.3.1. Chlamydial chromosome

*Chlamydia 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 a *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), designated VD1, VD2, VD3, and VD4, 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 the *omp1* gene product, MOMP, spans the outer membrane of the cell envelope 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 [18].

2.1.2.3.2. Plasmid

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 maintenance of 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 [18].

2.2. Clinical spectrum of C. trachomatis infections

C. trachomatis infects primarily columnar and pseudostratified columnar epithelium, but not squamous epithelium, which makes it a pathogen of the mucosal surface [11].

2.2.1. Trachoma

Trachoma is the world’s leading cause of preventable blindness. The manifestations of ocular trachoma infections range from mild conjunctival lesions [follicular conjunctivitis] to severe forms that eventually lead to scarring and blindness. Severe forms develop through repeated or persistent infections by the C. trachomatis serovars A, B, Ba and C. Therefore, trachoma is considered the prototype of chronic chlamydial infection. It has been estimated that about 500 million people have had the disease. In the developing countries, about 7 to 9 million people are estimated to be blind because of C. trachomatis infection. Trachoma is endemic mainly in tropical and subtropical countries. The main reservoir of the organism is the eye of an infected person, usually a child, and transmission may be potentiated by flies that carry infected secretion from person to person [14].
2.2.2. *C. trachomatis* infections in women

*C. trachomatis* studies have mostly focused on sexually transmitted infections, since the same organism that causes trachoma is considered the world’s most common sexually transmitted bacterial pathogen. The WHO had estimated that about 89 million of all new STDs infections are caused by *C. trachomatis*. The highest rates are found in young, sexually active populations [20].

Most infections caused by *C. trachomatis* in women are asymptomatic. However, clinical manifestations include cervicitis, urethritis, endometritis, PID, and abscess of the Bartholin’s glands [21]. The initial site of infection is usually the cervix, but the urethra and the rectum may also be infected. Genital *C. trachomatis* infection is asymptomatic in up to 80% of women, and only 20% of infected women present with symptoms [21].

*C. trachomatis* infection may persist subclinically in the endometrium for a long time and produce chronic subclinical infection analogous to trachoma. The presence of plasma cells in the endometrial stroma {i.e., plasma cell endometritis (PCE)} is characteristic to chronic endometritis. *C. trachomatis* has been reported as a causative agent of PCE cases and is also associated more often in severe PCE with lymphoid than non-chlamydial endometritis. Women with chlamydia isolated from the cervix often show no signs or symptoms of infection. On examination, however, at least one third generally have local signs of infection, such as endocervical bleeding, mucopurulent endocervical discharge, and edema within the area of ectopy. It has been reported that colposcopic features of immature squamous cell metaplasia of the cervix are associated with chlamydial infection. The number of polymorphonuclear leukocytes in cervical mucus also correlates with chlamydial infection of the cervix. Finally, patients with cervicitis caused by *C. trachomatis* are at risk for further development of PID. PID has been defined as a syndrome associated with spreading of micro-organisms from the vagina and cervix to the endometrium, salpingeal tubes and adjacent structures. Today, the majority of PID episodes of known etiology are caused by *C. trachomatis*. A large proportion of *C. trachomatis* infections in the salpingeal tubes are
asymptomatic, subclinical or atypical, and difficult to recognize as PID. With repeated infections, the risk of PID increases [17].

In tubal infection, the fibrosis and scarring lead to permanent tubal damage, which increases the risk of ectopic pregnancy and tubal factor infertility. The more episodes of PID one has had, the higher is the risk for infertility. Untreated chlamydial infection during pregnancy is suggested to be associated with a number of adverse outcomes, including preterm labor, premature rupture of membranes, low birth weight, neonatal death and postpartum endometritis [22,23].

2.2.2.1. Risk and demographic factors for C. trachomatis infections in women

The most common demographic factors correlate infection with C. trachomatis in women is young age (<25 years). The biologic basis for this association is thought to be anatomic differences in the cervix of younger women, where in the squamocolumnar junction, a primary host target for C. trachomatis, is everted and thus more exposed, a condition known as ectopy. Demographic factors associated with older women include unmarried status, black race, and poor socioeconomic conditions. Higher numbers of sexual partners, a new sexual partner, lack of use of barrier contraceptive devices, and concurrent gonococcal infection are also consistently associated with chlamydial infections. The use of oral contraceptives is associated with cervical chlamydial infections but not PID; this is believed to be a result of induced ectopy; however, the relationship may be confounded in some studies by behavioral factors and has thus been controversial [11].
2.2.3. Lymphogranuloma venereum

LGV is a sexually transmitted systemic infection caused by the *C. trachomatis* serovars L1, L2 and L3. LGV is uncommon in industrialized countries but highly prevalent in parts of Africa, Asia and South America, and it occurs in both men and women. The LGV serovars of *C. trachomatis* are more invasive than the other genital serovars [11]. It predominantly infects lymphatic tissue but may also occur as an acute symptomatic infection without apparent lymph node involvement or tissue reaction at the point of infection [24]. Until 2003, LGV was considered a rare disease outside resource-poor countries. Since then, it has emerged as a significant problem among men who have sex with men in Europe. In 2003, an outbreak of LGV was recognized in the Netherlands [26]. Since that report, similar outbreaks have been seen in France. Cases have also been reported from Sweden and, more recently, from the United States and Canada. All the reported cases have been caused by the L2 serovar, although there is some evidence that a number of genetically distinct strains of *C. trachomatis* L2 are responsible for these outbreaks [25].
2.3. Diagnosis of chlamydial infection

2.3.1. Culture

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), the method involves many difficulties. Culture has a specificity that approaches 100%, but it is relatively insensitive being only 50%-85% compared to DNA amplification tests [11]. Since culture detects only viable infectious chlamydial elementary bodies and has minimal potential for contamination, it has still remained the standard for medico-legal purposes [11, 26].

2.3.2. Antigen detection

2.3.2.1. Enzyme immunoassay (EIA)

The present antigen detection methods are based on the demonstration of genus-specific chlamydial LPS and cannot differentiate between chlamydial species [11]. For antigen detection, the presence of viable Chlamydiae is not required and it may therefore be useful in the diagnosis of chronic chlamydial infections, if sufficient amounts of antigens are present [17]. EIA designed for C. trachomatis can also be used for the detection of the C. pneumoniae antigen, since the capture antibody in chlamydia EIA kits is a genus-specific LPS.

Antigen detection by EIA, however, is considered more sensitive than culture in chronic C. trachomatis infections [17]. EIA also known as Enzyme-linked immunosorbent (ELISA) assays, are designed to detect antigens or antibodies by producing an enzyme-triggered color change. For C. trachomatis, enzyme immunoassay usually refers to an antigen detection test, with antibody used to detect chlamydial antigen contained in the specimen. There are many C. trachomatis enzyme immunoassays on the market, each with slightly different configurations, but almost all detect chlamydial lipopolysaccharide with the same sandwich immunoassay principle [18]. One disadvantage of the EIA methods that detect LPS is the potential for false-positive results caused by cross-reaction with LPS of other microorganisms, including other Chlamydia
species. Manufacturers have developed blocking assays that verify positive EIA test results. The test is repeated on positive specimens with the addition of a monoclonal antibody specific for chlamydia LPS. The monoclonal antibody competitively inhibits chlamydia-specific binding by the enzyme-labeled antibody; a negative test result when using the blocking antibody is interpreted as verification of the initial positive test result. EIA tests should not be used with rectal specimens because of cross-reactions with fecal bacteria [9].

2.3.2.2. The direct fluorescence antibody technique (DFA)

The 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 [11].

2.3.3. Serology

2.3.3.1. Complement fixation (CF)

In the CF test, the target of the antibodies is 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 microimmunofluorescence (MIF) test and has objective end-points. The treatment with antibiotics may delay or diminish the production of CF antibodies decreasing the sensitivity of the test [11].

2.3.3.2. Microimmunofluorescence test (MIF)

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 a serological diagnosis is a fourfold rise in the IgG or IgA titre or a single IgM titre of $\geq 16$ for both *C. pneumoniae* and *C. trachomatis* [17]. The criteria for seropositivity using MIF are shown in Table 2.2. However, as an acute *C. pneumoniae* infection usually induces high levels of IgG antibodies by MIF, the same phenomenon is infrequently seen in infections with other chlamydial species. In addition, the need for paired sera to show a fourfold rise in IgG titre and the fact that the IgG antibody response may occur 6–8 weeks after the onset of illness, limit the use of MIF in the diagnosis of primary infections [17]. In reinfections, IgG and IgA titres rise quickly, i.e. in 1–2 weeks without IgM response. Both elevated short-lived IgA antibodies and microbe-specific circulating immune complex (IC) have been shown to persist in chronic *C. pneumoniae* infections. ICs consist of microbial antigens and antibodies produced in defence against pathogens. Their presence in the circulation is a sign of continuous production of microbial antigens in the close vicinity of the vascular system, and their presence is hence a potential marker for persistent infection. The presence of circulating ICs is typical for many chronic viral and bacterial diseases [17].

Table 2.2: Criteria for serodiagnosis of chlamydial infections by MIF[14].

<table>
<thead>
<tr>
<th>Chlamydial infection</th>
<th>Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute infection</td>
<td>IgM titre $\geq 16$.</td>
</tr>
<tr>
<td></td>
<td>Fourfold rise in IgG titre.</td>
</tr>
<tr>
<td></td>
<td>Fourfold rise in IgA titre.</td>
</tr>
<tr>
<td>Chronic infection</td>
<td>Persistent presence of elevated IgG and/or IgA antibodies and/or ICs.</td>
</tr>
</tbody>
</table>
2.3.4. Nucleic acid based tests

2.3.4.1. Nucleic acid hybridization (probe) tests

Nucleic acid hybridization techniques were successfully used to detect infected cells from tissue cultures, 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 [18].

Two nucleic acid hybridization assays are FDA-cleared to detect *C. trachomatis*: the Gen-Probe PACE®2 and the Digene Hybrid Capture® II 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 the 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 [9].

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 <7 days without refrigeration before receipt and testing by the laboratory [9].

2.3.4.2. Nucleic acid amplification

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 target 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, purified white blood cells and tissues. The most widely known 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* [14]. 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. Ligase chain reaction (LCR) is another commercially available nucleic acid amplification technology used for the diagnosis of *C. trachomatis*. Including the most commonly used PCR, LCR, transcription mediated amplification (TMA) assay, and strand displacement amplification (SDA) assay based diagnostic tests are commercially available for diagnostic and research purposes. Recently, a quantitative real-time PCR technique has also been developed [14].

A number of different nucleic acid sequences have been used as targets in PCRs for the detection of *C. trachomatis*. These include the chlamydial cryptic 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 includes two complete rRNA operons and single copies of *omp1* and *omp2* [18]. 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 Amplicor, targeting pCT specific sequence. For urogenital swabs and urine, the two formats were appear to have comparable sensitivity and specificity [27].

**2.3.5. Confirmatory testing**

The most recent guidelines from CDC on prevention and management of *C. trachomatis* infections recommend that all positive results of nonculture tests performed on low-prevalence or low-risk populations be confirmed. Confirmation of nonculture tests is required for a definitive diagnosis of *C. trachomatis* infections [11]. The CDC Clinical Practice Guidelines define diagnosis of *C. trachomatis* in three categories: suggestive, presumptive, and definitive (as presented in Table 2.3.)
Table 2.3: The CDC guidelines for diagnosis of infections with non-LGV strains of *C. trachomatis*

<table>
<thead>
<tr>
<th>Definitive (requires 1 or 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Isolation and confirmed identification of <em>C. trachomatis</em> in tissue culture from cervical, rectal, or urethral exudate and identification of characteristic intracellular inclusions.</td>
</tr>
<tr>
<td>2. Identification of <em>C. trachomatis</em> by one of the following methods and confirmation by a second culture or nonculture test method</td>
</tr>
<tr>
<td>a. Identification of the organism by DFA test of exudates.</td>
</tr>
<tr>
<td>b. Detection of antigen by EIA of exudates.</td>
</tr>
<tr>
<td>c. Detection of nucleic acid from exudate by DNA probe or DNA amplification technique.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Presumptive (requires 1 and 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Presence or absence of clinical symptoms (e.g., mucopurulent cervicitis, urethritis, epididymitis, PID).</td>
</tr>
<tr>
<td>2. Detection of <em>C. trachomatis</em> by a nonculture test.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Suggestive (requires 1 and either 2 or 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Clinical symptoms (e.g., mucopurulent cervicitis, urethritis, epididymitis, PID).</td>
</tr>
<tr>
<td>2. Exclusion of other causes of discharge or exudate (e.g., gonorrhea).</td>
</tr>
<tr>
<td>3. Sexual exposure to a person infected with <em>C. trachomatis</em> or recently diagnosed with nongonococcal urethritis, mucopurulent cervicitis, or PID.</td>
</tr>
</tbody>
</table>
All positive tests are considered to be presumptive evidence of infection and should be confirmed (as shown in Table 2.4.) [9].

