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Endocervical miRNA Expression Profiles and Genotyping of *Chlamydia trachomatis* in Infected Women.

A Thesis

Submitted to the Council of the College of Medicine, University of Babylon, in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Science / Medical Microbiology

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Dedication

To my compassionate Father.... My role model, my ideal in life, he is the one who taught me how to live

.With dignity and glory

To my tender mother..... I do not find words that can give her due, it is the epic of love and the joy of a lifetime, and the example of dedication and giving

To my brothers and sisters.... My support, my support, and my share of my joys and sorrow

To my husband.... The highest symbol of sincerity and loyalty and the companion of the path

To my children..... The livers

To all the evacuees, I dedicate to you my scientific research…...

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Summary

Chlamydia trachomatis, which belongs to the family Chlamydiaceae, is the most commonly reported sexually transmitted bacteria that lead to infections of the human genital tract. *Chlamydia trachomatis* infects the columnar epithelial cells and causes endocervical infection, which is mainly asymptomatic.

Pelvic inflammatory disease was a leading cause of both tubal factor infertility and ectopic pregnancy. *Chlamydia trachomatis* is an important risk factor for Pelvic inflammatory disease.

The present case-control work aimed to study and analyze *Chlamydia trachomatis* genetically in patients with pelvic inflammatory disease, which included 200 endocervical swab samples collected from female patients suffering from signs and/or symptoms of Pelvic inflammatory disease; they were diagnosed as having Pelvic inflammatory disease by the gynecologist and had risk factors for this infection. In addition, 25 endocervical swab samples from females used as a control, which used in the second part of this work. Patients attained to the out-patient clinics of Gynecology and Obstetrics, in two hospitals of Babylon Province: Babylon Maternity and Pediatrics Hospital, and Al- hashemia General Teaching Hospital; as well as the samples taken from private clinics, during the period from March to August 2022.

All 200 samples were subjected for immunological diagnosis of *Chlamydia trachomatis* and only 30/200(15%) gave positive results for *Chlamydial trachomatis* with rapid strip serological test.

The samples were subjected to DNA extraction. DNA extracted from swabs were utilized for molecular detection of *Chlamydia trachomatis*, among Pelvic inflammatory disease patients, carried out by using

housekeeping *omp* genes (144bp) as genetic markers via conventional PCR; where gave approximate results 22/200 (11%).

From all 200 collected endocervical swab specimens, this study found that only 8/22(36%) were had bleeding between time with highest percentage according to other grouping, 5/22(23%) were had primary infertility and secondary infertility, and 4/22(18%) were had recurrent miscarriage

Only 100/200 (50%) women who were diagnosed by the gynecologists as having pelvic inflammatory disease with age group (20-30) found that 11/22(50%) were positive for *Chlamydia trachomatis*. 5/11(45.4%) were positive for Chlamydia had bleeding between time, while 2/22(18.1%) were positive for Chlamydia had primary infertility, secondary infertility, and recurrent miscarriage; while 100/200 (50%) women with age group (30- 40 years) found that 11/22(50%) were positive for *Chlamydia trachomatis*. observed that the primary infertility, secondary infertility, and bleeding between time had the same rate 3/11(27.2%) which had the high rate in this range of age, while recurrent miscarriage had 2/11(18.1%).

A total of (22) patient endocervical swab samples which gave positive results for *Chlamydial trachomatis* were RNA extraction and using to study the gene expression of microRNA by using (ΔΔcycle thrashed) PCR method. The level of expression to microRNA-142 and 520-a genes in the test sample as well as in control samples normalized with house- keeping gene. The study showed that the expression of the micro-142 and 520-a gene were elevated in *Chlamydia trachomatis* patients when compared to the control group, with the expression of the gene increasing more than 40% and 80% respectively when compared to the control group.

Also, only 20 tested isolates of *Chlamydia trachomatis* were successfully amplified in the *omp1* PCR with amplicon size 1134pb to Sequence analysis of the *omp1* gene from clinical isolates of *Chlamydia trachomatis* and the phylogenetic analysis of *omp1* gene showed that *Chlamydia trachomatis* were segregated in to two main clusters (A and B). The A cluster divided in to two sub cluster and contained 4 and these groups contained 9 isolates and only two isolates (CT2-CT1) showed 100% identical or similarity while other isolator characterized by small genetic distance with each Cluster. While cluster B contain two sub cluster which 4 group and these groups contain 11 isolates which have small genetic distance. Sequencing data analysis showed variability in *omp*A sequence. It was found that the most substitution mutation displayed C > T in 18.9612, then G > A in 17.3018 follow by A > G in 14.9856 and T > C in 13.5256.

The results of in silico PCR-RFLP for *omp*1 gene by using *Alu*1 enzymes. Show that 9 genotype was found from 20 clinical isolates and according to the phylogenetic analysis the results show that all isolates grouped into two cluster. The cluster (A) contained two subcluster and contained (15) isolates, While the subcluster (B) contain (5) isolates.

According to the data in this investigation, the study noticed that several potential biomarkers such as microRNA 142 and 520a may be associated with susceptibility to *Chlamydia trachomatis* and severity and progressive of diseases caused by *Chlamydia trachomatis*. Genotyping of *Chlamydia trachomatis* was important to study the pathogenesis and epidemiology of genital chlamydia infection.

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List of Abbreviations

Abbreviations	Key
µg	Microgram
µl	microliter
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate synthetase
bp	base pair
bv	bacterial vaginosis
CD	Cluster of Differentiation
Cds	Contact-dependent secretion
cg MLST	core genome Multilocus Sequence Typing
cHsp	chlamydial heat shock protein
Cop	Chlamydial outer protein
CPAF	chlamydial protease-like activity factor
CSCC	cutaneous squamous cell carcinoma
CTSS	Chlamydia trachomatis with signs and symptoms
CTNS	Chlamydia trachomatis with no signs and symptoms
DFA	Direct Fluorescent Antibodies
DNA	Deoxy Ribo Nucleic Acid
EB	Elementary body
EBV	Epstein-Barr virus
EDTA	Ethylene di-amine tetra acetic acid
EIA	Enzyme immunoassay

ELISA	Enzyme linked immunosorbent assays
EMT	epithelial-mesenchyme transition
H2O	Water
HCC	human hepatocellular carcinoma
hES	human embryonic stem
HIV	human immunodeficiency virus
HOCl	Hypochlorite
H_2O_2	hydrogen peroxide
HPV	human papillomavirus
HR-HPV	high-risk human papilloma virus
Hsps	Heat shock proteins
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Inc	Inclusion Membrane Protein
ISR	Intergenic spacer region
IUCD	intrauterine contraceptive device
Kbp	Kilo-base pair
kDa	Kilo-Dalton
LGV	lymphogranuloma venereum
LPS	Lipopolysaccharide
Mbp	Mega-base pair
MHC	Major histocompatibility complex
min	minute
MLST	Multi locus Sequence Typing

mM	miliMolar
Momp	major outer membrane protein
MPO	Myeloperoxidase
mRNA	messenger Ribo Nucleic Acid
miRNA	micro-RNA
NAATs	Nucleic acid amplification tests
NGU	Non-gonococcal urethritis
NK	Natural killer cell
Nm	Nanometer
OMP	Outer membrane protein
OR	Odds ratio
PBS	Phosphate Buffer saline
PCR	Polymerase Chain Reaction
qpcr	Quatitive polymerase Chain Reaction
PGP	plasmid glycoproteins
pH	Power of hydrogen
PID	Pelvic inflammatory disease
PMN	Polymorphonuclear neutrophils
RB	reticulate body
RBC	Red Blood Cell
RDTs	Rapid diagnostic tests
RFLP	Restriction fragment length polymorphism
RFU	relative fluorescence units.
RISA	Ribosomal RNA intergenic spacer analysis
ROS	Reactive Oxygen species

RSA	recurrent spontaneous apportion
rpm	Round per minute
RNA	Ribo Nucleic Acid
rRNA	ribosomal Ribo Nucleic Acid
ncRNA	non coding Ribo Nucleic Acid
s	second
S	Svedberg
Scc	Specific chlamydial chaperones
SNP	single nucleotide polymorphisms
SP1	Specificity protein 1
STI/ STD	Sexually transmitted infections/ diseases
T3SCs	Type III secretion chaperones
T3SS	type III secretion system
TAE	Tris-acetate-EDTA buffer
TBE	Tris- brotase- EDTA buffer
TE	buffer Tris- EDTA buffer
TFI	tubal fallopian infertility
TLRs	Toll-like receptors
TNF	tumor necrosis factor
U	Unit
UPGMA	unweighted pair group method with arithmetic mean
UTR	Untranslated region
UV	Ultra Violet

v/v	Volume/volume dilution
v/w	Volume/weight dilution
vs.	versus
WBC	White blood cell

Chapter One

Introduction and Literature Review

1.1 Introduction:

Chlamydia trachomatis is an important pathogen that causes various diseases and syndromes, including urogenital infection, trachoma and lymphogranuloma venereum, depending on the serovar involved (Manavi *et al.*, 2006).

Chlamydia trachomatis, which belongs to the family Chlamydiaceae, is the most commonly reported sexually transmitted bacteria that lead to infections of the human genital tract. *Chlamydia trachomatis* infects the columnar epithelial cells and causes endocervical infection, which is mainly asymptomatic (Banhart *et al.*, 2018).

Pelvic inflammatory disease (PID) is a leading cause of both tubal factor infertility and ectopic pregnancy. *Chlamydia trachomatis* is an important risk factor for PID, but the proportion of PID cases caused by *C. trachomatis* is unclear (Malcolm *et al.*, 2016).