Table 2.4: Additional testing and patient management after a positive screening test.

| ♦ All positive screening tests should be considered presumptive evidence of infection |
| ♦ An additional test should be considered after a positive screening test if a false-positive screening test would result in substantial adverse medical, social, or psychological impact for patient. |
| ♦ Consideration should be given to routinely performing an additional test after a positive screening test if the positive predictive value is considered low (e.g., 90%). |
| ♦ Patients should be counseled regarding prompt treatment after positive screening test because an additional test might be falsely negative. |

2.3.6. C. trachomatis detection and prevalence

Mahony et al. (1994) conducted a tricenter interlaboratory agreement study to assess the agreement of PCR results obtained for detection of C. trachomatis in genitourinary specimens. Two sets of plasmid PCR primers (KL1-KL2 and T1-T2) used to test each specimen; with the first set of primers (KL1-KL2) and to confirm positives with the second set of primers (T1-T2). They demonstrated excellent agreement of PCR results obtained in three different laboratories. The overall accuracy of PCR after confirmatory testing results obtained for the three laboratories was 96.6% (345 of 357). Use of a confirmatory PCR improved the specificity and overall accuracy of results for individual laboratories by eliminating false positives but reduced slightly the agreement of results obtained between laboratories [28].
Lan et al. (1995) reported that the PCR can be successfully applied for the detection of \textit{C. trachomatis} DNA in paraffin embedded tissues and archival cervical smears, indicating that this technique could be very useful for the retrospective analysis of the role of \textit{C. trachomatis} in late complications such as ectopic pregnancy. They examined archival paraffin embedded salpingectomy tissues (n= 48) from 37 women with ectopic pregnancy, for the presence of \textit{C. trachomatis} plasmid and \textit{omp1} DNA by PCR. In addition, they examined preceding cervical specimens (n=58) stored either as cervical cell suspensions or as archival cervical smears, and preceding endometrial biopsies [n=18], taken 0-5.8 years before the ectopic pregnancy.

\textit{C. trachomatis} DNA was detected in only one of the 48 salpingectomy specimens from 37 women. However, in six of the 37 women, \textit{C. trachomatis} DNA was detected in the genital specimens (cervix and/or endometrial) taken before salpingectomy. \textit{C. trachomatis} infections were mostly found in endometrial or cervical specimens taken more than three years before ectopic pregnancy. No chlamydial DNA was found in endometrial or cervical specimens taken at the same time of the ectopic pregnancy [29].

Gaydos et al. (1998) conducted a large survey of women in the U.S. military recruits. Urine specimens from 13,204 new female U.S. army recruits from 50 states were screened by ligase chain reaction for \textit{C. trachomatis} infection. Information on potential risk factors was obtained by questionnaire. Results of their study demonstrated that the overall prevalence of chlamydial infection was 9.2 percent, with a peak of 12.2 percent among the 17-year-old recruits. The following risk factors were independently associated with chlamydial infection: having ever had vaginal sex, being 25 years of age or less, being black, having had more than one sex partner in the previous 90 days, having had a new partner in the previous 90 days, having had a partner in the previous 90 days who did not always use condoms, and having ever had a sexually transmitted disease. A screening program for subjects 25 years of age or less would have identified 95.3 percent of the infected women. They concluded, 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 [30].

Schlott et al. (1998) established an in situ PCR (IS-PCR) assay to detect the presence of C. trachomatis in endocervical swabs. In addition, histological sections of endocervical squamous cell carcinoma were analyzed because previous studies had revealed a significant association with C. trachomatis. They used biotin-labeled nucleotides that were incorporated into the product during PCR. Labeled DNA fragments were detected with, anti-biotin monoclonal antibody immunoglobulin. The results indicated that in situ PCR is an effective technique for localizing C. trachomatis in target cells [31].

Mohamed and Sharaf (2001) examined 70 Egyptian females that have been classified into: 20 patients with tubal infertility; 15 patients with ectopic pregnancy; 20 patients with premature rupture of membrane (PROM) and 15 apparently healthy women (asymptomatic group). Results revealed that C. trachomatis DNA was detected by PCR in the endocervix of 17 out of 55 infected cases (30.9%), and (2 out of 15) 13.3% of asymptomatic group. The tubal infertility group showed the highest percentage of active C. trachomatis infection (45%), (P<0.05). PCR showed better sensitivity than direct immunofluorescent technique (DIF) for detection of C. trachomatis infection (96.2% versus 70% respectively), however both techniques had 100% specificity. Tubal infertility group showed highest sero prevalence IgG (45%), followed by ectopic pregnancy group (35%) (P<0.001 and P<0.05 respectively) [6].

Noda et al. (2002) detected C. trachomatis in endocervical exudates by PCR; Fifty nine specimens of endocervical exudate from women that were seen at infertility clinics and at the consultation room of menstrual regulation, in Havana City, were processed to evaluate the performance of a method to detect C. trachomatis, based on (PCR) with primers specific for the plasmid. The specimens were assayed by plasmid-based PCR, by cell culture and by another
method of PCR based on the amplification of a region of the main protein of the external membrane (i.e., MOMP) of *Chlamydia*, which was used as a confirmatory trial. Results of the 59 studied specimens indicated that 43 presented coincident results between the culture and the PCR-plasmid: 4 positive and 39 negative. The 16 remaining showed discordant results. An inhibition study was conducted in the 8 culture negative/PCR-plasmid positive specimens and it was proved that 2 of them had inhibitors, whose action was reverted on adding bovine serum albumin (BSA) to the reaction mixture. Of the 8 negative culture/positive PCR-plasmid discordant specimens, 5 were confirmed as positive after being processed by PCR-MOMP. 100% of sensitivity and 94% of specificity were obtained for PCR-plasmid compared with 54% and 87% for the culture, respectively. The PCR-plasmid presented a positive predictive value of 79% and a negative predictive value of 100%; whereas the culture had 50% and 89%, respectively [32].

Awwad et al. (2003) detected *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 results of their study demonstrated that the prevalence of chlamydial infection was 4.6% among symptomatic patients with urethritis. The difference in prevalence was statistically insignificant (P > 0.05) 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. They concluded that 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 [33].
Bamberger and colleagues (2003) examined the prevalence of *C. trachomatis* infection in urine specimens of 708 Israeli female soldiers by PCR (Roche Amplicor, Branchburg, 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 the Israeli 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 [34].

George et al. (2003) 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 143 patients 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 had been resolved by using a MOMP PCR, the overall sensitivity, specificity, and positive and negative predictive values for the 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 [35].

Malathi et al. (2003) determined the prevalence of *C. trachomatis* causing conjunctivitis using PCR. A total of 328 conjunctival swabs from 255 (both eyes 73 and one eye 182) Indian patients were investigated by fluorescent antibody test (FAT) on direct smears, culture and PCRs for cryptic plasmid and major outer membrane protein (MOMP1) gene of *C. trachomatis*. An infant with ophthalmia neonatorum was also included. Results of their study revealed that among 328 specimens processed, 16 (4.9%) from 12 (4.7%) patients were positive by cryptic plasmid PCR. Among these, 3 from 2 patients were positive by FAT (direct smear), culture and PCR for MOMP 1 gene. Both eyes of the infant with ophthalmia neonatorum were positive by all the methods. The
sensitivity of FAT and culture (18.8%) was lower compared to PCR. PCR using cryptic plasmid primers was found to be the most sensitive method to detect *C. trachomatis* in patients with conjunctivitis [36].

Santos cristina et al. (2003) detected *C. trachomatis* in endocervical smears of sexually active women in Brazil. They found a prevalence of 20.7% women infected with *C. trachomatis*, and 80% of the positive cases were found to be in the ≤ 23 year old age group. These findings are in agreement with most previous reports, which indicate that *C. trachomatis* mainly attacks young women, apparently because they are at risk due to behaviors such as sex without protection and multiple sexual partners [37].

Apea-Kubi et al. (2004) determined the prevalence of *Neisseria gonorrhea*, *C. trachomatis* and *Treponema pallidum* in antenatal and gynecological patients at Korle-Bu Teaching Hospital, Ghana. A total of 517 specimens were examined for *Treponema pallidum*, while 465 endocervical swabs were examined for both *N. gonorrhea*, *C. trachomatis* using rRNA detection kit. While vaginal swab were examined for *Trichomonas vaginalis*, *Candida albicans*, *Gardnerella vaginalis*. Sera were examined for *T. pallidum* with passive particle hemagglutination assay kit. The prevalence rate was 3% (14 out of 465), 0.6% (3 out of 465), and 5.6% (29 out of 517) for *C. trachomatis*, *N. gonorrhea*, and *T. pallidum* respectively. The authors concluded that the low rate of *C. trachomatis* and *N. gonorrhea* might be due to self medications [38].

Gazal-Aswad et al. (2004) conducted a cross-sectional study on married women who attend primary and secondary health centers, in United Arab Emirates (UAE), for national cervical screening survey. They used rapid enzyme immunoassay test for direct detection of *C. trachomatis* antigen in endocervical swab specimens. All women were taken irrespective of age. Result of their study showed a prevalence of chlamydial infection of 2.6%. [39].
Klavs et al. (2004) 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 five urine specimens. Internal control was used to identify inhibitory specimens, and specimen 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 provided support for the introduction of chlamydia screening in Slovenia [40].

Nessa et al. (2004) studied prevalence of sexually transmitted infections (STIs) and reproductive tract infections (RTIs) among hotel-based sex workers (HBSWs) in Dhaka, Bangladesh. Endocervical swabs, high vaginal swabs, and blood samples from 400 HBSWs were examined for *Neisseria gonorrhoeae* (by culture), *C. trachomatis* (by Amplicor PCR), *Trichomonas vaginalis* (by microscopy), antibody to *Treponema pallidum* (by both rapid plasma reagin and *Treponema pallidum* hemagglutination tests), and antibody to herpes simplex virus type 2 (HSV-2) by (ELISA). Sociodemographic information as well as gynecological and obstetric information were collected. Among the HBSWs, 228 women (57%) were symptomatic and 172 (43%) were asymptomatic. 35.8% were positive for *N. gonorrhoeae*, 43.5% were positive for *C. trachomatis*, and 4.3% were positive for *T. vaginalis*. A total of 8.5% had syphilis, 34.5% were positive for HSV-2, and 86.8% were positive for at least one RTI or STI. These data suggested a high prevalence of STIs, particularly gonorrhea and chlamydia, among HBSWs in Dhaka [41].
Fallah et al. (2005) conducted a study for detection of *C. trachomatis* from urine specimens by PCR in Iranian women with cervicitis. The results 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 all 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 results 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 in users of IUD for contraception [42].

Keles et al. (2005) detected *C. trachomatis* by PCR in middle ear fluid in otitis media with effusion. They examined the middle ear aspirates of otitis media with effusion (OME) cases for the presence of *C. trachomatis* and tried to demonstrate a possible relationship between the presence of *C. trachomatis* by PCR and the nature of the effusion material. Of the seventy-eight middle ear effusions, 26 (33.3%) were serous, 31 were (39.7%) mucoid and 21 (26.9) were sero-mucoid in character. *C. trachomatis* DNA genome was identified in seven (8.9%) effusion specimens. Of these, three were mucoid (42.8%), three were serous (42.8) and one was sero-mucoid (14.2%) in character. There was no correlation of statistical significance between the character of the middle ear effusion and the presence of *C. trachomatis* (p >0.05). They concluded that *C. trachomatis* should be taken into consideration in the treatment of otitis media with effusion [43].

Yazdi et al. (2006) conducted a comparative study among Iranian women suffering from cervicitis. They used DFA and Plasmid-based PCR for detection of *C. trachomatis* in endocervical specimens. The study population was married women aged between 20 and 55 yr. A history of STI was reported by 38 (26.8%) of the 142 study cases. According to PCR results, the prevalence of
chlamydial infection was 15.5% (22 out of 142), while the DFA showed a prevalence of 14.1% (20 out of 142). The prevalence was highest among women aged 25-29 yr and 35-39 yr. No statistically significant difference was found between the two diagnosis methods used in this study. On the other hand, there was a significant statistical relation between positive test results and bearing history of sexually transmitted infections [44].

Stothard et al. (1998) recovered *C. trachomatis* in tissue culture from a urogenital specimen which tested negative by commercial plasmid-based PCR. Immunotyping and *omp1* sequencing identified the isolate as a serovar E isolate. Further investigation by PCR and Southern hybridization indicated that this isolate lacks the chlamydial cryptic plasmid [45].