As an agent of urogenital infection, *C. trachomatis* is the most prevalent bacterial sexually transmitted disease (STD), with estimated 92 million new cases annually worldwide (Bom *et al.*, 2011).

Approximately 50% of men and 70% of women infected individuals are asymptomatic, making it difficult to diagnose and treat. When the infection remains untreated, complications such as pelvic inflammatory disease may occur, leading to severe sequelae like ectopic pregnancy and infertility (Quint *et al.*, 2007).

Nineteen serovars and related variants (A, B/Ba, C, D/Da, E, F, G/Ga, H, I/Ia, J, K, L1, L2, L2a and L3) of *C. trachomatis* have been identified by using polyclonal and monoclonal antibodies against the major outer membrane protein (MOMP) (Bandea *et al.*, 2001, *Hsu et al.*, 2006).

Serovars A, B and C have usually been associated with trachoma, whereas D through K have tended to correlate with urogenital infections. Serovars L1 though L3 are commonly associated with lymphogranuloma venereum (Beni *et al.*, 2010).

Typing of *C. trachomatis* strains remains an important goal in the field of epidemiology, as do clinical and basic research on *C. trachomatis* infections. The temporal and geographical distribution of strains throughout the world has significant implications for understanding of the epidemiology of this infectious agent and for vaccine development (Bandea *et al.,* 2001).

The traditional immunotyping methods were replaced by genotyping methods, such as restriction fragment length polymorphism (RFLP) or DNA sequence analyses of the major outer membrane protein (Momp) gene *omp*A (Ruettger *et al.,* 2011).

Chlamydia trachomatis is the most common bacterial cause of sexually transmitted infections (STI) worldwide, with over 1.7 million cases reported in the United States in 2018 (CDC 2018).

Infection rates are highest among adolescents and young adults aged 15 to 24 years. Additionally, higher rates are reported among women and African-Americans. Some *C. trachomatis* infections in women are asymptomatic and require screening efforts to diagnose. A proportion of Chlamydial infections will progress to pelvic inflammatory disease (PID). PID may be symptomatic, but may also present with vague or nonspecific symptoms or may be asymptomatic. Diagnosis is based on imprecise clinical findings, which may vary by provider and experience of the provider. Given these diagnostic limitations, mild or asymptomatic episodes of PID may not be recognized or treated, which may result in ectopic pregnancy, tubal factor infertility, and chronic pelvic pain (Workowski et al. 2015).

There are currently no known biomarkers in humans that predict risk for progression to upper genital tract disease. Furthermore, if there are specific biomarkers that are associated with progression of disease, it is not known how these could be implemented clinically. One potential biomarker of interest is noncoding

micro (~22-nucleotide) RNAs (miRNAs or miRs). These are stable, widely distributed molecules associated with inflammatory responses to human pathogens (Eulalio *et al.*, 2012).

The role of miRNAs in chlamydial pathogenesis has been demonstrated in both animal models and chlamydia-infected humans (Arkatkar *et al.*, 2015). Additionally, in a murine model of chlamydial genital infection, demonstrated that a unique set of miRNAs present in cervical tissue early in infection is associated with progression to upper tract disease (hydrosalpinx). These particular miRNAs were associated with infection by virulent but not avirulent chlamydial strains (Yeruva *et al.*, 2017).

However, it is not known if miRNAs can be measured from a human cervical swab and whether miRNA patterns detected in cervical samples from women with chlamydial infection are associated with signs and symptoms. Therefore, study evaluated the patterns of miRNAs in women with Chlamydial infection with symptoms and/or signs. Moreover, Other utilized material obtained directly from the endocervical region, which is more likely to accurately reflect the physiological processes (Batteiger *et al.*, 2020).

The aim of the study:

This work aimed to investigate the role of miRNA expression profile during Chlamydia infection and genotyping of *Chlamydia trachomatis* in Hilla city.

Steps of work:

1-Endocervical swab samples were collected from symptomatic women with PID and endocervical swab samples used as control group from healthy women.

2- Immunological Diagnosis of *Chlamydial trachomatis* by using rapid strip test.

3-Genomic DNA and RNA extracted from Endocervical specimens from woman with PID.

4- Molecular identification of *Chlamydia trachomatis* by using house-keeping gene *omp* (outer membrane protein) gene.

5- Study the role of micro-RNA in positive patients with Chlamydia and control like miR-142, and -520a by using real-time PCR (relative gene expression).

6- Genotyping of *Chlamydia trachomatis* by using sequencing of *omp*-1 gene and insilico PCR-RFLP.

1.2 Literature review:

1.2.1. General Characters of *Chlamydia*:

Members of the Chlamydiales are classified into one of the following eight families: Parachlamydiaceae, Criblamydiaceae, Waddliaceae, Simkaniaceae, Rhabdochlamydiaceae, Clavichlamydiaceae, Piscichlamydiaceae, and Chlamydiaceae (Gupta *et al.*, 2015).

With the exception of the Chlamydiaceae, most members of this order infect various hosts in the environment and are collectively referred to as "environmental" Chlamydia. In contrast, the members of Chlamydiaceae, which contains the single genus Chlamydia, are considered pathogenic and contribute to disease burdens in humans and animal species of commercial importance. The genus Chlamydia comprises nine species: *Chlamydia trachomatis, C. muridarum, C. pneumoniae, C. pecorum, C. suis, C. abortus, C. felis, C. caviae, and C. psittaci* (Cheong *et al.*, 2019).

Chlamydia are microorganisms exhibiting characteristics intermediate between bacteria and viruses. It is widespread in the natural world, intracellular parasites of humans and animals. They are capable of independent reproduction, because they do not synthesize ATP, in its development cycle using the host cell metabolic pathways. The life cycle of these microorganisms is original, unique among bacteria and lasts from 24 to 48 hours (Omsland *et al.*, 2014).

Chlamydia antigens consist of 4 groups: group-specific, species-specific, type-specific and subspecies-specific. The group of species-specific antigens consists of major outer membrane protein (Momp) and heat shock proteins. *C. trachomatis* is a potent immunogen, stimulating the immune processes of microorganisms. In the course of *C. trachomatis* infection, the response mechanisms involved are: non-specific, specific, humoral and cellular (Choroszy *et al.*, 2012).

Chronic infection is characterized by maintenance of microorganisms in the host cell. Inflammation is formed in less time and with increased intensity and has a

rapid immune response on the part of previously sensitized lymphocytes. *C. trachomatis* infections are the most common bacterial sexually-transmitted infections (Unemo *et al.,* 2017).

Sexual transmitted disease represents an important clinical problem for doctors in many areas of medicine such as dermatology, venereology, ophthalmology, gynecology and obstetrics, rheumatology and others. Chlamydial infections are important pathogens in medical practice, not only because they cause disease in various fields of medicine, but also because of the large proportion of the population suffering and exposed to these microbial infections (Unemo et al. 2017).

Chlamydial infections are characterized by multifocality and polymorphism changes. Chlamydia causes inflammation in the adult urethra and cervix with the possibility of serious complications, and can cause perinatal infections in infants (Tímea, 2020).

Chlamydial infection is the most frequently reported bacterial infectious disease in the United States, and prevalence is highest among persons aged ≤24 years (CDC. 2019).

Multiple sequelae can result from *C. trachomatis* infection among women, the most serious of which include PID, ectopic pregnancy, and infertility (Hoenderboom *et al.,* 2019).

Certain women who receive a diagnosis of uncomplicated cervical infection already have subclinical upper genital tract infection. Asymptomatic infection is common among both men and women. To detect chlamydial infection, health care providers frequently rely on screening tests. Annual screening of all sexually active women aged <25 years is recommended, as is screening of older women at increased risk for infection (e.g., women aged ≥25 years who have a new sex partner, more than one sex partner, a sex partner with concurrent partners, or a sex partner who has an STI) (LeFevre, 2014).

In a community-based cohort of female college students, incident chlamydial infection was also associated with bacterial vaginosis (BV) and high-risk of human papilloma virus (HR-HPV) infection (Aghaizu *et al.,* 2014).

Although chlamydia incidence might be higher among certain women aged ≥25 years in certain communities, overall, the largest proportion of infection is among women aged <25 years, Chlamydia screening programs have been demonstrated to reduce PID rates among women (*CDC.2019*).

Although evidence is insufficient to recommend routine screening for *C. trachomatis* among sexually active young men because of certain factors (i.e., feasibility, efficacy, and cost-effectiveness), screening of sexually active young men should be considered in clinical settings with a high prevalence of chlamydia (e.g., adolescent clinics, correctional facilities, or STD specialty clinics) or for populations with a high burden of infection (LeFevre, 2014).

Among women, the primary focus of chlamydia screening should be to detect and treat chlamydia, prevent complications, and test and treat their partners, whereas targeted chlamydia screening for men should be considered only when resources permit, prevalence is high, and such screening does not hinder chlamydia screening efforts for women (van Bergen *et al.,* 2021).

Undoubtedly, the best-characterized species in this genus is *C. trachomatis*, which infects humans and is a major cause of ocular and urogenital diseases. This species is classified into serovars (serological variants) and two human biovars (trachoma and lymphogranuloma venereum [LGV]), dictated by the nature of the diseases that they cause (Harris *et al.,* 2012). Based on high-resolution genome-wide single-nucleotide polymorphism (SNP) analysis, the population structure of the trachoma biovar is further subdivided into two lineages: lineage 1 comprises clinically prevalent urogenital serovars (D, E, and F), whereas lineage 2 includes uncommon urogenital serovars (G, Ia, J, and K) (Robert *et al.,* 2016).

These serovars are the primary cause of sexually transmitted diseases, such as cervicitis and urethritis, which can further progress into pelvic inflammatory disease in women and epididymitis in men (da Silva *et al.,* 2017).

The ocular serotypes (A, B, and C) cause trachoma, a chronic inflammatory disease resulting in infectious blindness, and cluster into a lineage that likely evolved from a urogenital ancestor (Satpathy *et al.,* 2017).

The LGV biovar contains invasive serovars L1 to L3, including the epidemic L2b strain, which can disseminate to regional lymph nodes and cause invasive disease, including ulcer formation, inguinal lymphadenopathy, and hemorrhagic proctitis (Desclaux *et al.,* 2018).

1.2.2. General Characters of *Chlamydia trachomatis*:

Chlamydia trachomatis is a Gram-negative, obligate intracellular pathogen that can lead to a broad spectrum of clinical diseases in human populations. It is known to cause a significant burden of preventable blindness in third world countries. *Chlamydia trachomatis* is also known as the most common bacterial sexually transmitted infection worldwide (Newman *et al.,* 2012).

Chlamydia trachomatis infections represent a major problem worldwide. Urogenital infections with *C. trachomatis* have been associated with a wide range of genitourinary conditions including cervicitis and salpingitis in women as well as epididymitis and urethritis in men. Infection with the pathogen is however often asymptomatic and hence frequently remains undiagnosed, leading to an array of severe long-term consequences (Hsu, 2019).

Studies indicate that chlamydial infections can lead to severe impairments such as pelvic inflammatory disease, tubal damage and ultimately tubal factor infertility in women if they are not treated in a timely and adequate fashion (David *et al.,* 2021).

Infections with *C. trachomatis* can severely impact the reproductive health of women, causing severe conditions such as ectopic pregnancies, repeated and spontaneous abortions and stillbirths (Thomas *et al.,* 2019).

The asymptomatic nature of the disease also requires evidence-based guidelines for the implementation of population-wide screening programs. In fact, studies have claimed that, due to the low prevalence of Chlamydia at the population level, screening in the general population may not be cost-effective (Brunton *et al.*, 2016). The identification of high-risk groups or chlamydial infections is however a public health issue (Evenden *et al.*, 2018).

Chlamydia trachomatis causes the world's most common non-viral sexually transmitted disease. As a result of the varying levels of specificity and sensitivity of the diagnostic tools utilized in the clinical settings, the choice of method is of primordial importance (Pierre *et al.,* 2017).

1.2.3. Pathogenesis of *Chlamydia trachomatis*:

Chlamydia trachomatis, an obligate intracellular Gram-negative bacterium, is characterized by a wide range of different serotypes responsible for several local or systemic human diseases, including genital tract manifestations (D–K), trachoma (A–C), and lymphogranuloma venereum (L1–3). Among them, *Ch. trachomatis* genital infections are the most common sexually transmitted diseases of bacterial origin, with more than 130 million new cases per year worldwide (Rowley *et al.*, 2016).

Symptoms of *C. Trachomatis* include abnormal vaginal discharge and dysuria (Wiesenfeld, 2017). *C. trachomatis* infections can also occur at extragenital sites, such as pharynx and rectum, and these infections are often asymptomatic (Chan *et al.*, 2016).

In a large study performed in the USA, the prevalence of *Ch. trachomatis* was 9% in rectum and 3% in pharynx among women (Trebach *et al.,* 2015).

Untreated or repeated *C. trachomatis* infections can ascend from the cervix to the uterus and fallopian tubes and lead to PID (Brunham *et al.,* 2015).

The PID was a risk factor for tubal damage which can lead to ectopic pregnancy and tubal fallopian infertility (TFI). Ectopic pregnancy can be a life-threatening condition and it occurs when the blastocyst implants outside the uterus in either the fallopian tubes, ovaries or abdomen (Adachi *et al.,* 2016).

The C. trachomatis infection during pregnancy can lead to miscarriage, premature rupture of membranes, stillbirth, preterm delivery and low birth weight of the infant by either direct fetal infection, placental damage or severe maternal illness (Adachi *et al.*, 2016).

The mechanisms by which a *C. trachomatis* infection can lead to adverse pregnancy outcomes are only partially known. *C. trachomatis* might directly infect the fetus, triggering a harmful inflammatory response, or the maternal inflammatory might induce embryonic rejection due to homology of the chlamydial and human (HSPs) (Adachi *et al.*, 2016).

The pathogenesis of *C. trachomatis* disease is a multi-step process that includes: (1) infectivity and exposure to the organism (2) Susceptibility to infection and sickness related to the host's genetic makeup. Recurrence and chronic infections are also common in at-risk teenage and young adult groups. Antibiotic resistance to the primary medications used to treat *C. trachomatis* is becoming increasingly widespread, even with the correct diagnosis (Parashar *et al.,* 2021).

Chlamydial infection can prevent tumor necrosis factor (TNF)-an-induced physiological apoptosis. Failure to adequately prevent, identify, treat, and remove infection increases the risk of pathogenicity and illness. The plasmid glycoproteins

1–8 (pGP1–8) encode eight open reading frames and most Chlamydia species. (Parashar *et al.,* 2021).

The C. trachomatis genital infection is still a relevant public health problem due to the high prevalence of asymptomatic infections, in both women and men (80–90% and 50%, respectively) that, untreated, may lead to chronic complications, such as pelvic inflammatory disease (PID), ectopic pregnancy, as well as reactive arthritis and infertility (Di Pietro *et al.,* 2019). Pelvic inflammatory disease (PID) is an outcome measure for the evaluation of chlamydia screening programs (Grahame *et al.,* 2021).

However, it should be noted that reliable monitoring systems and population-based data are *Chlamydia trachomatis* 4 gitis. *Chlamydia trachomatis* is made plasma or accessible; moreover, it's weakened in the vaginal canal of the mouse and nonhuman primate ocular tissue. The plasmid free organisms 'in vivo but not in vitro traits were completely mimeographs when pGP3 was inadequate, demonstrating that plasmid-encoded pGP3 is a critical virulence factor in vivo (Parashar *et al.*, 2021).

Many women are left untreated and are prone to chlamydia-related sequelae such as pelvic inflammatory disease (PID), ectopic pregnancy and tubal factor infertility (TFI). PID comprises a spectrum of inflammatory disorders of the upper female genital tract, including any combination of endometritis, salpingitis, tubo-ovarian abscess, and pelvic peritonitis. Sexually transmitted organisms, especially *N. gonorrhoeae* and *C. trachomatis*, often are implicated. Recent studies report that the proportion of PID cases attributable to *N. gonorrhoeae* or *Ch. trachomatis* is decreasing; of women who received a diagnosis of acute PID, approximately 50% have a positive test for either of those organisms (Wiesenfeld *et al.,* 2021).

Proportions of PID following chlamydia were found between 3.0% and 30.0%, for ectopic pregnancy between 0.2% and 2.7%, and for TFI between 0.1% and 6.0% (Davies *et al.,* 2016).

Pelvic inflammatory disease (PID) is a syndrome that causes substantial morbidity, including chronic pelvic pain, to women globally. While limited data are available from low- and middle-income countries, national databases from the United States and Europe suggest that PID incidence may be decreasing but the rate of decrease may differ by the etiologic cause. Recent studies of women with PID have reported that fewer than half of women receiving a diagnosis of PID have gonococcal or chlamydial infection (Sharon *et al.*, 2021).

Alongside *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, and *Treponema pallidum*, genital *Chlamydia trachomatis* infection is one of the four most common curable sexually transmitted infections (STIs) in the world, (Unemo *et al.*, 2017).

Chlamydial infection also occurs frequently among women with fertility disorders and gynecological problems in Malaysia (22.7%-51.1%), indicating widespread infection within the country (Yeow *et al.*, 2016).

Although a cure for *C. trachomatis* infection is achievable using appropriate antibiotics for most cases (≥ 97%), a large proportion of asymptomatic cases (50–70%) combined with high rates of reinfection remain the significant challenges to ongoing efforts targeted at preventing bacterial dissemination and reducing damages related to infection (Walker *et al.*, 2012).

C. trachomatis infections of the genital tract in females are characterized by a vast spectrum of genital tract pathologies that include mucopurulent cervicitis, urethritis, and salpingitis, which could further lead to pelvic inflammatory disease (PID), infertility, ectopic pregnancy and cervical cancer (Mackern et al. 2013). Further, genital chlamydial infection is also linked to preterm delivery and spontaneous abortion, as well as neonatal conjunctivitis (Rours *et al.*, 2011).

An increasing number of studies have highlighted disruption of vaginal microbiome as a predisposing factor for infection by urogenital pathogens. A

healthy cervicovaginal microbiota is typically dominated by bacteria of the genus *Lactobacillus* (Kaminska and Gajecka, 2017).

Lactobacillus spp. exert their protective role in the female reproductive tract against the invasion of pathogenic microorganisms by maintaining the acidity of the mucosal environment, inhibiting the adhesion of pathogens, and producing bactericidal compounds such as hydrogen peroxide (H_2O_2) (Gong *et al.*, 2014). Under *in-vitro* condition, *Lactobacillus* spp. are potent inhibitors of *Ch. trachomatis* largely due to their lactic acid producing capacities (Nardini *et al.*, 2016).