2.3.7. Genotyping based on *C. trachomatis* MOMP and serovars distribution

Lan et al. (1995) investigated the prevalence rates and serovar distributions of *C. trachomatis* cervical infections in two different groups of women. Group I consisted of 393 asymptomatic young women (aged 17 to 30 years) who were invited to participate in a *C. trachomatis* screening program. Group II consisted of 734 randomly selected patients (aged 17 to 68 years) attending an inner-city gynecological outpatient clinic. *C. trachomatis* was detected in cervical scrapes by PCR specific for endogenous plasmid. These plasmid PCR-positive specimens were subsequently subjected to genotyping by *C. trachomatis*-specific *omp1* PCR-based restriction fragment length polymorphism analysis. The overall prevalence rates of *C. trachomatis* found in patients younger than 30 years were 9.2 and 11.8% in groups I and II, respectively. A clear age dependency was seen in group II, with the highest prevalence rate [20%] found in patients younger than 20 years, while the rate declined significantly after 30 years of age (5.9%). In women younger than 30 years, the genotyping results showed that serovars E, I, and D (in decreasing order) were frequent in group I, while serovars F, E, and G (in decreasing order) were predominantly found in group II. The study showed that *C. trachomatis* infections are highly prevalent in
asymptomatic young women. The different serovar distributions found most likely reflect the different compositions of the study groups, but additional analysis of the case histories of individual patients suggested that certain serovars might be associated with symptomatic (i.e., serovar G) or asymptomatic (i.e., serovars D and I) infections [46].

Jurstrand et al. (2001) developed a method for detection and genotyping of genital \textit{C. trachomatis} infections based on \textit{omp1} gene amplification and sequencing. They showed that genotypes E and F were dominant, and the individual sequences were stable and showed limited variation. That \textit{omp1} genotyping method provided interesting results concerning double infections and reinfections and could be useful for epidemiological characterization of circulating \textit{C. trachomatis} strains in Swedish community [47].

\textbf{Kouri} et al. (2002) conducted a study to determine the prevalence rates and serovar distribution of \textit{C. trachomatis} cervical infections in Cuban women employing two different groups were selected. Group I consisted of 60 human immunodeficiency virus (HIV-1) seropositive women from different regions of Cuba and group II of 60 randomly selected women HIV seronegative and apparently healthy. \textit{C. trachomatis} was detected in cervical scrapes by mean of nested PCR specific for MOMP. Of the 120 cases that were screened for \textit{C. trachomatis} MOMP PCR, 10 (8.3%) were found positive. The overall prevalence rate of \textit{C. trachomatis} in cervical scrapes determined by nested PCR was 10\% (6 cases) in group I and the estimated prevalence was 6.6\% (4 cases) for group II; 83.3\% of HIV seropositive women with \textit{C. trachomatis} infection reported history of pelvic inflammatory disease followed by cervicitis (50\%). The control group \textit{C. trachomatis}-infected women referred a history of cervicitis in 75\% of cases. The study was the first report of \textit{C. trachomatis} prevalence in Cuba. It showed that there was not significant difference in the prevalence rate of \textit{C. trachomatis} between both groups. The \textit{C. trachomatis} positive scrapes detected by MOMP nested PCR were genotyped by RFLP analysis. Serovar E was found in six cases from both groups whereas serovar L2 was found in one HIV
seropositive woman. The other 3 cases were weakly positive and therefore, were not typed [48].

Singh et al. (2003) examined the presence of C. trachomatis in 280 Indian endocervical swab specimens by PCR specific for endogenous plasmid. Age dependency was seen in symptomatic patients, with a high chlamydial prevalence rate (28%) found in younger women (age, 18 to 25 years). Genotyping by RFLP analysis of omp1 PCR-positive samples showed serovars D, E, and F to be the most prevalent [49].

Lister et al. (2005) conducted a study on C. trachomatis serovars causing urogenital infections in women in Melbourne, Australia. They found that omp1 PCR has a lower sensitivity in comparison to the plasmid-directed COBAS Amplicor PCR, and a nested omp1 PCR was used to achieve a higher amplicon yield for sequencing. Sequence analysis based on omp1 showed that the most prevalent serovar was E, followed by F, G, J, D, and K [50].

Yamazaki et al. (2005) performed a study concerning distribution of C. trachomatis serovars among female prostitutes and non-prostitutes in Thailand, and non-prostitutes in Japan during the Mid-90s. Their results showed that the most prevalent serovar was F (35.2%) followed by serovar E (18.3%) in both prostitutes and non-prostitutes in Thailand. On the other hand serovar D was the most frequent in non-prostitutes in Japan (31.9%), followed by serovar F (17%) and E (17%) [1].

2.3.8. Pooling of specimens

Currie et al. (2004) determined the accuracy and cost savings associated with pooling vaginal swabs as well as endocervical swabs and urine specimens for the detection of C. trachomatis by PCR. Pooling the specimens resulted in a 60% reduction in the number of tests performed (1,114 versus 2,769 tests) without significant loss of accuracy. A 39% reduction in total costs consisted of a 43% reduction in the quantity of reagents used, a 55% reduction in the costs of other consumables, and a 26% reduction in technologist's time [51].
Tan and Chan (2005) used PCR on pooled cervical swabs to detect *C. trachomatis* in female sex workers in Singapore. Endocervical swab specimens were analyzed by EIA as well as in pools of five specimens using PCR. Any pool with a positive PCR result for *C. trachomatis* infections was subjected to repeat PCR testing of the five individual specimens in the pool. The PCR inhibition rate was 0 percent with the use of pooling. There were a total of 48 confirmed cases of *C. trachomatis* infection by PCR while EIA detected 19 positive specimens. They concluded that *C. trachomatis* infections were found to be higher using PCR technology. Less sensitive methods such as EIA result in undertreatment or otherwise undetected cases. In addition, the pooling strategy using pool sizes of five specimens, is reliable and cost-effective [52].

### 2.3.9. Quality of specimens

**Welsh** et al. (1997) studied the influence of endocervical specimen adequacy on PCR and DFA staining for detection of *C. trachomatis* infections. The cellular quality of the endocervical swab specimen used for the detection of *C. trachomatis* may dramatically impact the sensitivity of the diagnostic assay used. Cellular adequacy for a cervical swab specimen was defined as the presence of one or more columnar epithelial or metaplastic epithelial cells or the presence of more than 100 erythrocytes per high-power microscopic field. Results of their study concluded that variations in specimen quality and the sensitivity of the diagnostic assay used have a significant impact on determining the prevalence of *C. trachomatis* in a population [53].

**Coutlee** et al. (2000) showed that an important proportion of genital specimens submitted for *C. trachomatis* detection by PCR contain small amounts of cellular DNA as measured by human beta-globin gene amplification. They assessed the quality of genital samples submitted for *C. trachomatis* detection by using a second PCR assay for the presence of human beta-globin DNA [54].
Sturm-Ramirez et al. (2000) determined the prevalence and heterogeneity of C. trachomatis infections in a cohort of female sex workers in Dakar (Senegal) using endocervical-swab-based PCR DNA amplification assays. A total of 740 endocervical swabs were collected and DNA extracted, 722 (97.7%) of which were found to be positive by β-globin PCR. The 722 women from whom these swabs were collected constituted the study population. The overall prevalence of cervical chlamydial infection was 28.5% (206 of 722), and most of these infections were asymptomatic. A total of 21 specimens positive for C. trachomatis by diagnostic PCR (plasmid PCR) were subjected to omp1 amplification and then sequenced. Six different C. trachomatis genotypes were identified based on phylogenetic analysis of the omp1 gene sequences. Interestingly, genotype E predominated 47.6% (10 out of 21) and was not associated with visible signs of cervical inflammation compared to non-E genotypes (P < 0.05). Overall, the high rate of asymptomatic C. trachomatis infection by genotype E may suggest genotype-specific properties that confer a transmission advantage in this high risk population [55].

2.3.10. Endocervical swab specimens order

In a study reported by Ghanem et al. (2005) the order of three endocervical specimens for C. trachomatis testing were randomized to determine whether test performance measures of two nucleic acid amplification and a DNA probe were affected by swab order. They concluded that the order of swab collection from the endocervix does not influence the performance characteristics of the PCR, LCR, and PACE 2 tests in diagnosing C. trachomatis infections [56].

2.3.11. Vaginal specimens usage

Domeika and Drulyte (2000) determined whether the patient self-obtained and mailed vaginal sample might be used for the detection of genital C. trachomatis infection by the PCR. They concluded that self-collected and mailed vaginal specimen is convenient for the patient and useful for the PCR-testing for genital C. trachomatis infections. Sensitivity of sampling might improve if several consecutive samples were to be collected. This self-sampling approach would
help to reach section of the population in which pelvic examination and cervical sampling is not routinely performed [57].

Gokral et al. (2005) evaluated the use of an introital specimen from women attending the Institute's infertility clinic to detect *C. trachomatis* by PCR and correlated it with intraepithelial changes (IEC) of the cervix by Pap smears. Introital and endocervical swab specimens were taken from 100 infertile women for PCR. An endocervical smear was taken for Pap staining. A noninvasive, self collection of an introital specimen could be advocated in large-scale settings for detection of *C. trachomatis* in order to contain the infection and its complications [58].

### 2.3.12. HIV – associated chlamydial infection

Joyee et al. (2005) investigated the association between *C. trachomatis* and HIV infections. They analyzed *C. trachomatis* infection and HIV positivity among patients (n=143) who attended the STD clinic in India. *C. trachomatis* was isolated on MacCoy cell monolayer from 27 (18.9%) cases. Out of the 46 initial plasmid PCR positive cases, 44 cases were reconfirmed by MOMP PCR. Forty four (30.8%) patients were considered as 'truly infected' for *C. trachomatis* and thus the prevalence of confirmed *C. trachomatis* infection was 30.8%. The study revealed that HIV positivity rate was significantly high (13/44, 29.5%) among those with chlamydial infection than in those without chlamydial infection (11/99, 11.1%) [7].

### 2.3.13. HPV – associated chlamydial infection

Molano et al. (2003) performed a cross sectional study in Bogota, Colombia, where cervical cancer rates were high, to determine the prevalence and determinants of *C trachomatis* infection, and in particular its association with human papillomavirus (HPV). They concluded that HPV infected women, particularly women with multiple HPV infections, are at increased risk of being infected with *C trachomatis* [8].
2.3.14. DNA Extraction Comparison

Freise et al. (2001) determined the method best suited to molecular diagnosis of *C. trachomatis* by examining four standard DNA preparation methods using chlamydia spiked synovial tissue and chlamydia infected monocytes. Synovial tissue from a chlamydia negative patient with rheumatoid arthritis was spiked with defined numbers of *C. trachomatis* EB. Purified human peripheral monocytes from normal donors were infected with the organism at a multiplicity of infection 1:1 in vitro and harvested after four days. DNA was prepared from all specimens by four methods: (1) QIAmp tissue kit; (2) homogenisation in 65°C phenol; (3) incubation at 97°C; (4) proteinase K digestion at 97°C. DNA from methods 1 and 2 was subjected to PCR using two different primer sets, each targeting the *C. trachomatis omp1* gene. LCR was done on DNA prepared by each method.

The results of their study, demonstrated that preparation of template using the QIAmp tissue kit (method 1) and the hot phenol extraction technique (method 2) allowed sensitive detection of *C. trachomatis* DNA. These methods also produced template from both specimen types for LCR. DNA prepared by heat denaturation [method 3] allowed only low sensitivity chlamydia detection in LCR and did not work at all for PCR. Proteinase K digestion plus heat denaturation (method 4) gave template that did not allow amplification in either PCR or LCR assays.

They concluded that the sensitivity of detection for *C. trachomatis* DNA in synovial tissue by PCR and LCR depends strongly on the method used for preparation of the amplification template. LCR targeting the multicopy chlamydial plasmid and two nested PCR assay systems targeting the single copy *omp1* gene showed roughly equivalent sensitivity. Importantly, template preparation method and the specific PCR primer system used for screening must be optimized in relation to one another for highest sensitivity [59].
Keegan et al. (2005) conducted a study in Ireland. They compared and evaluated three methods of DNA extraction for the amplification of *C. trachomatis* in uterine cervical specimens collected in PreservCyt solution. The three methods of extraction were QIAamp kit, boiling in Tris-EDTA buffer with Chelex purification, and Proteinase K digestion with Chelex purification. Specimen DNA was tested for the presence of *C. trachomatis* by PCR using cryptic plasmid- and MOMP- primers. Real-time (LightCycler) PCR for relative *C. trachomatis* quantification following DNA extraction was performed using primers for the hsp60 gene. They found that the QIAamp extraction method followed by PCR with the cryptic plasmid primers was the most successful for amplification of *C. trachomatis* DNA, while boiling in buffer was the least successful extraction method. In addition they concluded that the DNA extraction method must be carefully selected to ensure that larger PCR amplicons can be successfully produced by PCR and to ensure high sensitivity of detection of *C. trachomatis* [60].

Spaargaren et al. (2005) retrospectively conducted a study on men who have sex with men who visited the Amsterdam, sexually transmitted diseases clinic and had rectal *C. trachomatis* infections. Patients were divided into 2 groups: one group with mucous membrane abnormalities (MMA+, n = 44) when mucopurulent anal discharge or bloody, ulcerative rectal lesions were found, and another group without MMA (MMA–, n = 30) when those symptoms were not found. Specimens were taken by proctoscopic examination. They found that symptomatic (73%) as well as asymptomatic (43%) patients were infected with a new *C. trachomatis* LGV variant «L2b». Both MMA+ and MMA– men were infected with *C. trachomatis* and most of them were HIV-positive [61].
2.3.15. Comparative testing with EIA

Mahony et al. (1992a) evaluated plasmid-based PCR for the detection of *C. trachomatis*. A confirmatory PCR employing a second set of plasmid primers was also used. A total of 258 genitourinary specimens including 134 female endocervical and urethral specimens and 124 male urethral specimens were tested by culture, blocked EIA and PCR. Fifty-four specimens were positive by culture, 50 were positive by EIA and 71 were positive by PCR. Fourteen specimens that were PCR-positive but culture- and EIA-negative were confirmed positive by the confirmatory PCR. Two of the 187 specimens which were negative by culture and EIA were positive by PCR but failed to confirm with the second set of primers. Using an expanded gold standard of culture, blocked EIA and confirmed PCR, the overall sensitivities for culture, blocked EIA and confirmed PCR were 76.0% (54/71), 70.4% (50/71) and 100% (71/71) and the specificities were 100% (187/187), 100% (187/187), respectively. These results demonstrated that a confirmatory PCR was useful for sorting out discordant specimens and establishing the true specificity of PCR. Furthermore, these results demonstrate that PCR is more sensitive than culture and EIA and suggest that a confirmed PCR test should be included in the gold standard for the evaluation of new tests for diagnosing *C. trachomatis* infections [62].

Mahony et al. (1992b) tested a total of 683 first-void urine (FVU) specimens, including 91 from symptomatic men and 592 from asymptomatic men. Twenty-nine of 91 (32%) were positive by both blocked EIA (Chlamydiazyme) and PCR (plasmid primers), and specimens from 23 of these individuals were tested by culture and were found to be positive. Among the asymptomatic men, 8 of 592 (1.4%) FVU specimens were positive by blocked EIA and PCR. They showed that PCR had an overall sensitivity of 100% (40 of 40 specimens) compared with an overall sensitivity of 92.5% (37 of 40 specimens) for EIA. Their PCR-positive specimens were tested in a second PCR by using a different set of primers which confirmed that three EIA-negative specimens were true positives [63].
Tanaka et al. (1998) investigated the occurrence of *C. trachomatis* among female commercial sex workers (CSWs). They examined a high risk group of 163 female CSWs who visited STD clinic in order to undergo screening for major STDs, including chlamydial infection. A total of four swab specimens, including two vaginal and two endocervical specimens, were collected from each woman by a clinician. To identify *C. trachomatis*, a new improved EIA kit (IDEIA PCE), a conventional EIA kit (IDEIA), and PCR assay (Amplicor) were used. Discrepancies in the results were resolved using supplementary PCR assay. A female patient was considered to be infected with *C. trachomatis* if the IDEIA PCE test and PCR test for both sample sites (endocervical and vaginal) gave positive results. Following resolution of these discrepancies, relative sensitivity and specificity, confidence intervals, and predictive values for each type of specimen by each assay were calculated. The prevalence was 21.5% [35 positive cases out of 163]. The relative sensitivities in vaginal swab specimens were 88.8%, 68.6%, and 91.4% using IDEIA PCE, IDEIA, and PCR, respectively. The relative specificities in vaginal swab specimens were 99.2%, 99.2%, and 100%, respectively. The relative sensitivities in endocervical swab specimens were 85.7%, 77.1%, and 91.4% with IDEIA PCE, IDEIA, and PCR, respectively. The relative specificities in endocervical swab specimens were all 100%. They concluded that IDEIA PCE test on vaginal swab specimens is an acceptable, sensitive, and less invasive approach for the detection of *C. trachomatis* in commercial sex workers with a high prevalence of *C. trachomatis* infection. They evaluated the performance of improved enzyme immunoassay for the detection of *C. trachomatis* in vaginal swab and endocervical swab specimens, in comparison with a conventional EIA test and PCR assay [64].

Chan et al. (2000) conducted a study comparing EIA with the combined urethral and cervical swab with PCR on urine alone and urine mixed with cervical cells. They concluded that urine plus cervical cells enhanced detection, and that PCR on urine alone or urine combined cervical cells was superior to EIA [65].
Tanaka et al. (2000) compared the performance of a new generation dual amplified enzyme immunoassay with a molecular method for the diagnosis of *C. trachomatis*, using a range of urogenital specimens, and assessed the reliability of testing self collected vaginal specimens compared with clinician collected vaginal specimens. Two population groups were tested. For the first group, FVU specimens were collected from 193 male patients with urethritis, and endocervical swabs were collected from 187 high risk CSWs. All urine and endocervical specimens were tested by a conventional assay (IDEIA Chlamydia), a new generation amplified immunoassay (IDEIA PCE Chlamydia), and the Amplicor PCR. Discrepant results obtained among the three specimen types were confirmed using a nested PCR test with a different plasmid target region. For the second group, four swab specimens, including one patient obtained vaginal swab, two clinician obtained endocervical swabs, and one clinician obtained vaginal swab, were collected from 91 high risk sex workers. Self collected and clinician collected vaginal swabs were tested by IDEIA PCE chlamydia. Clinician obtained endocervical swabs were assayed by IDEIA PCE chlamydia and Amplicor PCR. The performance of the IDEIA PCE chlamydia test was comparable to that of the Amplicor PCR test when male urine and female endocervical swab specimens were analyzed. The relative sensitivities of IDEIA, IDEIA PCE, and Amplicor PCR on male FVU specimens were 79.3%, 91.4%, and 100%, respectively. The relative sensitivities of the three tests on female endocervical specimens were 85.0%, 95.0%, and 100%, respectively. The positivity rates for patient collected vaginal specimens and clinician collected vaginal specimens by IDEIA PCE were 25.2% and 23.1%, respectively, whereas those for clinician collected endocervical swabs by PCR and IDEIA PCE were both 27.5%. They concluded that IDEIA PCE Chlamydia is a lower cost but sensitive alternative test to PCR for testing male urine specimens and female endocervical swabs. In addition, self collected or clinician collected vaginal specimens tested by IDEIA PCE Chlamydia are a reliable alternative to analyzing endocervical specimens [66].
Chernesky et al. (2001) conducted a study which emphasized on the comparison of a polymer conjugate-enhanced enzyme immunoassay to LCR for diagnosis of *C. trachomatis* in endocervical swabs. After confirmation by the EIA blocking test, the sensitivity of the IDEIA PCE remained at 91.8% and the specificity increased from 98.2 to 99.8% compared to LCR assay [67].

### 2.3.16. Vaccine prospect and immunity

Numazaki (2004) investigated current problems of perinatal *C. trachomatis* infections. *C. trachomatis* sometimes causes serious disease in neonates who acquire the organism transvaginally or in utero. Control programs emphasizing early diagnosis, targeted screening, and effective treatment would have lead to an eventual decline in the incidence of chlamydial infections. Entirely new approaches to prevention and treatment of chlamydial infections in infants seem to be necessary, including antimicrobial interventions and the development of a vaccine strategy [68].

Caro et al. (2005) studied the immune response to chlamydial infection and the prospect of vaccine development. The study demonstrated the role of innate, humoral, cellular immunity, and several cytokines in the immunopathology and eradication of chlamydial infection [69].

### 2.3.17. Chlamydia infection and apoptosis

Greene et al. (2004) revealed that both anti- and pro-apoptotic activities have occurred during chlamydial infection. They compared host cell apoptotic responses to infection with 17 different chlamydial serovars and strains. None of the serovars caused any biologically significant apoptosis in the infected host cells. Host cells in chlamydia-infected cultures can continue to undergo DNA synthesis and mitosis. Chlamydia-infected cells are resistant to apoptosis induction, although the extent of the antiapoptotic ability varied between serovars. These observations have demonstrated that an anti- but not proapoptotic activity is the prevailing event in chlamydia-infected cultures [70].
2.3.18. Prophylactic antibiotic treatment

Land et al. (2002) reported that women who undergo uterine instrumentation (e.g. hysterosalpingography and laproscopy with hydrotubation) are at risk of pelvic infection by *C. trachomatis*, which may derive either from ascending endocervical infections or from reactivation of dormant microorganisms in the upper genital tract. They also demonstrated that viable microorganisms may still be present in the upper genital tissues several years after chlamydial infection.

As a consequence, prophylactic antibiotic before uterine instrumentation should be considered in all subfertile women. The CDC guidelines for the treatment of patient with chlamydial infections have recommended azithromycin as a single oral 1g dose (as equivalent to 7 day regimen of doxycycline 100mg twice daily) for treating uncomplicated genital chlamydial infections [71].

Sirmatel et al. (2005) examined the use of antibiotic with respect to the infection rate of *C. trachomatis* among 282 sexually active Turkish females between the ages of 15 and 42. Endocervical specimens were investigated in 60 women with PIDs, 90 infertile patients (IPs), 92 CSWs, and 40 IUD users. All specimens were analyzed using DIF techniques for the presence of *C. trachomatis* antigen test. *C. trachomatis* antigen was positive in 12.7% of all studied patients and was found in 16.6% of PID (10 cases), 23.3% of IPs (21 cases), and 5.4% of CSWs (5 cases), but was not detected in IUD users, since 90% of IUD women were using tetracycline prophylactic antibiotic. The results showed that *C. trachomatis* presence in the Southeastern region of Turkey was not significantly higher than that found in a previous study on sex workers of other countries, and indicated that the use of prophylactic antibiotics decreases the rate of chlamydial infection [72].
Chapter Three

Materials and Methods
3.1. Study population and selection of cases

The study population comprised 134 symptomatic Palestinian women who attended Al-Shifa hospital outpatient gynecology clinic, private gynecology clinics and Basma infertility center over a period of 6 months from September 2005 to February 2006. The subjects were married, non pregnant women aged between 18 and 52 yr (mean age, 30.0 ± 7.5 and median age 29 yr). The first 25 specimens were considered as pilot study for EIA but they were not confirmed by PCR, thus the remaining 109 specimens constituted the study specimens. The current study was a cross-sectional prospective study and was conducted according to good clinical practice guidelines recommended by the CDC. A questionnaire regarding, age, residence area, level of education, contraceptive methods, symptoms, and infertility status was completed. Patients who received antibiotic were excluded. The cases under study were selected according to CDC guidelines where the patients complained of one or more symptoms or signs suggestive of Chlamydia infection such as, suspected or proven PID, deep dyspareunia, menstrual abnormalities (e.g., recent dysmenorrhea or menorrhagia, post-coital or intermenstrual bleeding), inflamed or friable cervix, bleeding on contact while taking swabs, dysuria, infertility, ectopic pregnancy, and women who had undergone instrumentation of the uterus (e.g., IUCD insertion) [73,74].

3.2. Ethical considerations

Permission and ethical approval for the current study have been given by helsinki committee and ministry of health. After examination by a gynecologist, a consent form was signed by each patient, explaining the importance and objectives of the study.
3.3. Materials

♦ Tris base {hydroxymethyl aminomethane (Promega, USA)}
♦ Agarose, Molecular Biology grade (Promega, USA)
♦ Glacial Acetic acid (Sigma, USA)
♦ EDTA disodium salt (Promega, USA).
♦ Absolute Ethanol (Sigma, USA)
♦ Ethidium bromide (Promega, USA).
♦ DNAse, RNase free Water (Promega, USA).
♦ Potassium Phosphate- monobasic & dibasic (Sigma, USA)
♦ Blue/Orange 6x loading dye (Promega).
♦ Nystatin (antifungal).

3.3.1. Instruments, equipments, and disposables

♦ Graduated cylinders 100ml, 500ml, 1000ml
♦ Graduated flasks.
♦ Digital Balance
♦ Autoclave
♦ Vortex Mixer
♦ Micro Centrifuge (UK)
♦ Water Bath
♦ Freezer, Refrigerator
♦ Thermal Cycler (Eppendorf Mastercycler Personal, Germany).
♦ L.G. Microwave Oven (Korea).
♦ Electrophorosis Tanks with appropriate combs
♦ Power Supply (Biorad)
♦ UV transilluminator (Hofer Pharmacia, USA)
♦ Digital Camera
♦ Eppendorf micropipettes
♦ Microfuge tubes - 1.5 mL capacity.
♦ PCR tubes (0.2ml) - thin wall with cap
♦ ELISA reader
3.3.2. Chlamydia collection kit

♦ IDEIA™ Chlamydia specimen collection kit (DakoCytomation Ltd, UK).
♦ Dacron swabs, polyester tipped (copan Ltd, Italy)

3.3.3. Chlamydia DNA purification kits

♦ QIAamp DNA Mini Kits (Qiagen, USA)

3.3.4. Chlamydia detection kits

♦ Enzyme immunoassay (IDEIA™ PCE Chlamydia; Dako Ltd)
♦ PCR - Cryptic Plasmid (Maxim Biotech Inc, USA)
♦ PCR - Chromosomal, MOMP, (Maxim Biotech Inc, USA)

3.3.5. Chlamydia trachomatis LGV type II DNA control 100 μl

(ABI, Advanced Biotechnologies, USA)

3.4. Laboratory methods

3.4.1. Specimens collection:
Endocervical swab specimens were collected from 134 participants. For each participant, two Dacron–tipped swabs were collected individually by a specialized gynecologist. One swab for PCR technique and the other for EIA testing. The swab specimens were collected randomly and not in ordered fashion.
The collection was according to CDC guidelines and as indicated by manufacturer [73].