In case of bacterial vaginosis, a state of microbial imbalance within the vaginal environment where *Lactobacillus* spp. were replaced by other anaerobic bacteria has been linked to the increase transmission of STIs, including infections caused by *C. trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, human papillomavirus (HPV), and human immunodeficiency virus (HIV) (Gosmann *et al.*, 2017).

The involvement of *C. trachomatis* in these pathologies is related to its ability to infect and reproduce within different cell types beside the epithelial cells of the genital tract, including synovial and testicular cells (Filardo *et al.*, 2021; Filardo *et al.*, 2022; Di Pietro *et al.*, 2020).

Following chlamydial infection, target tissue activates mechanisms of cell-autonomous immunity, undergoes cellular changes, and produces proinflammatory cytokines, recruiting innate immune cells (Finethy *and Coers,* 2016).

As the infection proceeds, antigen-presenting cells, in turn, activate adaptive immunity, leading to the production of anti-Chlamydia antibodies and the migration of Chlamydia-specific CD4 and CD8 T-cells, resulting in an inflammatory environment that frequently clears the infection but also damages the infected tissue (Helble, 2021).

It was of at most importance to shed light on the pathogenetic mechanisms underlying host–Chlamydia interaction and influencing the clinical outcomes of chlamydial mediated genital diseases. Over the course of the last decades, as for example, the injection of chlamydial virulence factors in host cells via the type-3 secretion system, the escape from the endocytic pathway via chlamydial Incs proteins, have been described as mechanisms responsible for chlamydial adhesion, invasion and intracellular survival (Pekmezovic *et al.,* 2019).

The importance of the multi-faceted interaction between the host and the resident microflora of the female genital tract has also emerged, as a first line of defense against *C. trachomatis* infection (Di Pietro *et al.,* 2019). Indeed, several studies have characterized the cervico-vaginal microbiota via metagenomic approaches and advanced statistical algorithms, evidencing networks of specific bacterial species as potential biomarkers of chlamydial genital infection (Filardo *et al.,* 2019).

Furthermore, the information hidden in the 16s rDNA sequencing data have allowed researchers to describe distinct microbial community states of the cervico-vaginal microbiota associated with the risk of acquiring a *C. trachomatis* genital infection. Peculiar cervico-vaginal microbial signatures were also described in *C. trachomatis*-positive pregnant women, or in women after *Ch. trachomatis* treatment (Tamarelle *et al.,* 2020).

1.2.4. Life cycle of *Chlamydia trachomatis*:

Chlamydia trachomatis are obligate intracellular parasites that depend on infection of a host cell and transition through a biphasic developmental cycle. Following host cell invasion by the infectious elementary body (EB), the pathogen transitions to the replicative but noninfectious reticulate body (RB). Differentiation of the RB back to the EB is essential to generate infectious progeny (Lee *et al.,* 2018).

The EB form has historically been regarded as metabolically inert, maintenance of infectivity during incubation with specific nutrients has revealed active maintenance of the infectious phenotype. Using transcriptome sequencing, results show that the transcriptome of extracellular EBs incubated under metabolically stimulating conditions does not cluster with germinating EBs but rather with the transcriptome of EBs isolated directly from infected cells. In addition, the transcriptional profile of the extracellular metabolizing EBs more closely resembled that of EB production than germination (Grieshaber *et al.,* 2018).

Maintenance of infectivity of extracellular EBs was achieved by metabolizing chemically diverse compounds, including glucose 6-phosphate, ATP, and amino acids, all of which can be found in extracellular environments, including mucosal secretions. The study found that the EB cell type actively maintains infectivity in the inclusion after terminal differentiation. Overall, these findings contribute to the emerging understanding that the EB cell form is actively maintained through metabolic processes after terminal differentiation to facilitate prolonged infectivity within the inclusion and under host cell fre**e** conditions, for example, following deposition at mucosal surfaces (Tan and Ramamurthi, 2014).

1.2.5. Biological Structure of *Chlamydia trachomatis*:

Chlamydia were approximately 350 nm spherical or ovoid, non-motile, Gram negative, compulsory intracellular-living bacteria, which cannot be cultured on artificial growth culture media, but they need cell-culture supplemented with exogenous source of these high energy compounds, as Chlamydia does not have the capacity to synthesize high-energy compounds (e.g. ATP), amino acids, vitamins and other vital compounds and it is termed an energy parasite, for these causes it is thought previously to be a virus, as it could only be present in living cells, since they use them for energy (Schachter *et al.,* 2006).

Dissimilar with viruses, Chlamydia was susceptible to antibiotics (tetracyclines, sulfadiazine and macrolides) and therefore are considered bacteria. Since they are very small (approximately 350 nm) thus they cannot be imagined using the Gram stain and the standard magnification of the light microscope used in the microbiology lab (Frohlich *et al.,* 2014).

They have unique rigid bacterial cell wall but do not have typical peptidoglycan layer with absence of muramic acid, thus with a greater magnification, Chlamydiae seem as red on Gram staining like Gram-negative, and they are not affected by penicillin, the peptidoglycan may be autolyzed by amidases during differentiation of reticulate body into elementary body (Carlson *et al.,* 2005).

Its outer membrane complex is encompassed primarily of three proteins, of which the major outer membrane protein (Momp) is the most important. It has an important role in serotyping of *C. trachomatis,* which is based on the serological differentiation of antigenic species-specific epitopes on Momp into 19 human serovars (Hafner *et al.,* 2014).

Genome sequencing have revealed that Chlamydia has a somewhat small chromosome between (1.04-1.23) Mbp and comprehends between 894-1130 protein coding genes, with few repetitive elements and pseudogenes, expressing their long-term coadaptation to life within eukaryotic hosts (Collingro et al., 2011), plus an extrachromosomal plasmid of approximately 7.5 k bp, with a total of approximately 900 protein-coding genes, some *C. trachomatis* strains lack these plasmids (Thomson and Clarke, 2010).

Since chlamydial genomes are small, and many genes essential for the biosynthesis of vital metabolites have been lost, doubtless these are directly acquired from the host. The absence of genes encoding DNA competence, conjugation, phages, or DNA adaptation systems in pathogenic Chlamydia was comparable with

that of an organism submitting to very restricted genetic exchange (Gomes *et al.*, 2007).

Chlamydial infection activates both CD4+ and CD8+ -bearing T-cell, but in spite of this immune response *C. trachomatis* can persist immunity as it modifies host immune response and inhibits apoptosis and has ability to downregulate MHC (major histocompatibility complex) expression and inhibit T-cell functions, in addition to other different evasion mechanisms (Paavonen, 2012). Combined with this, its role in the induction of inflammation and metaplasia to act as a cofactor in carcinogenesis, this is proved by finding of antibodies to chlamydial heat shock protein 60 kDa and chlamydial EBs in addition to diagnosed *Ch. trachomatis* infection have been concomitant with cervical carcinoma (Lehtinen *et al.*, 2011)

1.2.6. Virulence factor of *Chlamydia trachomatis*:

1.2.6.1. Proteins and antigens of *Chlamydia trachomatis*:

Lipopolysaccharide (LPS) is a thermostable antigen common to all members of the family Chlamydiaceae, and as an endotoxin it has low activity. In the absence of LPS, *Ch. trachomatis* is unable to make the transition from RB to EB, which is the infective form (Favaroni, 2017).

Outer membrane proteins dividing in to: **A.** Momp (major outer membrane protein): Which encoded by the ompA gene It is the major outer membrane protein (Momp), representing 60% of the dry weight of the membrane. It is exposed on the surface of the membrane and therefore is highly immunogenic (de Haro-Croz *et al.*, 2019).

Momp presents two types of structures: Trimeric in EBs, acting as an adhesin, providing non-specific interactions and enabling penetration of the EB into the eukaryotic cell and Monomeric in RBs, acting as a porin, facilitating permeability of nutrients and ATP. (Luis *et al.*, 2015).

B. polymorphic membrane proteins (Pmps): which are a family of nine membrane proteins (PmpA-I), with a high degree of diversity between them (>50%), which have an important role in the biology of *C. trachomatis*. They have a transport function and act as adhesins. Phylogenetic reconstructions of Pmps allow *C. trachomatis* to be grouped in different pathotypes, for which reason it has been suggested that these proteins might modulate tissue tropism (Patricia *et al.*, 2022).

Cellular process proteins (Hsp proteins) these are proteins with a structure that has been highly conserved during evolution. They are classified into different families according to their molecular weight. They have two principal functions: they act as chaperones and they are induced in response to stress. Hsp60 or "GroEL-like" appears during persistent chronic infections. Hsp70 or "Dnak-like," is located in the cytoplasm and in the outer membrane of the EB (Sottile, and Nadin 2018).

Type III secretion system (TTSS) consists of >40 ORFs that release effector proteins directly into the cytosol or in the inclusion membrane. They facilitate the interaction between the host and the bacterial pathogen. Chlamydia spp. use a highly conserved type III secretion system (T3SS) composed of structural and effector proteins which is an essential virulence factor (Katerina *et al.*, 2017).

Some of the most important proteins are the Inc proteins (IncA-G), related to the transition from EB to RB (Blessy *et al.*, 2017); TarP, related to invasion (Keb *et al.*, 2021); CrpA, which seems to activate the CD8+ cells (Jennifer *et al.*, 2021); and the recently identified CPAF (chlamydial protease-like activity factor), which interferes with the ability of the host to respond to the chlamydial infection (Li *et al.*, 12019).

Cytotoxin is a putative chlamydial cytotoxin has been described, with amino acid sequence. Significantly, cytotoxin gene variations are useful for defining *C. trachomatis* disease phenotypes. Genital biovars contain a single gene with a large central deletion, while lymphogranuloma venereum (LGV) strains lack the toxin

gene. In addition, cytotoxin gene polymorphisms have been useful in distinguishing ocular and genital *C. trachomatis* isolates (Bonner *et al.*, 2014).

1.2.7. MicroRNAs (miRNAs) role during *Chlamydial* Infection:

Micro-RNAs (miRNAs) are a class of small non-coding RNA fragments with a length of about 22 nt, which play an important role in regulating gene expression after transcription. Chlamydia infection can cause changes in host cell miRNA expression (Liu *et al.*, 2021).

Micro-RNAs have been shown to be immune modulators, playing an important link between innate and adaptive immune responses, and dysregulation of miRNA expression has been linked to a number of diseases such as cardiovascular disease, cancer, and fibrosis (Honarpisheh *et al.*, 2018).

Proposed mechanisms for the inflammatory response and sequalae in Chlamydia infected host cells includes presence of chlamydia proteins and small non-coding RNAs (miRNAs) belonging to the host expressed during *Chlamydia trachomatis* infection (Stallmann and Hegemann, 2016).

One potential biomarker of interest is noncoding micro (~22-nucleotide) RNAs (miRNAs or miRs). These are stable, widely distributed molecules associated with inflammatory responses to human pathogens (Eulalio *et al.*, 2012). The role of miRNAs in chlamydial pathogenesis has been demonstrated in both animal models and chlamydia-infected humans (Arkatkar *et al.*, 2015).

Micro-RNAs are evolutionarily conserved, endogenous non-coding RNAs of approximately 22 nucleotides that play important regulatory roles in human, animals and plants by targeting miRNAs for degradation, cleavage, translational repression or occasional enhancement. miRNAs have been reported to account for approximately one-third of mammalian gene expression by regulating the expression of genes involved in cellular differentiation, maintenance of cellular integrity,

development, functions and normal metabolism, reproduction, fibrosis and oncogenesis (Weber *et al.,* 2017).

Small noncoding RNAs such as miRNAs have emerged as regulators of host cell apoptotic pathways by influencing gene expression of several key modulators, including p53 (Lekka and Hall .2018) .

The miRNAs have also been implicated in the regulation of host cell bioenergetics and metabolism (Goedeke *et al.,* 2013; Ramírez *et al.,* 2013).

Additionally, several studies showed that bacterial infections directly and indirectly affect the host immuno-metabolic, apoptotic and autophagic responses by modulation of miRNA and gene expression profiles (Wang *et al.*, 2013). MiRNAs have been shown to be differentially expressed during ocular chlamydia infection (Derrick *et al.,* 2016).

In addition, other authors have shown an association between genital chlamydia infection and differential expression of miRNAs (Benyeogor *et al.,* 2019).

The development of PID due to chlamydial infection with virulent and attenuated phenotypes can be predicted using miRNAs with the Chlamydia virulence associated with the miRNA expression profile of the host (Yeruva *et al.,* 2017).

The miRNAs have also been associated with regulating mitochondrial function which is necessary for *C. trachomatis* development (Chowdhury *et al.,* 2017).

These studies buttress the importance of miRNAs as important factors in host cell changes and response during chlamydial infection. MicroRNAs (miRNAs), are expressed in various tissues and cells that play key part in various physiological and pathologic processes. Increasing evidence implies roles for miRNAs in bacterial infectious diseases by modulating inflammatory responses, cell penetration, tissue remodeling, and innate and adaptive immunity (Zhou *et al.,* 2018).

The miRNAs are readily available from several sources like tissue, blood, and even urine (Arab et al. 2017). These short RNA molecules are stable in bodily liquids even at room temperature making them superior to unstable RNA. Aberrant expression of miRNAs in cervical cancer and its precursor lesions was previously investigated (Kawai *et al.,* 2018).

The miRNAs contribute to normal immune system development and function. In particular, miRNAs can tightly regulate innate immune response by targeting key signaling components of inflammatory shapes such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and type I-III interferon (IFN-I-III) pathways. Moreover, they can finely modulate distinct immune signaling pathways involved in the T and B cell development, differentiation, tolerance (central and peripheral), and function (Frasca *et al.,* 2022).

MiRNAs expression showed the importance of determining the outcome of chlamydial infections, but they do not provide a full picture of the role miRNAs may play during chlamydial infection. For instance, they do not show the effect of Chlamydia trachomatis on miRNA expression over an extended period (De Clercq *et al.,* 2013).

Although previous studies have identified chromosomal variations, abnormal expression of oncogenes or tumor-suppressor genes and aberrant promoter methylation in cutaneous squamous cell carcinoma (CSCC), few are relevant to microRNAs (miRNAs) (Li *et al.,* 2020).

MiRNAs play important regulatory roles in various biological processes e.g., differentiation, development, proliferation, apoptosis, cell cycle control and metabolism. Changes in miRNA expression have been identified in many diseases, such as cardiac and autoimmune disorders, schizophrenia and cancer (Kabekkodu *et al.,* 2018).

The miRNAs are a class of small non-coding RNAs (ncRNAs) ~19–25 nucleotides (nts) in length that can regulate gene expression by known classic mechanisms such as binding to messenger RNA (mRNA) at the 3′-untranslated region (3-UTR) to downregulate protein-coding genes by increasing mRNA degradation. They also act through other newly found mechanisms such as binding to promoters, proteins, and by directly interacting with other ncRNAs (Berindan-Neagoe *et al.,* 2014).

The miRNAs have been found to be differentially expressed between almost all types of analyzed benign and malignant tumors and non-tumor tissues, including cutaneous squamous cell carcinoma (CSCC). In addition, miRNAs participate in regulating a variety of neoplastic biological capabilities acquired during the multistep process of tumor development (Lin et al. 2017).

There was an association of cervical miR-142 and miR-147 expression on day 4 to pathology on day 35 (Yeruva *et al.,* 2017). The miR-155 may be a marker of infection and that miR-142 and/or miR-147 may be markers for signs or symptoms (Batteiger *et al.,* 2020). Other study has been shown that miR-142 and miR-147 bind to TLR7/TLR8 and induce inflammation in cancer, arthritis, and ischemia models (Yelamanchili et al., 2015).

Batteiger *et al.,* (2020). saw that miR-142 and -147 are upregulated in the CTSS but not in the CTNS group of women. It is possible that these miRs play a role in inflammation in human infection. These data, along with the published literature from the animal model and clinical studies, suggest that inflammatory and fibrotic genes are tightly regulated by a complex network of transcription and posttranscriptional events. Additionally, these data warrant future studies to determine if miR-16 along with miR-142 and miR-147 could serve as marker(s) of progression for fibrosis.

Chlamydia are a group of intracellular bacterium that infect a range of hosts and are responsible for the most common sexual transmitted infections, which could be the result of a plethora of factors leading to varied pathological outcomes and Chlamydia possibly manipulates host defenses through microRNAs interaction (Eledge *et al.,* 2018).

1.2.8. Genotyping of *Chlamydia trachomatis*:

Chlamydia trachomatis DNA is comprised of a ~ 1 million base pairs (bp) long single circular chromosome, as well as multiple copies of a 7.5 kb long highly conserved plasmid (Jelocnik *et al.,* 2016). Based on the antigenic properties of the major outer membrane protein (Momp), *C. trachomatis* is typically divided into 17 distinct serovars, or 19 serovars which include subtypes (Gallo Vaulet *et al.*, 2016).

The serovars include: trachoma serovars (A-C) that are the major etiological agents of preventable blindness; genital tract sexually transmitted serovars (D-K); and serovars L1–L3 that cause invasive urogenital infection or anorectal infection (lymphogranuloma venereum, LGV). Characterized by ulcerative proctitis, the LGV disease is of high concern (Levy *et al.*, 2018).

Genotypic variation within the *omp*A gene exhibits a great degree of polymorphism that cannot be identified by serotyping (Kapil et al.2015). However, *omp*A variability does not provide sufficient discriminatory power for epidemiological purposes (Herrmann *et al.*, 2015).

Although *C. trachomatis* genome harbor regions with highly nucleotide diversity and high events of recombination, it is considerably conserved (Abdelsamed *et al.,* 2013).

Serotyping has traditionally been used for typing *Ch. trachomatis*; it uses specific antibodies directed against the outer membrane protein (Momp). However, this technique is considered laborious, takes too long and has low sensitivity (Nunes and Gomes, 2014).

Some molecular techniques used for typing *Ch. trachomatis* have been restriction fragment length polymorphism (RFLP), DNA hybridisation-based techniques, polymerase chain reaction (PCR) and DNA microarrays based on analysis of the *omp*A gene (encoding Momp) (Gallo Vaulet *et al.*, 2016). These have led to 19 variants being identified (Xia and Xiong, 2014), grouped into 3 clusters; one includes variants L1-L3 and L2a, associated with Lymphogranuloma venereum (LGV), another covers variants A, B, Ba, and C, associated with trachoma and another covers variants D-K, Da, Ga, Ia, and Ja, associated with genital-urinary infections (Petrovay *et al.*, 2017).

The low discrimination power of some of the techniques mentioned above and their multiple disadvantages have led these techniques to be replaced by other typing methods especially those based on sequencing, which are much more specific and enable *C. trachomatis* intra- specific typing (de Vries *et al.*, 2015).

Among sequencing techniques emerges the Multilocus Sequence Typing (MLST) that has provided a portable, reproducible and scalable typing system and is performed easily by different laboratories (Xia and Xiong, 2014; de Vries *et al.*, 2015).