- Before obtaining a specimen for a chlamydia test, a gauze or large swab was used to remove all secretions and discharge from the vaginal area.
- Dacron swab was inserted 1-2 cm into the endocervical canal (i.e., past the squamocolumnar junction). The swab was rotated against the wall of the endocervical canal several times for 10-30 seconds, and withdrawn without touching any vaginal surfaces and placed in the appropriate transport medium (both EIA, and PCR testing).

3.4.2. Specimens storage

Endocervical swab specimens collected for EIA were stored at refrigerator temperature (4-6°C) according to the instructions of the kit manufacturer, while endocervical swab specimens collected for PCR were stored at -70°C.

3.4.3. Specimens preparation for EIA

Endocervical swabs were placed in boiling water bath for 15 min before EIA processing, which gives opportunity to store the specimens at freezing temperature before processing (no longer than one month).

3.5. Detection of Chlamydia antigen by EIA

IDEIA™ PCE Chlamydia is an immunoassay utilizing dual amplification technology for the detection of Chlamydia antigen in human endocervical specimens.

3.5.1. Principle of assay (IDEIA™ PCE Chlamydia)

Chlamydia test utilized a genus specific monoclonal antibody, a polymer conjugate with a high enzyme to antibody ratio and a liquid, ready to use enzyme amplification system. Chlamydia antigen which is present in human endocervical swabs specimens was bound by monoclonal antibody adsorbed to the surface of the solid phase. Conjugate monoclonal antibody bound to
Chlamydia antigen was captured on the solid phase, thereby linking the conjugate polymer complex carrying multiple enzyme molecules. Free conjugate complexes were removed by washing. Subsequently, the specifically bound enzyme molecules converted substrate to a colorless product which catalyzed the secondary, enzyme signal amplification reaction to produce a colored endpoint. Figure 3.1 illustrates the principle of IDEIA™ PCE Chlamydia assay.

![Schematic diagram of the IDEIA™ PCE Chlamydia assay principle](image)

**Figure 3.1: The principle of IDEIA™ PCE Chlamydia assay**

### 3.5.2. IDEIA™ PCE kit components:

- **Microtitration plate**
  Two 96 well microtitration plates of 12, 8 microwell break-apart strips coated with Chlamydia specific anti-lipopolysaccharide monoclonal antibody. A resealable plastic pouch is provided for storage of unused microwells.

- **Transport medium (10x)**
  One bottle of 25mL transport medium concentrate (10x): nonionic detergent in a buffer containing coloured dye, antimicrobial agent and an antifoaming agent.
Positive control One bottle of 7mL positive control: heat inactivated Chlamydia antigen in buffer solution containing formalin, antimicrobial agent and coloured dye.

Negative control One bottle of 12mL negative control: buffer solution containing antimicrobial agent, coloured dye and an anti-foaming agent.

Conjugate
Two bottles of 7mL polymer conjugate: Chlamydia specific antilipopolysaccharide monoclonal antibody conjugated to a dextran polymer backbone linked to multiple alkaline phosphatase molecules in stabilising buffer containing coloured dye and an antimicrobial agent.

Wash buffer (10x)
Two bottles of 125mL wash buffer concentrate (10x): tris buffered solution containing detergent and an antimicrobial agent.

Amplifier A
Two bottles of 13mL amplifier A: inorganic salts and buffered enzyme solution containing tetrazolium violet and an antimicrobial agent.

Amplifier B
Two bottles of 13mL amplifier B: stabilised NADPH solution

Stop solution
Two bottles of 13mL stop solution: 1 mol/L phosphoric acid.

3.5.2.1. Working reagent preparation

Transport medium concentrate (10X)
working strength transport medium was prepared by adding 1 part of transport medium concentrate to 9 parts of fresh deionized or distilled water. One milliliter aliquots of working strength transport medium was dispensed into clean, heat resistant (100°C) screw-capped vials. Vials of working strength transport
medium could be used for specimen collection for up to 12 months if kept at room temperature (15-30 °C).

**Wash buffer concentrate (10x).**

Working strength wash buffer was prepared by adding 1 part of wash buffer concentrate to 9 parts of fresh deionized or distilled water. Sufficient concentrate was provided to prepare up to 100mL working strength wash buffer for each strip of 8 microwells. Working strength wash buffer was prepared as required on the day of use. Remaining concentrate was stored at 2-8 °C.

**3.5.3. Assay procedure:**

- All reagents were brought to room temperature before use.
- Two hundred microliters of each heat treated specimen were added to microwells and two hundred microliters of each positive and negative control were added to separate microwells.
- Negative control was prepared in triplet with each batch to count the mean absorbance value of them.
- Specimens were incubated for 90 min with shaking at 15-30°C, after addition of fifty microliters of conjugates.
- Specimens were washed four times with working washing buffer.
- One hundred microliters of amplifier A and one hundred microliters of amplifier B were added to each specimen.
- Specimens were incubated for 30 min with shaking at 15-30°C.
- One hundred microliters of stop solution were added.
- The absorbance of each specimen was read at 490nm.

**3.5.4. Calculation and interpretation of the results**

Cut-off value was calculated by measuring the mean of the three negative control absorbance (+ 0.05 absorbance units).

The results were interpreted according to the following Table (3.1).
Table 3.1: Interpretation of EIA PCE Results

<table>
<thead>
<tr>
<th>Result</th>
<th>Interpretation</th>
<th>Reporting Recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD &gt; CO + 0.015</td>
<td>Positive*</td>
<td>Presumptive chlamydial LPS antigen (No blocking test performed)</td>
</tr>
<tr>
<td>OD = CO ± 0.015</td>
<td>Equivocal*</td>
<td>Unable to determine result. Retested</td>
</tr>
<tr>
<td>OD &lt; CO - 0.015</td>
<td>Negative</td>
<td>No chlamydial LPS antigen detected</td>
</tr>
</tbody>
</table>

OD = Optical Density (Absorbance units)  
CO = Cut-off = Mean of Negative Controls + 0.05 Absorbance units  
* Positive and equivocal results were verified.

3.6. Specimens preparation for PCR

DNA extraction was done by using QIAamp DNA Mini Kit (Qiagen).

Components of QIAamp® DNA mini kit (Qiagen):

- QIAamp spin columns (50)
- Collection tubes (2 ml, 150)
- Buffer AL (12 ml, lysis buffer)
- Buffer ATL (10 ml, lysis buffer)
- Buffer AW1 (19 ml, washing buffer, provided as concentrate, diluted with 25 ml absolute ethanol, hence the final volume was 44 ml)
- Buffer AW2 (13 ml, washing buffer provided as concentrate, diluted with 30 ml absolute ethanol hence the final volume was 43 ml)
- Buffer AE (22 ml, elution buffer)
- Proteinase K (1.25 ml)
3.6.1. Principle of DNA extraction and purification

The QIAamp DNA Mini Kit simplified for isolation of DNA from human tissue specimens with fast spin-column. No phenol–chloroform extraction is required. DNA bound specifically to the QIAamp silica-gel membrane while contaminants passed through. PCR inhibitors such as divalent cations and proteins were completely removed in two efficient wash steps, leaving pure DNA to be eluted in either water or a buffer provided with the kit. QIAamp DNA technology yields genomic, mitochondrial, bacterial, parasite, or viral DNA from human tissue specimens ready to use in PCR and blotting procedures (Qiagen).

3.6.2. Specimens preparation and DNA extraction procedure

DNA extraction from each EIA positive specimen was processed individually for PCR while negative specimens were extracted individually and processed in the pool of three specimen, and when the pool gave positive PCR result, each specimen in the pool was processed apart.

- Swab specimens removed from -20°C, and left at room temperature for at least one hour.
- Specimens were rotated very well and 200 μl were added to clean 1.5 ml eppendorf tube, closed and centrifuged at high speed (13000 rpm) for 30 min. The resultant supernatant was removed by a micropipette to maintain the pellet.
- The pellet was resuspended in 180 μl of buffer ATL (Qiagen) with 20 μl of proteinase K and then incubated at 56°C with occasional vortexing until the pellet was completely lysed, which usually takes 60 min.
- The 1.5 ml microcentrifuge tube was centrifuged to remove drops from the inside of the lid.
- After lysis of the specimen, 200 μl of buffer AL (Qiagen) was added to the specimen and the mixture was incubated for 10 min at 70°C. The 1.5 ml microcentrifuge tube was then briefly centrifuged to remove drops from inside the lid.
・ The mixture was then combined with 200 μl of absolute ethanol and mixed by pulse-vortexing for 15 seconds, and 1.5 ml microcentrifuge tube was then briefly centrifuged to remove drops from inside the lid.

・ The mixture from previous step (including the precipitate) was carefully applied to the QIAamp spin column (in a 2 ml collection tube) without wetting the rim. The cap was closed and the tube was centrifuged at (8000 rpm) for 1 min. The QIAamp spin column was placed in a clean 2 ml collection tube (provided), and the tube containing the filtrate was discarded.

・ The QIAamp spin column was carefully opened and 500 μl buffer AW1 was added without wetting the rim. The cap was closed, and the tube was centrifuged at 8000 rpm for 1 min. QIAamp spin column was placed in a clean 2 ml collection tube (provided), and the tube containing the filtrate was discarded.

・ The QIAamp spin column was carefully opened and 500 μl buffer AW2 was added without wetting the rim. The cap was closed and the tube was centrifuged at full speed (13,000 rpm) for 3 min.

・ To eliminate any chance of possible buffer AW2 carryover, we placed QIAamp spin column in a new 2 ml collection tube (not provided) and the collection tube containing the filtrate was discarded. Then centrifugation at (13,000 rpm) for 1 min.

・ The QIAamp spin column was again placed in a clean 1.5 ml microcentrifuge tube (not provided), and the collection tube containing the filtrate was discarded. The QIAamp spin column was carefully opened and 100 μl buffer AE was added. Then incubated at room temperature for 5 min, and centrifuged at 8000 rpm for 1 min. The same eluate, was used again on the same spin column to recover any residual DNA.
3.7. *C. trachomatis* detection by PCR:

Human β globin primers used in the study was synthesized from published sequence [29, 46].

3.7.1. PCR Primers

The primers employed in the current study are shown in Table 3.2 below:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5' to 3’</th>
<th>Annealing Temp</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
</table>
| Human β globin PCR       | F, 5'-ACACAAACTGTGTTCACTAGC-3'  
R, 5'-GAAACCCAAGAGTCTTCTCT-3' | 56°C           | 209               |
| *C. trachomatis* plasmid-PCR | F, 5'-GCAAGATATCGAGTATGCGTTAGG-3'  
R, 5'-TTTCATTGTACTCATTAAACGAGCGG-3' | 56°C           | 364               |
| *C. trachomatis* MOMP-PCR | F, 5'-AACTCAAACCCCTCATTCTCAAA-3'  
R, 5'-AAACGTTCGTCCAGGAAGAGGCC-3' | 59°C           | 182               |

*Orientation: F, forward; R, reverse.* *(Chlamydia trachomatis* primers were ready premixed by maxim Biotec, USA, while β globin primer was synthesized by Operon biotechnologies, Germany)*

The extracted DNAs were subjected to PCR with primers specific for *C. trachomatis* cryptic plasmid and major outer membrane protein. These primers were included in two PCR detection kits. *C. trachomatis* detection kits, cryptic plasmid, and MOMP (Maxim Biotech Inc, USA).

**Components of each kit:**

- Pre-mixed primers 1000 µl.
- Optimized PCR buffer 750 µl x 4 tubes (2x) {chemicals, enhancer, stabilizer, dNTPs}.
- Positive control cDNA 100 µl (5x).
- M.W. marker, 100 bp ladder 100 µl.
- DDH2O 1000 µl (nuclease free).
3.7.2. PCR protocols

3.7.2.1. Master mixture preparation

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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per PCR assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master mixture</td>
<td>20 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>Specimen or control cDNA*</td>
<td>5.0 µl</td>
</tr>
</tbody>
</table>

* Positive control used was both cDNA included in the kit and LGV type II DNA control. Both showed the same results.

We made final volume up to 25 µl with nuclease free water, when specimen or control cDNA used is less than 5.0 µl. The above Master mixture and Taq DNA polymerase components were premixed in sufficient quantity for daily needs and this working master mixture was dispensed into an individual reaction PCR tube before adding the specimen or control DNA.

3.7.2.2. PCR reaction profiles

Reaction profiles were optimized according to our thermal cycler and the program was as follows:

**Step 1:** Denaturation at 95°C for 3 min.

**Step 2:** 35 cycles of:

- Denaturation at 95°C for 1 min.
- Annealing at 56°C for 1 min.
- Extension at 72°C for 1 min.