Additionally, other studies using whole-genome sequencing (WGS), have allowed expanding the knowledge about the epidemiology, evolutionary history and diversity of members of *C. trachomatis* based on approaches defined as core genome MLST (cgMLST) (Rawre *et al.*, 2017).

The WGS (Whole genome MLST and cgMLST) has demonstrated to be a tool with a high discriminatory power. This technique presents some disadvantages due to its higher costs and requirement of big computational capacity (Versteeg *et al.*, 2018).

Several Multilocus Sequence Typing (MLST) schemes have been developed for *Chlamydia trachomatis*. Bom's MLST, scheme for MLST is based on nested PCR amplification and sequencing of five hypervariable genes and *omp*A. In contrast to other Chlamydia MLST schemes, Bom's MLST scheme gives higher resolution and phylogenetic trees that are comparable to those from whole genome sequencing. However, poor results have been obtained with Bom's MLST scheme in clinical samples with low concentrations of Chlamydia DNA. Two groups developed multilocus sequence typing, based on the sequences of seven housekeeping genes (MLST-7) of *C. trachomatis* (Pilo *et al.*, 2021).

Regarding molecular characterization of sexually transmitted *C. trachomatis* strains, the knowledge gap is even more prominent. Globally, the emergence of new sexually transmitted strains and variants and their rapid spread indicate the need for more comprehensive molecular studies to better understand the *C. trachomatis* tissue tropism as well as epidemiological network structures (Versteeg *et al.*, 2018).

Molecular characterization of *C. trachomatis* is important for understanding the pathophysiological mechanisms of chlamydial disease and its transmission dynamics in sexual networks. Traditionally, strain typing of *C. trachomatis* was based on serotyping methods characterizing the major outer membrane protein (Momp). With the advent of polymerase chain reaction and sequencing the era of molecular typing began. Molecular characterization of *C. trachmatis* strains is based on sequence analysis of *omp*A gene encoding Momp (Rawre et al. 2017).

New high-resolution genotyping methods using multiple loci such as multilocus sequence typing (MLST) and multiple loci variable number of tandem

repeats (MLVA) were developed but were unable to differentiate mixed infections (MIs). The development of DNA-hybridisation methods emerged as a major breakthrough in detecting MIs. Although MLST and MLVA are more discriminative than other genotyping methods, they are laborious and expensive (Rawre et al. 2017).

DNA microarray technique is an affordable alternative for genotyping. Since recombination is widespread in the *C. trachomatis* genome, *ompA* is not a reliable marker for phylogenetic studies; hence, whole genome sequencing may provide maximum phylogenetic resolution of *C. trachomatis* strains (Rawre *et al.,* 2017).

Chapter Two

Materials and Methods

Materials and Methods:

2.1. Materials:

2.1.1. Laboratory Apparatuses and Instruments:

Table **(2.1.A):** Scientific Laboratory Apparatus.

Item	Company	Country
Gynecological Cusco speculums	TTN	Iran
Millipore filters	Sigem	Spain
PCR tubes 1.5 ml (Eppendrof)	Biobasic	Canada
PCR tubes 200µl (Eppendrof)	Biobasic	Canada
Plain tubes 10ml	Dolphin	Syria
Sterile swabs	Sigem	Spain
Syringes	Dolphin	Syria
Tips	Dolphin	Syria
Transport swabs	Sigem	Spain
Wooden sticks	Supreme	China

Table **(2.1.B):** Scientific Laboratory Instruments.

Item	Company	Country
Autoclave	Herayama	Japan
Bacteriological cabinet	Labogene	Denmark
Benson burner	Dolphin	Syria
Centrifuge	Gemmy	Taiwan
Conventional PCR system	Clever Scientific	UK
RT-qPCR System	Promega	USA
In silico PCR-RFLP (uniplex PCR) System	Promega	USA
Cool box	Gemmy	Taiwan
Distillator	GFL	Germany
Electrophoresis	Clever Scientific	UK

Nano drop	Clever Scientific	UK
Microcenterfuge	Beckman	USA
Oven	GS	Taiwan
Refrigerator	Concord	Italy
Sensitive electric balance	Kern	Germany
UV-transilluminator	Clever Scientific	UK
Vortex	Gemmy	Taiwan
Water bath	GFL	Germany
Micropipettes 5-50 µl ,100-1000 µl, 2-20 µl	TopDragon	Europe
	Ecopipette	Denmark

2.1.2. Chemical Materials:

Itemized down in Table (2-2), the main chemicals utilized in this study.

Table (2-2): Chemical Materials and Reagents.

Item	**Company**	**Country**
Ethanol absolute	GCC	UK
Isopropanol absolute	GCC	UK
Normal saline	Pharmaline	Egypt
Phosphate buffer saline tablet	Himedia	India

2.1.3. Immunological and Molecular Materials:

Categorized and detailed down in Table (2-3), the materials and kits employed in the immunological and molecular study. While Table (2-4-A-B-C) contains all primer sets used in this study, with their sequences, PCR conditions and their amplicon size.

Table (2-3): Immunoassay and Molecular Related Materials.

Item	Company	Country
Rapid test device(swab/urine) for chlamydia (immunoassay test for detection of chlamydia)		Chaina
100 bp Ladder, consists of: 11 double-stranded DNA fragments ranging in sizes from 100 to 1,500 bp with 100 bp increments. The 500, 1,000 and 1,500 bp bands are double to triple of the intensity of other fragments and brighter, for easier identification and comparison of molecular weight). While all other fragments seem with equal intensity on gel.	Bioneer Promega	Korea USA
Agarose	Promega	USA
Blue/Orange Loading Dye, 6X containing: 1-0.4% orange G. 2-0.03% bromophenol blue. 3-0.03% xylene cyanol. 4-15% Ficoll. 5-10mM Tris-HCl (pH 7.5). 6-50mM EDTA (pH 8.0). It is used for loading DNA samples into wells and tracking migration during gel electrophoresis.	Biobasic	Canada
Red Safe Stain, (10mg/ml).	Bioneer	Korea
Geneaid Genomic DNA Isolation Kit	Geneaid	UK
Geneaid Genomic RNA Isolation Kit	Geneaid	UK
Gel/PCR DNA Fragments Extraction Kit	Geneaid	UK

Green master mix 2X Kit, consist of: 1-Taq DNA polymerase. 2- dNTPs, 400µM for each. 3-Tris-HCl (pH 8.5-9.0), 10 mM. 4-KCl, 30 mM 5-MgCl2, 3mM. 6-2eppendroffs of Nuclease free water 7-Stabilizer and tracking dye	Promega	USA
BRYT Green® dye	Promega	USA
Nuclease free water.	Bioneer	Korea
Primer pairs	Bioneer	Korea
TBE Buffer (Tris-Borate-EDTA), 10X (pH 8.3) Composition: 890mM Tris-borate, 890mM boric acid, 20mM EDTA.	Promega	USA
TAE Buffer (tris-acetate-EDTA),1X (PH 8.6) Cmposition: 40 mM tris-acetate, 20 mM acetate and 1 mM EDTA.	Promega	USA
TE Buffer, 1X (pH 8.0) composed of 10mM Tris-HCl containing 1mM EDTA•Na2.	Bioneer	Korea

Table (2-4-A): The primer sequences and PCR conditions with their amplicon size (Base pair (bP)) for chlamydia.

Gene's Name	**Primer Sequence (5´- 3´)**	**Size (BP)**	**Conditions**	**Reference**
Identification Genes				
Chlamydia *Omp* A	F- 5'CCTGTGGGGAATC CTGCTGAA3' R- 5'GTCGAAAACAAAG TC ACCATAGTA 3'	144	94°c, 5min 1x 94°c, 30s 56°c, 30s 35x 72°c, 30s 72°c, 7min 1x	(Joolayi et al. 2017)

Table (2-4-B-1): The primer sequences and PCR conditions with their amplicon size (Base pair (bp)) for MiR-142 Coding Genes.

MiR-Coding Genes (RT-qPCR)				
Genes Name	**Primer Sequences (5´- 3´)**		**Condition**	**References**
MiR-142	F-5'GGTGGGTCATAAAGTAGAAAG3' R-5'GAGCAGGGTCCGAGGT3'		37c, 15min 1x 95°c, 10min 1x	(Livak KJ, and Schmittgen, 2001)
GAPDH-F	GTCTCCTCTGACTTCAACAGCG		95°c, 10s	
GAPDH-R	ACCACCCTGTTGCTGTAGCCAA		60°c, 30s 40x 72°c. 30s	

Table (2-4-B-2): The primer sequences and PCR conditions with their amplicon size (Base pair (bP)) for MiR- 520a Coding Genes.

MiR-Coding Genes (RT-qPCR)				
Genes Name	**Primer Sequences (5´- 3´)**		**Condition**	**References**
MiR-520a	F-5'AAAGTGCTTCCCTTTGGACTGT3' R-5'GTGCAGGGTCCGAGGT3'		37c 15min 1x 95°c 10min 1x 95°c 10s	(Livak KJ and Schmittgen, 2001)
GAPDH-F	GTCTCCTCTGACTTCAACAGCG		60°c 30s 40x	
GAPDH-R	ACCACCCTGTTGCTGTAGCCAA		72°c 30s	

Table (2-4-C): The primer sequences and PCR conditions with their amplicon size (Base pair (bP)) for genotyping.

Gene's Name	Primer Sequence (5´- 3´)	Size (BP)	Conditions	Reference
omp-1	F- 5'GCCGCTTTGAGTTCTGCTTCCTC3' R- 5'ATTTACGTGAGCAGCTCTCTCAT3'	1134	95c 5min 95°c 1min 35x 59°c 1min 35x 70°c 1min 35x 72°c 5min	Design in this study

2.2. Methods:

2.2.1. Subjects of the Study:

Clinical samples were collected from patients admitted to the out-patient clinics of Gynecology and Obstetrics, in two hospitals of Babylon Province: Babylon Hospital for Maternity and Pediatrics, and Al-Hilla General Teaching Hospital; in addition, samples taken from private clinics, during the period from March to August 2022.

The study involved 200 female patients were subjected for sampling which include two endocervical swab from each female. The age of patients ranged from 20 to 40 years. These females were diagnosed by the gynecologist as having pelvic inflammatory disease, according to the characteristic criteria of national guidelines for pelvic inflammatory disease (Workowski and Berman, 2006) and according to the signs and symptoms, abdominal and pelvic ultrasound, in addition to be having risk factors that were determined by the information about patients were taken according to formula listed in the questionnaire at (Appendix 1).

In this study, females with recent usage (within 7days) of antibiotic treatment; unmarried females; those who pregnant and patients having vaginal bleeding are excluded from sampling. Also, 25 healthy females were employed as a control group; for analysis of genetic association between miRNA in healthy and those with *Chlamydia trachomatis*.

2.2.2. Ethical Approval:

A valid consent was achieved from each female (patients and controls) before their inclusion in the study. For every female or her followers, the procedure had been informed before the samples were collected, making absolutely sure that they understood the procedure that

was to be carried out. The subjects were sentient that they had the right to reject to be included in the study without any detrimental effects.

2.2.3. Study Design:

(Case-control) study.

2.2.4. Sample Collections:

2.2.4.1. Endocervical Swabs:

After obtaining the permission from the subjects for examination and sampling, patient was rested in lithotomy-dorsal position. A methodical inspection was done for lesions and vaginal/cervical discharge. Sterile vaginal speculum was introduced and fixed by the clinicians, using proper lightening and environment.

After cleaning the exocervix with cotton swab, cervical mucus or inflammatory exudates were removed before the introduction of swab into the endocervical canal.

Later on, two cotton swabs were used for each patient; as the specimens were collected by inserting swab about 1 cm into the endocervical canal and were rubbed by rotating it against the wall of endocervical canal vigorously or scraping to get more cells from the endocervix. Then swabs were removed carefully to avoid any contact with vaginal secretions and immersed in plain tube-containing 1 ml of phosphate buffered saline (PBS) normal saline. The speculum was withdrawn. The sample was labeled with the patient's data or number. The sample was transported to the laboratory using iced-box.

This method is rapid, inexpensive and fairly specific, but its disadvantage is difficulty in collection (Abdella *et al.*, 2015).

2.2.5. Preparation of Materials:

2.2.5.1. Phosphate Buffer Saline (PBS) (pH=7.2):

Buffer preparation was done by dissolving one buffer tablet in 100 ml of distilled water, then sterilized by autoclave (according to the orders of the company), after that was kept at 4oC until using.

2.2.5.2. Preparation of 1X Tris-borate- EDTA buffer (TBE) Buffer:

The preparation of 1X TBE buffer was performed by dilution of a concentrated 10X TBE buffer, this dilution was accomplished as 1:10 (v/v); 1 volume of 10X TBE: 9 volumes of distilled water. This solution was used to prepare agarose gel and as a transmission buffer in electrophoresis process. Thus, each 100ml of 10X TBE added to 900ml of sterile distal water to produce final concentration,1X TBE (Sambrook and Russel, 2001).

2.2.5.3. Preparation of Agarose Gel:

This gel was prepared by adding agarose powder in 1X TBE buffer to be dissolved by boiling, then it was left to cool to 50ºC. The dissolved amount of agarose powder is depending upon the aim for which agarose is used.

For DNA profile (visualization of the DNA after extraction), 1% agarose is used. While for visualization of PCR product (amplicon), 1%-1.5% of agarose was employed.

Red safe stain solution with a concentration 10mg/ml was used. Only 5µl of this stock solution were supplemented to 100ml of melted agarose gel to get final concentration 0.5µg/ml (Boner/ Korea). Then after the addition of red safe stain, mixed well and dispensed to the tray of gel electrophoresis.

2.2.5.4. Rehydration of Primers:

Lyophilized primer pairs were rehydrated by DNA rehydration solution 1X (pH 8.0) Tris- EDTA buffer (TE-buffer). Initially, primer storage-stock tube prepared and then the working solution would prepare from primer stock tube. Consistent with the instructions of the producer (Bioneer / Korea), TE buffer was added to produce 100 picomole/microliter concentration of primer stock solution. The working solution prepared from stock as 1:10 (v/v) by dilution with TE buffer to get 10 picomole/microliter.

2.2.6. Immunological Method for detection of *Chlamydial trachomatis*:

In this study, the collected samples; endocervical swab from patients were subjected to rapid test device (swab), which was a rapid visual immunoassay for the qualitative presumptive detection of chlamydia in female cervical swab. the kit is intended for use as an aid in the diagnosis of chlamydia infection. The material which provided in kit was show in table (2-5). The procedure of the kit was as illustrated:

Table (2-5) The material which provided in kit

Numbers	Component
1	Individually packed test devices
2	Reagent A
3	Reagent B
4	Package insert
5	Extraction tubes and tips
6	Sterilized swab
7	Workstation

1- The test samples and reagent were allowed to reach room temperature (15-30°C).

2- The test device was removed from the sealed foil pouch and use it as soon as possible.

3- The chlamydia antigen was extracted according to the type.

4- The clean extraction tube was placed in the workstation and then add 8 drops of reagent A to extraction tube.

5- The patient swab was Immersed in to the extraction tube and wait 2 minutes. While waiting, use a circular motion to roll the swab against the side of the extraction tube so that the liquid is expressed from swab and can reabsorb.

6- 8drops of reagent B was added. squeeze the swab firmly against the tube to expel as much liquid as possible from the swab for 1 minute. Discard the swab following guidelines infectious agents. Fit the dropper on top of the extraction tube.

7- The extraction specimen can remain at room temperature for 60 minutes without affecting the test results.

2.2.7. Genetic Methods:

2.2.7.1. Genomic DNA Extraction:

In this study, the collected samples; both endocervical swab from patients and controls were subjected to DNA extraction procedure. It was performed according to protocols recommended by manufacturer (Geneaid/UK). The achieved DNA was stored at 2-8°C for further applications and processing.

2.2.7.1.1. Genomic DNA Extraction from Endocervical Swab Sample:

The collected endocervical swab samples as mentioned at (2.2.4.1.); after their wash in PBS to draw out epithelial cells from swabs by centrifugation (5000 rpm for 10 minutes) for three times. Then the supernatant was removed completely and the pellet was subjected to DNA extraction as illustrated is:

1- 200 µl of the samples swab pellet was placed in a 1.5ml microcentrifuge tube.

2- A volume of 500µl of S1 buffer and 20 µl of proteinase K were added to the tube and mix vortex for 10 seconds then the tube was incubated for at least 10 minutes at 60 ºC

3- A volume of 500 µl of Lysis S2 buffer was added then vortex immediately and then incubate for 10 minute to ensure the sample lysate was cleared.

4- During incubation, the tube was inverted each 5 minutes. Meanwhile, Elution buffer (200µl per sample) was pre-heated at 60 ºC (to be used in step 17 DNA Elution).

5- A volume of 500µl of absolute ethanol was added for DNA binding step and immediately mixed by shaking vigorously for 10 seconds.

6- The GD column was placed in 2 ml collection tube.

7- All the mixture was transferred to the GD column.

8- Centrifugation process was achieved at 14000-16000 rpm for 1 minutes.

9- The 2 ml collection tube containing the flow-through was discarded and the GD column was placed in a new 2ml collection tube.

10- A volume of 400µl of W1 Buffer was added to the GD column.

11- Centrifugation process was achieved at 14000-1600 rpm for 30 seconds.

12- The flow-through was discarded and the GD column was placed back in the 2 ml collection tube.

13- A volume of 600µl of Washing Buffer **(**ethanol added**)** was added to the GD column.

14- Centrifugation process was done at 14000-16000 rpm for 30 seconds.

15- The flow-through was discarded and the GD column was placed back in the 2 ml collection tube.

16- Centrifugation process was done at 14000-16000 rpm for 3 minutes to dry the column matrix.

17- The dried GD column was transferred to a clean 1.5 microcentrifuge tube.

18- A volume of 100 µl of pre-hated Elution Buffer was added to the center of the column matrix and let to stand for at least 3 minutes to ensure the Elution Buffer was absorbed by the matrix.

19- Centrifugation process was achieved at 14000-16000 rpm for 30 seconds to elute the purified DNA.

20- The extracted DNA was stored at 2-8°C.

Later on, the extracted DNA from swabs was used for molecular identification of *Chlamydia trachomatis* and its sequencing.

2.2.7.1.2. Detection of DNA Concentration and Purity by Nanodrop:

The extraction DNA was checked by using nanodrop spectrophotometer, which measured DNA concentration (ng/ µl) and checked the DNA purity by reading the absorbance at (260/280nm) as following steps:

1. The appropriate application (Nucleic acid, DNA) was chosen after opening nanodrop software,

2. A dry wipe was taken to clean instrument pedestals several times, then are fully pipette 2 µl of ddH2o on the surface of the lower measurement pedestals for blank system.