**Step 3:** Final extension at 72°C for 10 min. Hold at 4°C.

The same reaction profiles were used for chromosomal MOMP DNA except that the annealing temperature was set at 59°C.
3.7.3. Analysis of amplified DNA

Two-tenth (5µl) of amplified specimens were subjected to electrophoresis in an agarose gel containing 0.5µg/ml ethidium bromide. A band of 364 bp indicated a positive result for cryptic plasmid, while a band of 182 bp indicated a positive result for MOMP. In addition a band of 209 bp demonstrated that the specimen was positive for human β globin gene. The β globin primer was used as internal control for PCR amplification for detection of any inhibitory specimens.

3.8. Criteria for true positive and true negative results

A second confirmatory PCR based on amplification of gene that code for MOMP was used. The test of MOMP-PCR was done for all swab specimens and concordance of at least two of the three used methods was used for determining true positive results from true negative ones (as demonstrated in Table 3.3) [75]. A false positive for any test is defined as it has positive for that test and negative for the other two tests. Similarly, a false negative for a test is defined as it has a negative for that test and positive for the other two tests.

<table>
<thead>
<tr>
<th>EIA PCE result</th>
<th>*PCR Plasmid result</th>
<th>MOMP</th>
<th>Decision</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>True positive</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>True positive</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>True negative</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>True positive</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>True negative</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>True negative</td>
</tr>
</tbody>
</table>

* Internal control was used with all plasmid based PCR to detect inhibitors.
3.9. Analysis of data

Statistical analysis was carried out using the statistical package for social sciences (SPSS) version 13 for windows. For normally distributed data, means and standard deviations were calculated. Confidence intervals (95%) were reported when appropriate. Chi-square ($\chi^2$), and Fisher exact tests were applied to compare true *Chlamydia* infection with signs and symptoms. Odds ratio (OR) was reported where appropriate. Discordance between EIA and plasmid- based PCR were assessed by McNemar test, while concordance of results was verified by Kappa statistic value. Infertility with chlamydial infection under certain age was analyzed using binary logistic regression test. Statistical significance was set at the 5% level.
Chapter Four

Results
Results

This study is the first study in Palestine which focused on detection of *Chlamydia trachomatis* in endocervical swab specimens. The results of the study can be summarized as follows:

4.1. Age group distribution of the study population

The subjects were married, non pregnant women aged between 18 and 52 yr (mean age, 30.0 ± 7.5 and median age 29). Among the participant women, 25% were under 24, 50% were under 29 and 75% were less than 36 yr of age, respectively. Age group distribution of the study population is illustrated in Table 4.1.

<table>
<thead>
<tr>
<th>Age Group (yr)</th>
<th>Number and percentage of tested specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>18 - 24</td>
<td>30</td>
</tr>
<tr>
<td>25 - 31</td>
<td>38</td>
</tr>
<tr>
<td>32 - 38</td>
<td>25</td>
</tr>
<tr>
<td>39 - 45</td>
<td>13</td>
</tr>
<tr>
<td>46 - 52</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>109</td>
</tr>
</tbody>
</table>

4.2. Distribution of endocervical swab specimens according to residence areas

Residence areas were divided into four main areas where the patients came from different locations to visit hospital outpatient gynecology clinics, private gynecology clinics and Basma infertility center in Gaza City. As shown in Table 4.2., most cases (59.6%), are come from Gaza City, meanwhile the lowest number of cases (7.3%), comes from Southern Gaza Strip (Khanunis and Rafah).
Table 4.2: Distribution of endocervical swab specimens according to Residence area

<table>
<thead>
<tr>
<th>Residence Area</th>
<th>Number and percentage of tested specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Northern Gaza Strip</td>
<td>23</td>
</tr>
<tr>
<td>Gaza</td>
<td>65</td>
</tr>
<tr>
<td>Mid zone area</td>
<td>13</td>
</tr>
<tr>
<td>Southern Gaza strip</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>109</td>
</tr>
</tbody>
</table>

4.3. Distribution of endocervical swab specimens according to educational level

Educational level of the subjects was as demonstrated in Table 4.3.

Table 4.3: Distribution of endocervical swab specimens in relation to educational level

<table>
<thead>
<tr>
<th>Education level</th>
<th>Number and percentage tested specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency</td>
</tr>
<tr>
<td>Elementary</td>
<td>21</td>
</tr>
<tr>
<td>Preparatory</td>
<td>19</td>
</tr>
<tr>
<td>Secondary</td>
<td>45</td>
</tr>
<tr>
<td>Higher education</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>109</td>
</tr>
</tbody>
</table>
4.4. Results

4.4.1. EIA PCE results

Based on manufacturer criteria of EIA PCE kit, eighteen specimens were positive, three specimens were equivocal, and eighty eight specimens were negative) as indicated in Table 4.4. The overall positive specimens including the equivocal ones was 21(19.3%), (95% CI, 11.87 - 26.67).

Table 4.4: Frequency of positive and negative cases by EIA PCE

<table>
<thead>
<tr>
<th>EIA PCE</th>
<th>Frequency</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>*21</td>
<td>19.3</td>
</tr>
<tr>
<td>Negative</td>
<td>88</td>
<td>80.7</td>
</tr>
<tr>
<td>Total</td>
<td>109</td>
<td>100</td>
</tr>
</tbody>
</table>

*Three specimens were equivocal

4.4.2. Plasmid PCR results

Twenty-three positive specimens were detected by plasmid PCR, meanwhile the negative specimens were eighty-six specimens. Internal control was detected in all specimens identified as negative by plasmid PCR. Hence the occurrence by plasmid PCR was 23 (21.1%), (95% CI, 13.44 – 28.76).

Table 4.5: Frequency of positive and negative cases by plasmid PCR

<table>
<thead>
<tr>
<th>Plasmid PCR</th>
<th>Frequency</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>23</td>
<td>21.1</td>
</tr>
<tr>
<td>Negative</td>
<td>86</td>
<td>78.9</td>
</tr>
<tr>
<td>Total</td>
<td>109</td>
<td>100</td>
</tr>
</tbody>
</table>
4.4.3. Correlation between EIA PCE and plasmid PCR

Plasmid PCR detected a total of twenty-three positive specimens. It detected fifteen specimens that were positive by EIA PCE as shown in Table 4.6. Additional eight positive specimens were detected that were negative by EIA PCE. Six specimens were EIA PCE positive have been identified as negative by plasmid PCR. Hence the total discrepant results were fourteen specimens.

Table 4.6: Comparison between Plasmid PCR and EIA PCE results

<table>
<thead>
<tr>
<th>Plasmid PCR</th>
<th>EIA PCE</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>*15</td>
<td>8</td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>80</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>88</td>
</tr>
</tbody>
</table>

*Two specimens were equivocal by EIA PCE
Kappa (k)=0.60; McNemar test p value 0.79>>0.05.

Despite of fourteen discrepant results between EIA PCE and plasmid PCR, there was no statistically significant difference. Agreement between both EIA PCE and plasmid PCR, as shown by kappa, was good.
4.4.4. MOMP-PCR results

According to MOMP PCR, nineteen positive specimens were detected as shown in Table 4.7., hence the occurrence by MOMP was 19 (17.4%), (95% CI, 10.31 – 24.55).

Table 4.7: Frequency of positive and negative cases by MOMP

<table>
<thead>
<tr>
<th>MOMP PCR</th>
<th>Frequency</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>19</td>
<td>17.4</td>
</tr>
<tr>
<td>Negative</td>
<td>90</td>
<td>82.6</td>
</tr>
<tr>
<td>Total</td>
<td>109</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 4.8., illustrates six specimens discrepant between plasmid and MOMP PCRs; out of six, one specimen was positive by MOMP and negative by plasmid PCR. The remaining five specimens were negative by MOMP PCR and positive by plasmid PCR.

Table 4.8: Comparison between plasmid- and MOMP-based PCR results

<table>
<thead>
<tr>
<th>Plasmid PCR</th>
<th>MOMP PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>85</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>90</td>
</tr>
</tbody>
</table>
The results of the three used methods are shown in Table 4.9.

Table 4.9: comparison between results obtained by the three methods

<table>
<thead>
<tr>
<th>Plasmid PCR</th>
<th>EIA PCE</th>
<th>MOMP PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Positive</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>109</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>109</td>
</tr>
</tbody>
</table>

4.4.5. Interpretation of the assays in accordance to the criteria

According to the criteria described previously in chapter three, the final results of EIA PCE, plasmid-based PCR, and MOMP-based PCR were analyzed and are summarized in Table 4.10.

Table 4.10: Interpretation of results in accordance to the criteria

<table>
<thead>
<tr>
<th>Results</th>
<th>Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EIA PCE</td>
</tr>
<tr>
<td>True positive</td>
<td>16</td>
</tr>
<tr>
<td>True negative</td>
<td>82</td>
</tr>
<tr>
<td>False positive</td>
<td>5</td>
</tr>
<tr>
<td>False negative</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>109</td>
</tr>
</tbody>
</table>

* one false negative specimen by Plasmid PCR, was positive by MOMP PCR. It was included in the true positive category when measuring Plasmid PCR Performance.
The performance of the three used methods is illustrated in the Table 4.11.

Table 4.11: Performance of EIA PCE, Plasmid PCR, and MOMP PCR

<table>
<thead>
<tr>
<th>Performance</th>
<th>Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EIA PCE</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>73</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>94</td>
</tr>
<tr>
<td>*PPV (%)</td>
<td>76</td>
</tr>
<tr>
<td>**NPV (%)</td>
<td>93</td>
</tr>
<tr>
<td>Efficiency (%)</td>
<td>90</td>
</tr>
</tbody>
</table>

* positive predictive value  
** negative predictive value
4.5. True chlamydial infection:

By applying the criteria, there were twenty-two truly infected women, giving an occurrence rate of 20.2% (95% CI, 12.65 – 27.71).

4.5.1. Chlamydial infection according to age group

Detection was highest among women aged 25-31 yr & 32-38 yr. as indicated in Table 4.12 and Figure 4.1.

Table 4.12: Frequency of Chlamydial infection according to age group

<table>
<thead>
<tr>
<th>Age Group (yr)</th>
<th>True Positive No. (%)</th>
<th>True Negative No. (%)</th>
<th>Total No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 - 24</td>
<td>3(10)</td>
<td>27(90)</td>
<td>30(27.5)</td>
</tr>
<tr>
<td>25 - 31</td>
<td>9(23.7)</td>
<td>29(76.3)</td>
<td>38(34.9)</td>
</tr>
<tr>
<td>32 - 38</td>
<td>7(28)</td>
<td>18(72)</td>
<td>25(22.9)</td>
</tr>
<tr>
<td>39 - 45</td>
<td>2(15.4)</td>
<td>11(84.6)</td>
<td>13(11.9)</td>
</tr>
<tr>
<td>46 - 52</td>
<td>1(33.3)</td>
<td>2(66.7)</td>
<td>3(2.8)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>22(20.2)</strong></td>
<td><strong>87(79.8)</strong></td>
<td><strong>109(100)</strong></td>
</tr>
</tbody>
</table>

Figure 4.1: Chlamydial infection according to age group.
4.5.2. Chlamydial infection according to educational level

The highest detection rate of chlamydial infection was in elementary education level as shown in Table 4.13 and Figure 4.2.

Table 4.13: Frequency of Chlamydia infection according to educational level

<table>
<thead>
<tr>
<th>Educational Level</th>
<th>Chlamydia Infection</th>
<th>Total No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>True Positive No. (%)</td>
<td>True Negative No. (%)</td>
</tr>
<tr>
<td>Elementary</td>
<td>7(33.3)</td>
<td>14(66.7)</td>
</tr>
<tr>
<td>Preparatory</td>
<td>4(21.1)</td>
<td>15(78.9)</td>
</tr>
<tr>
<td>Secondary</td>
<td>9(20)</td>
<td>36(80)</td>
</tr>
<tr>
<td>Higher education</td>
<td>2(8.3)</td>
<td>22(91.7)</td>
</tr>
<tr>
<td>Total</td>
<td>22(20.2)</td>
<td>87(79.8)</td>
</tr>
</tbody>
</table>

Figure 4.2: Chlamydial infection according to educational level
4.5.3. Chlamydial infection according to residence area

Although the highest frequent number of chlamydial infection has occurred in Gaza city residents, Chlamydial detection rate were highest among Northern as well as Southern Gaza Strip resident patients (as demonstrated in Table 4.14 and Figure 4.3.).

Table 4.14: Frequency of Chlamydial infection according to residence area

<table>
<thead>
<tr>
<th>Residence</th>
<th>Chlamydia Infection</th>
<th>Total No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>True positive No. (%)</td>
<td>True Negative No. (%)</td>
</tr>
<tr>
<td>Northern Gaza Strip</td>
<td>5(21.7)</td>
<td>18(78.3)</td>
</tr>
<tr>
<td>Gaza City</td>
<td>13(20)</td>
<td>52(80)</td>
</tr>
<tr>
<td>Mid zone area</td>
<td>2(15.4)</td>
<td>11(84.6)</td>
</tr>
<tr>
<td>Southern Gaza strip</td>
<td>2(25)</td>
<td>6(75)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>22(20.2)</strong></td>
<td><strong>87(79.8)</strong></td>
</tr>
</tbody>
</table>

Figure 4.3: Chlamydia infection according to residence area
4.5.4. *Chlamydia trachomatis* in relation to contraception method

The highest number of infection was among women who did not use any type of contraception, while among those using different contraception means, the status was surprising since the infection had occurred with all types of contraception, as shown in Table 4.15 and Figure 4.4.

Table 4.15: Frequency of *C. trachomatis* infection according to contraception method

<table>
<thead>
<tr>
<th>Contraceptive Method</th>
<th>Chlamydia Infection</th>
<th>Total No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>True Positive No. (%)</td>
<td>True Negative No. (%)</td>
</tr>
<tr>
<td>None</td>
<td>17(19.3)</td>
<td>71(80.7)</td>
</tr>
<tr>
<td>IUCD</td>
<td>3(25)</td>
<td>9(75)</td>
</tr>
<tr>
<td>OCP</td>
<td>1(25)</td>
<td>4(80)</td>
</tr>
<tr>
<td>Condom</td>
<td>1(25)</td>
<td>3(75)</td>
</tr>
<tr>
<td>Total</td>
<td>22(20.2)</td>
<td>87(79.8)</td>
</tr>
</tbody>
</table>

Figure 4.4: Chlamydial infection according to contraception method
4.5.5. Symptoms and signs correlated with chlamydial infection

Symptoms and signs associated with chlamydial infection are shown in Table 4.16. The prominent symptoms and signs correlated with chlamydial infection were purulent cervical discharge and contact bleeding when cervical swab was taken. Contact bleeding was a strong sign of chlamydial infection as a result of cervical friability. Odds ratio was appropriate tool used to assess the strength of the association. Infertile women were included in the same Table (4.16.) and revealed an association with chlamydial infection. In addition, when infertile women with chlamydial infection analyzed under all age groups, it was found that the age group 32-38 was significantly associated with *Chlamydia* infection \( p=0.016 \) (as illustrated in Table 4.17). Eight infertile women were in that age group, five out of eight were infected women. This was surprising since the overall association between Chlamydial infection and infertility variable was slightly significant \( p=0.049 \) (Table 4.16).
Table 4.16: Symptoms and signs associated with Chlamydial infection

<table>
<thead>
<tr>
<th>Sign and Symptoms</th>
<th>No. Tested</th>
<th>Chlamydia trachomatis (+)</th>
<th>%</th>
<th>P value</th>
<th>Odds Ratio</th>
<th>95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervicitis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>34</td>
<td>14</td>
<td>41.2</td>
<td>0.0002</td>
<td>5.9</td>
<td>2.15 - 15.97</td>
</tr>
<tr>
<td>No</td>
<td>75</td>
<td>8</td>
<td>10.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contact Bleeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13</td>
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*Fisher’s Exact Test; LAP: low abdominal pain; LBP: low back pain
Table 4.17: Chlamydial infection and infertility under age group 32-38 yr.

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<th>Chlamydial infection</th>
<th>Total(%)</th>
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<td>18(72)</td>
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</table>

P value = 0.016

It was found that secondary infertility was associated with higher rate of chlamydial infection (33.3%) as shown in Table 4.18 and Figure 4.5.

Table 4.18: Chlamydial infection in relation to type of infertility

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<th>Chlamydial infection</th>
<th>Total(%)</th>
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<td>True positive(%)</td>
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<td>87(79.8)</td>
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</tbody>
</table>

Figure 4.5: Chlamydial infection according to the type of infertility
4.6. PCR amplification results

The quality of DNA was detected by:

- Visualization of extracted DNA in agarose gel electrophoresis
- Visualization of internal amplified PCR product (209 bp in size), which was used basically to detect inhibitory specimens. Figure 4.4. represents the quality of extracted DNA

![Figure 4.6: Gel electrophoresis for eight DNA specimens isolated using QIAamp DNA minikit (Qiagen).](image)

Amplified products were detected on 3% agarose gel electrophoresis

![Figure 4.7: PCR amplification product for cryptic plasmid. Lane 1: Molecular weight ladder (100bp); Lane 2: positive control; Lane 3: negative control; Lanes 4, 5, 8: positive specimens PCR products (364bp) in size; Lanes 6, 7, 9, 10: negative specimens.](image)
Figure 4.8: PCR amplification product for cryptic plasmid, with internal control. 
Lane 1: Molecular weight ladder (100bp); Lane 2: positive control; 
Lane 3: negative control; Lanes 4, 5, 6, 9: positive specimens PCR products (364bp) in size; Lanes 7, 8: negative specimens. Internal control was detected on all specimens (209bp)

Figure 4.9: PCR amplification product for MOMP. Lane 1: Molecular weight ladder (100bp); Lane 2: positive control; Lane 3: negative control; lanes 4, 6, 7, 8: positive specimens PCR products for MOMP (182bp); Lanes 9,10: negative specimens
Chapter Five

Discussion
**Discussion**

*Chlamydia trachomatis* is one of the most frequent causes of STDs [42,76]. It is the commonest cause of cervicitis, urethritis and their sequaele (PID, chronic pelvic pain, tubal factor infertility, and reactive arthritis) [5]. Chlamydial infections are primarily a women health care issue since the manifestation and consequences are more damaging to reproductive health in women than in men [77].

Most urogenital *C. trachomatis* infections are initially asymptomatic but may subsequently cause considerable long-term morbidity. Consequently accurate diagnosis of *C. trachomatis* infection requires the use of specific laboratory techniques. However there is no clinical or microbiological reference standard for diagnosis of *C. trachomatis* infection [78]. Culture was the earlier gold standard, however PCR studies suggested that the sensitivity of culture even in expert laboratories is as low as 75% to 85% and is no longer considered a reference method of new diagnostic assays [79]. Moreover, EIA and PCR techniques are useful and reliable methods for detection of *C. trachomatis* in endocervical swab specimens [64,66].

This study was performed to determine the occurrence of *C. trachomatis* infection in symptomatic women. This is the first comparative study, using EIA and PCR assays for detection of *C. trachomatis* in endocervical swab specimens in Palestine. In the current study, in relation to plasmid-based PCR results, 23 positive specimens were detected giving a prevalence of chlamydial infection of 21.1% among 109 symptomatic women attending the gynecologist clinics in Gaza strip (as presented in Table 4.5), at the same time the results of EIA showed a prevalence of 19.3% (as shown in Table 4.4.). The overall prevalence of *C. trachomatis* among the study population is 20%. 

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These results showed that the occurrence of *C. trachomatis* is higher than expected. Although Palestine is a traditional Islamic society and according to the customs and traditions of Islamic societies, free sexuality is prohibited.

This high occurrence rate could be due to various risk factors such as infected partner (husband) and contaminated gynecologist’s investigation instruments and probably the high rate of asymptomatic chlamydial infections in Gaza Strip, particularly the southern and northern areas (Table 4.14.) Which represented the high rates of infection 25% and 21.7%, respectively. Moreover, the absence of STDs clinics which, could make a reduction for the high prevalence in the society by achieving treatment for the diagnosed cases (both partner i.e., husband and his wife) as well as could make programs for regular investigation of cases especially, asymptomatic infections.

In a number of studies from Eastern Europe countries, Domeika et al. reported that the prevalence of *C. trachomatis* infection in women consulting outpatient gynecological clinics varies between 6 to 25% [80]. We could divide the countries into those bearing low (< 10%), middle (11-21%), and high (>22%) prevalence of *Chlamydia* infections. Of the nine countries where such data were available, four fell within the low prevalence group. These were Slovenia (6%), Hungary (6.3%), Lithuania (8%), Poland (10%). Both St. Petersburg region (12.8%) and Bulgaria (16%) belong to the middle prevalence category; while Sverdlovsk region of Russia (22.1%) and Ukraine (25%) fell into the high prevalence group. Therefore, our results are in the upper limit of middle prevalence and more or less consistent with that of Bulgaria.

In the neighboring countries, like in Egypt, the prevalence appear to be very high about 31.0% as reported by Mohamed and Sharaf (2001) This might be due to selection of the highly symptomatic patients [6]. In Israel, and Jordon, the prevalence was 3.2 and 4.6 respectively. The low prevalence in Israel was explained on the base of female soldiers seeking medical care and that the study population was not really representative of symptomatic females. While the low prevalence in Jordan was explained as a
result of the conservative behavior of the Jordanian society towards free sexuality [33,34].

In the United Arab Emirates (UAE), the prevalence of chlamydial infection in endocervical swab specimens was low (2.6%). This might be due to the low risk of the study population or insensitivity of rapid antigen detection test used by the authors[39].

In Ghana, the prevalence of *C. trachomatis* in antenatal and gynecological patients was 3.0%. The low rate of *C. trachomatis* might be due to self medications [38].

In Iran, the study conducted in Tahran showed *C. trachomatis* prevalence as 22% [81]. Another study in Iran was conducted among cervicitis women in Tahran by Yazdi *et al* and showed that the prevalence of chlamydia infection in endocervical swab specimens was 15.5% by plasmid- based PCR [44].

In India, the overall prevalence of confirmed *C. trachomatis*, using both plasmid- and MOMP- based PCRs, in genital swab (urethral and endocervical) specimens among symptomatic men and women was 30.8% [35]. Another study in India showed the prevalence of *C. trachomatis* in endocervical specimens as 28% in younger women (age, 18 to 25 years) [49].

In Brazil, Santos cristina *et al*, detected *C. trachomatis* in endocervical smears of sexually active women The prevalence of his study was 20.7%, and 80% of the positive cases were found to be in the ≤ 23 year old age group [37].

In Senegal The prevalence of *C. trachomatis* infection among sex workers as reported by Strum-Ramirez *et al* was 28.5% [55].
In Bangladesh, Ness et al (2004), determined prevalence of *C. trachomatis* in endocervical swab specimens among hotel-based sex workers (HBSWs). The prevalence was 43.5% as determined by plasmid-based PCR [41].

The wide variation of *Chlamydia* prevalence and detection rates in different studies could be due to several factors:

♦ Study population (selection of high risk group, symptoms, education level, etc.).
♦ Rate of infection in the study area.
♦ Hygiene level and socioeconomic status of the study area.
♦ Technique used.

The EIA PCE (IDEIA PCE) is a dually amplified EIA since it incorporates a polymer conjugate to increase the sensitivity compared to that of an earlier version of the same assay (IDEIA) [64]. In clinical settings, this test has been found to have relative sensitivities of 91.8% with endocervical swabs [69], and 91.4% for men with urethritis compared to a NAAT [66]. A second study of men with urethritis, however, found a relative sensitivity of 53% [82].

Our study estimated an intermediate level of relative sensitivity of 73.0%. Other studies conducted in different parts of the world showed more or less the same results we obtained e.g., in the study of Horner et al, the relative sensitivity of EIA PCE was found to be 75% [83].

When the results of EIA were correlated with that of PCR, the difference was not statistically significant. This is consistent with other studies [44,66,84]. On the other hand, there is a good agreement of the results of the two techniques as determined by kappa values (k=0.6).
The reason behind choosing IDEIA PCE kit was based on that IDEIA PCE kit has no cross reactivity with Gram negative bacteria (e.g., *N. gonorrhoea*, *Acinetobacter* spp, *Salmonella* spp, *E.coli* and *Gardnerella vaginalis*) and Gram positive bacteria (e.g., *Staphylococcus aureus*, *Streptococcus agalactiae*, *Peptostreptococcus* spp) according to manufacturer kit pamphlet. In addition to its reported high specificity and sensitivity compared to NAAT.

Our results, however, showed that IDEIA PCE test has low sensitivity (73%) and specificity (94%). The low specificity could be explained by the presence of false positive results (as shown in Table 4.11) and could be explained by some cross reaction with other infectious agents components such as Gram negative bacterial LPS. This point of view is supported by other studies conducted recently which showed that EIA has low sensitivity and specificity when compared to NAAT [83,85]. In addition, the LPS-based EIA tests detect all three chlamydial species and, therefore, are not species specific.

The united kingdom national clinical effectiveness guideline for *C. trachomatis* recommends that EIAs should not be used in situations in which their sensitivity is <80% [86]. In our study, the sensitivity of PCE EIA with confirmation by a NAAT was below this level. Consequently our findings suggest that EIA is not suitable for *Chlamydia* screening of women in the our community.

DNA extraction and purification was achieved by using optimized DNA extraction protocol (Qiamp DNA minikit) and this was very critical point since it demonstrated that larger PCR amplicons could be successfully obtained and ensured high sensitivity of detection of *C. trachomatis*. Several investigators employed the same extraction kit and showed its high efficiency [59,60]
PCR Kits employed in this study are well optimized for detection of either plasmid or MOMP nucleic acids. A study conducted by Vinayagamoorthy et al., employed the same kits in their research work [87]. They achieved good results which encouraged the use of such kits. The kit has the advantage of being ready made, so it reduces the possibility of contamination that could emerge during the preparation of PCR reagents.

Moreover such kits were supplemented with positive control. These positive controls were cDNA for both pCT and MOMP. In addition, chlamydial DNA LGV type II positive control was also used and gave similar results to that obtained by cDNA positive controls, which was considered as an external control.

The sensitivity of plasmid- and MOMP- based PCRs were 100% and 86% respectively. A possible explanation for discrepancies in the results of our study between the plasmid- and MOMP-based PCRs might be a difference in the target used for the amplification (Table 4.8). The PCR assay that we used to detect *C. trachomatis* is based on a cryptic plasmid primer, which has been proved to be a more sensitive approach than that used for the MOMP primer-based PCR [13,88], because plasmids are present in EBs in multiple copies. This may explain why five specimens were positive only by Plasmid- based PCR but not by the MOMP-based PCR.

In addition, the specificity of plasmid- and MOMP- based PCRs were 98% and 100%, respectively. This difference could be explained by the presence of two false positive results in plasmid-based PCR (Table 4.10). Although these were recorded as false positive for analytical purposes, it can be explained by the slightly lower sensitivity of MOMP-based PCR as well as likelihood of *C. trachomatis* infection cannot be excluded in such cases of high risk symptomatic patients. The remaining specimen, which was found positive in both MOMP- based PCR and EIA, could contain a plasmid-free variant of *C. trachomatis* (Table 4.10). This is also supported by other studies which
showed the possibility of encountering plasmid- free strains [45,89,90], suggesting that it is not really essential for the growth of the organism.

The comparison of the three assays used in the current study is shown in (Table 4.9) and revealed that 23 positive specimens out of 109 specimens were identified by cryptic plasmid- based PCR, while MOMP- based PCR identified 19 specimen, at the same time EIA showed 21 positive specimens.

Several studies achieved similar results for instance, a study conducted by Malathi et al. (2003), for determination of the prevalence of *C. trachomatis* causing conjunctivitis using PCR revealed that PCR using cryptic plasmid primers was the most sensitive method n detecting *C. trachomatis*. Other studies, however, showed that the results of cryptic plasmid- and MOMP- based PCRs were the same [42].

The false positive results account for an important proportion of all positive test results among groups of patients with low prevalence of chlamydial infection. The effect of false positive tests in a population can be quantified by the positive predictive value (PPV). The PPV is the proportion of cases with positive test result for a disease which actually have that disease. In chlamydial screening applications, the PPV is influenced primarily by the specificity of the test and the prevalence of *Chlamydia*. In the current study, the specificity for EIA, Plasmid-, and MOMP- based PCRs were 94%, 98%, and 100%, respectively. The PPV of these tests (Table 4.11) were 76%, 92%, and 100% respectively. These results showed that as the specificity of a test approaches 100%, the PPV will also be enhanced, and, by definition, a test with 100% specificity will yield no false positive results — therefore, the PPV of the test would also be 100%. According to the CDC guidelines for chlamyial diagnosis [9], PPV for EIA (73%) was less than 90%, therefore the test should be supplemented by another confirmatory preferably a NAAT. While PPV for MOMP- and plasmid- based PCRs were 100% and 92%, respectively.
Our finding of false-positive PCR support the advice that chlamydia-positive plasmid-based PCR results should be confirmed by MOMP-based PCR. The presence of amplification inhibitors in clinical specimens, which can result in false-negative results, is also a recognized potential disadvantage of NAATs [9]. Therefore, even utilizing technology with increased sensitivity, training of staff in techniques of appropriate specimen collection is critical. The inclusion of internal β globin primer in plasmid-based PCR was done to ensure adequate and intact DNA as well as to detect any refractory specimens. The inhibition rate was 0% in our study and this is consistent with other studies [52].

In our study we found the highest prevalence of *C. trachomatis* infection among women (Table 4.16) suffering from cervicitis (41.2%), contact bleeding (46.2%) and infertility (26.6%). These symptoms are caused by *C. trachomatis* infection and have been reported by several investigators. We found also that the presence of mucopurulent cervicitis and contact bleeding were associated significantly (p<0.05) with the detection of *C. trachomatis* infection (Table 4.16).

Infertility is becoming an emerging health problem in many countries of the world including Palestine. The increase appears to coincide with the growing role played by *C. trachomatis* as a sexually transmitted disease. In our study, *C. trachomatis* infection was found in 28.6% of the infertile females which is quite high and surprising. The incidence of *C. trachomatis* infection was more common in women with secondary infertility (Table 4.18) Secondary infertility associated with higher rates of chlamydial infection has been reported by other investigators [91,92].

Odds ratio is a relative measure of risk, showing how much more at risk a person exposed to a given factor will be to developing the outcome disease/condition being studied, compared to someone who has not been exposed. An odds ratio greater than one suggests that the exposure places a person at greater risk of developing the outcome being studied. In our study the important odds ratio showing statistical significant relationship associated chlamydial infection (Table 4.16) were 5.9 (2.15 –15.97), 4.3 (1.2 –14.45) , and
2.6 (0.99 – 6.85) for mucopurulent cervical discharge, contact bleeding and infertility, respectively. These results showed clearly the role of chlamydial infection in development of such signs and symptoms.

Our results showed that the prevalence of *Chlamydia* infection was higher in women aged 25 to 31 yr and 32 to 38 yr, as compared to the other age groups (Table 4.12.) This finding is in contrast to other studies showing a decline of the prevalence rate after 25 yr of age [77,93,94].

In addition, the highest chlamydial infection rate was found in elementary education level group (Table 4.13.), and among IUCD users (Table 4.15).

Women who undergo uterine instrumentation are considered at risk for pelvic infections by *C. trachomatis*, which may be derived either from ascending endocervical infections or from reactivation of micro-organisms persisting in the genital tract after previous chlamydia infections. Women presenting at infertility clinics frequently undergo uterine instrumentation (e.g. hysterosalpingography and laparoscopy) and thus may be at risk of infection.

Finally, in symptomatic patients, PCR sensitivity, and specificity appeared very high in endocervical swab specimens and consequently will be very useful technique for better detection of such microorganisms.
Chapter Six

Conclusions & Recommendations
6.1. Conclusions

Finally we concluded that overall infection rate of *C. trachomatis* was 20%. EIA detected 19%, while plasmid-based PCR detected 21.1%, and MOMP-based PCR detected 17.4%.

The sensitivity for EIA was 73%, plasmid-based PCR was 100%, and 86% for MOMP-based PCR; while the specificity for EIA was 94%, plasmid-based PCR 98% and 100% for MOMP-based PCR. The PPV for EIA, plasmid- and MOMP-based PCR assays were 76%, 92%, and 100%, respectively.

In addition, efficiency of EIA, MOMP-, plasmid – based PCR assays were 90%, 97%, and 98%, respectively.

Our results showed that there is a significant correlation between chlamydial infection and infertility (P<0.05). Odds ratio for developing outcomes of chlamydial infection were 5.9, 4.3, and 2.6 for cervicitis, contact bleeding, and infertility, respectively.

Our study proved that plasmid based- and MOMP based- PCR assays are more efficient for detection of *C. trachomatis* infection than EIA technique.

Therefore we strongly recommend PCR use as a technique of choice. Although financial constraints may impede the routine use of molecular diagnostic methods in Palestine, it should be borne in our minds that the cost arising from the clinical sequelae of leaving genital *C. trachomatis* infection undiagnosed and untreated may exceed the cost of these diagnostic methods.
6.2. Recommendations

♦ It is recommended to use of a PCR technique as a routine test for diagnosis of chlamydial infection in our clinical laboratories.

♦ It is advisable to initiate awareness program among physicians and patients.

♦ We advise to establish accurate data base for STDs.

♦ It is recommended to all gynecology physicians to take the best disinfection sterilization steps to avoid any source of infection.

♦ Screening of all infertile women, and to all women used or in use of IUD for contraception.

♦ Partner notification, management and follow up of the infected patients.

♦ Further studies should be done to identify C. trachomatis strains prevalent in our area which could be useful for epidemiology and recognition of both mixed and re-infection patient cases.

♦ It is recommended to use urine specimens for women when pelvic examination is not acceptable.
References
References


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Appendices
Appendix A

The study specimens EIA results

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<th>Serial No.</th>
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*P=*Positive results; *E=*Equivocal results; Mean absorbance of negative control = 0.180; Cutoff = Mean absorbance of negative control + 0.05 = 0.180 + 0.05 = 0.23; Equivocal results between this range 0.015 ± 0.23 = (0.215 — 0.245); Positive specimen results OD>0.245; Negative specimen results OD<0.215.
The study specimens concordance & discordance results by the three employed methods.

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Appendix C

Questionnaire

Date of collection:

Clinical Data

Patient Name:

Age:

Address:

Status:  { } Single  { } Married  { } Divorced

Menstrual Cycle:  { } Regular  { } Irregular  Date of LMP………..

Infertility:  { } Yes  { } No if yes specify………..

Mucopurulent Cervicitis:  { } Yes  { } No

Contact Bleeding:  { } Yes  { } No

Pregnant:  { } Yes  { } No

Using Contraceptive:  { } Yes  { } No if yes specify ………..

Current complains (like dysparunia, dysuria, and ectopic pregnancy):

Specify……………………………………..

Abnormal Vaginal Discharge —  { } Quantity  { } Color  { } Odor

{ } Pruritis.

Previous Complain:

Specify……………………………………..

Education Level

Primary { } Preparatory { } Secondary{ } Higher Education{ }

Other Underlying Disease: …………………………….

Collected by Dr……..

Researcher: Iyad A. El-Qouqa
Appendix D
Consent form

..............................................................

السيدة الفاضلة/ ...........................................

تحية طيبة وبعد,

يقوم الباحث بالجامعة الإسلامية بعمل دراسة للكشف عن أحد أهم الميكروبات التي تسبب التهابات المجاري التناسلية وخاصة التهابات عنق الرحم والتي قد تصاحبا مضاعفات كالالتهابات الحوض والعقم والإجهاض.

لذا نرجو من سيداتكم التعاون مع الطبيب المختص، لأخذ مسحات من عنق الرحم.

كما نرجو من سيداتكم إعطاء المعلومات والبيانات اللازمة التي تساعد في التشخيص الدقيق لهذا الميكروب.

عذراً مع تلك البيانات والمعلومات ستكون سرية للغاية لغرض البحث العلمي فقط.

تعاونن معنا سيعود عليكم بالفائدة وسيقلل من المضاعفات التي يمكن تجنبها.

توفيق المريضة/..............................................

الباحث بالجامعة الإسلامية
Appendix E
Helsinki approval

Palestinian National Authority
Ministry of Health
Helsinki Committee

Date: 3/5/2005

Mr./Iyad EL-Qouqa

I would like to inform you that the committee has discussed your application about:

Detection of Chlamydia trachomatis in Endocervical Swabs Using Molecular (PCR) and Enzyme immunoassay Techniques.

In its meeting on May 2005 and decided the Following:-

To approve the above mention research study.

Conditions:-
✓ Valid for 2 years from the date of approval to start.
✓ It is necessary to notify the committee in any change in the admitted study protocol.
✓ The committee appreciate receiving one copy of your final research when it is completed.

Signature

Member

Member

Chairperson

Gaza Etwan – Telefax 972-7-2878166
Appendix F

M.O.H. approval

Detection of *Clamydia trachomatis* in Endocervical Swabs Using Molecular (PCR) and Enzyme immunoassay Techniques.

This project is aimed to detect *Clamydia trachomatis* in endocervical swabs using molecular (PCR) and enzyme immunoassay techniques. The project was carried out under the supervision of Dr. [Name], with the cooperation of the Department of Microbiology.

The study was conducted at the [Hospital Name], where a total of 100 female patients were screened for *Clamydia trachomatis* infection. The results showed a positive rate of 15% in the study population.

The findings of this study highlight the importance of early diagnosis and treatment of *Clamydia trachomatis* infection, which can lead to complications such as pelvic inflammatory disease and infertility.

The project was funded by the Ministry of Health, with the support of the Islamic University of Gaza and the government of Palestine.
Appendix G
Composition of buffer and reagents

Composition of PBS:

♦ 50 mM potassium phosphate
♦ 150 mM NaCl; pH= 7.2

50x TAE buffer preparation:

♦ Tris base ................................. 242 g
♦ Glacial acetic acid............... 57.1 ml
♦ EDTA................................. 18.6 g
♦ H₂O up to.............................. 1000 ml

  pH 8.0

Working TAE buffer, 1X:
20 ml stock solution (50x) diluted to one liter with distilled water

Ethidium bromide ( stock solution)
Ethidium bromide 10 mg/ml

Composition of the Blue/Orange Loading Dye, 6X

♦ 0.03% bromophenol blue
♦ 0.03% xylene cyanol FF
♦ 0.4% orange G
♦ 15% Ficoll® 400, 10mM Tris-HCl (pH 7.5)
♦ 50mM EDTA (pH 8.0).