3. The sampling wipe was lowered and clicked OK to initialized the nanodrop, the cleaning off the pedestals and 1 µl of extracted DNA carefully pipette onto the surface of the lowered measurement pedestals, then concentration and purity of extracted DNA was checked (Wilfinger *et al.*, 1997).

2.2.7.1.3. Amplification of *omp* A(144BP) Primers:

All primer pairs used in the study, product size and amplification condition were listed in Table (2-4), amplification was carried out by Polymerase Chain Reaction (PCR), that was performed in a total volume of 20 µl.

2.2.7.1.4. Identification of *Chlamydia trachomatis*:

PCR for amplification of supposed specific gene belong to *Chlamydia trachomatis* that suspected to be present intracellularly inside the epithelial cells.

Omp A gene was using in PCR with conditions mentioned in **Table (2-4-C)**. A protocol and mixture as mentioned at **Table (2-6)**.

Table (2-6): Polymerase Chain Reaction Mixture.

Mixture. **No.**	**Mixture Contents**	**Volume (µl)**
1	Master Mix	5 µl
2	Forward Primer	2.5 µl
3	Reverse Primer	2.5 µl
4	Template DNA	5 µl
5	Nucleasr-Free Water	5 µl
6	Total	20 µl

2.2.7.1.5. Detection of Amplified Products by Agarose Gel Electrophoresis:

Successful PCR amplification was confirmed by agarose gel electrophoresis by visualization against UV light (Sambrook and Russell, 2001).

Agarose gel was prepared according to **(2.2.5.3.)**. Then the comb was fixed at one end of the tray for making wells used for loading DNA sample. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber filled with TBE buffer that covered the surface of the gel, 5µl of DNA

sample was transferred into each well of agarose gel, and in one well put the 5µl DNA ladder with 1µl loading dye.

The electric current was allowed to pass at 70 volts for 50min. UV trans-illuminator was used 280 nm for the observation of DNA bands,

The amplified products were determined by comparison with the ladder mentioned at **Table (2-3).**

2.2.7.2. **Genomic RNA Extraction:**

In this study, the collected samples; both endocervical swab from patients and controls were subjected to RNA extraction procedure. It was performed according to protocols recommended by manufacturer (Geneaid/UK). The achieved RNA was stored at -20°C for further applications and processing.

2.2.7.2.1. Genomic RNA Extraction from Endocervical Swab Sample:

The collected endocervical swab samples as mentioned at (2.2.4.1.); after their wash in PBS to draw out epithelial cells from swabs by centrifugation (5000 rpm for 10 minutes) for three times. Then the supernatant was removed completely and the pellet was subjected to RNA extraction as illustrated is:

1. 200 µl of liquid sample was Transferred up to a 1.5 ml of microcentrifuge tube (RNase-free). Mixed by inversion.

2. 3 volumes of GENEzol™ Reagent was added per 1 volume of sample (3:1) then mix well by vortex.

3. The sample mixture was incubated for 5 minutes at room temperature.

4. Centrifuge the sample at 12-16,000 x g for 1 minute to remove cell debris then transfer the clear supernatant to a new 1.5 ml microcentrifuge

tube (RNase-free).

5. 1 volume of absolute ethanol was added directly to 1 volume of sample mixture (1:1) in GENEzol™ Reagent.

6. The mixture was mixed well by vortex then place RB Column in a 2 ml Collection Tube.

7. 700 µl of the sample mixture was transferred to the RB Column. Centrifuge at 14-16,000 x g for 1 minute then discard the flow-through.

8. The RNA Binding Step was repeat by transferring the remaining sample mixture to the RB Column.

9. Centrifugation process was achieved at 14-16,000 x g for 1 minute then discard the flow-through. Place the RB Column in a new 2 ml Collection Tube.

10. 400 µl of Wash Buffer (make sure ethanol was added) was added to the RB Column then centrifuge at 14-16,000 x g for 30 seconds.

11. the flow-through was discarded and placed the RB Column back in the 2 ml Collection Tube.

12. DNase I solution was Prepared in a 1.5 ml microcentrifuge tube (RNase-free) as follows:

Table (2-7): The DNase I prepared contents

Content	**Volume**
D Nase 1	5µl (2 U/µl)
DNase 1 Reaction Buffer	45µl
Total Volume	50µl

13. By pipette DNase 1 solution was mixed carefully.

14. A volume of 50 µl DNase 1 solution was added into the RB column and incubated for 15 minutes at room temperature (20-30ºC).

15. A volume of 400 µl of W1 Buffer was added into the RB Column, centrifuge at 14000 x g for 30 seconds. Flow-through was discarded then the RB Column was placed in the 2 ml collection Tube.

16. A volume of 600 µl of Wash Buffer was added into the RB Column. Centrifuge at 14000 x g for 30 seconds.

17. A volum of 600 µl of Wash Buffer was added into the RB Column, Centrifuge at 14000 x g for 30 seconds . The RB Column was placed back in the 2 ml collection tube and centrifuge at 14000 x g for 3 min to dry the column.

18.The dried RB Column was place in a clean 1.5 ml microcentrifuge tube.

19. A volume of 50 µl of RNase-free Water was added into the column. It was left for at least 1 minute to ensure that the RNase-free water was absorbed. Centrifuge at 14,000 x g for 1 minute to elute the purified RNA.

20. DNA digestion in Solution the DNase 1 reaction in a 1.5 ml microcentrifuge tube (RNase-free) was prepare as follows:

Table (2-8) The DNase I reaction contents

Content	**Volume**
RNA in RNase-free Water	1-40 µl
DNase I	0.5 µl/µg RNA
DNase I Reaction Buffer	5 µl
RNase-free Water	add to final volume = 50 µl
Total Volume	50 µl

21. By pipette DNase 1 solution was mixed carefully.

22. The microcentrifuge tube was incubated at 37ºC for 15-30 minutes.

23. The tube containing RNA was kept at -20 °C.

2.2.7.2.2. Gene expression of microRNA 142 and 520a:

The real-time qPCR reactions were performed by using specific primers targeting reference gene GAPDH and the target genes microRNA 142 and 520a (Table:2-4-B). Conversion the total RNA to cDNA and amplification of DNA was done according to instructions provided by GoTaq® 1-Step RT-qPCR System (Promega) using BRYT Green® dye, where RT-qPCR Mixture and conditions were summarized in tables (2-4-B) and (2-9), where the final volume of RT-qPCR reaction was 20 µl. Relative expression fold was calculated by delta delta method (2^-(ΔΔCt) according to Livak and Schmittgen, 2001.

Table (2-9): RT-qPCR Mixture

Component	**Volume (µl)**
Go Taq QPCR Master Mix, 2X	10 µl
Go Script RT Mix for 1-step RT-qPCR (50)	0.5 µl
Forward primer (20x)	1 µl
Reverse primer (20X)	1 µl
RNA Template	5 µl
Nuclease- Free Water	2.5 µl
Total	20µl

2.2.7.3. Genotyping of *Chlamydia trachomatis*:

2.2.7.3.1. DNA Amplification of *Chlamydia trachomatis* for Sequencing using *omp-1* gene:

The acquired DNA was processed for sequences of *C. trachomatis omp1 gene*. using one pair of *omp*-1 gene primers by conventional PCR and store at 2 – 8°C for later applications. Table (2-4-C) lists the gene sequences and the associated circumstances.

A 5 µl of the PCR products amplicon were loaded into 2% agarose gels in 1 X TAE, and run at 100 v in 1X TAE for 40 minutes.

2.2.7.3.2. Gel/PCR DNA Fragments Extraction Kit to Using in Sequencing:

All PCR products from positive samples were purified by Gel Extraction Kit from Geneaid, and the steps for extraction as illustrated is.

1. Excise the agarose gel slice containing relevant DNA fragments and remove any extra agarose. Using TAE buffer for gel formation is recommended for optimal DNA recovery.
2. 300 mg of the gel slice was transfer to a 1.5 ml microcentrifuge tube then 500 µl of DF Buffer was added and vortex.
3. Incubation was achieved at 55-60ºC for 10-15 minutes to ensure the gel slice has been completely dissolved. During incubation, invert the tube every 2-3 minutes.
4. The dissolved sample mixture was cooled to room temperature.
5. The DF Column was Placed in a 2 ml Collection Tube then transfer 800 µl of the sample mixture to the DF Column.
6. Centrifugation was achieved at 14-16,000 x g for 30 seconds.

7. The DF Column back was Placed in the 2 ml Collection Tube. If the sample mixture is more than 800 µl, repeat the DNA Binding Step.

8. 600 µl of Wash Buffer (make sure ethanol was added) was added into the DF Column and let stand for 1 minute.

9. Centrifugation was achieved at 14-16,000 x g for 30 seconds then discard the flow-through.

10. The DF Column back was Placed in the 2 ml Collection Tube.

11. 600 µl of Wash Buffer (make sure ethanol was added) was added into the DF Column and let stand for 1 minute.

12. Centrifugation was achieved at 14-16,000 x g for 30 seconds then discard the flow-through.

13. The DF Column back was Placed in the 2 ml Collection Tube.

14. Centrifugation was achieved at 14-16,000 x g for 3 minutes to dry the column matrix.

15. the dried DF Column was Transferred to a new 1.5 ml microcentrifuge tube.

16. 20-50 µl of Elution Buffer or TE was added into the center of the column matrix.

17. For at least 2 minutes was stood to ensure the Elution Buffer was completely absorbed.

18. Centrifugation was achieved for 2 minutes at 14-16,000 x g to elute the purified DNA. Using pre-heated Elution Buffer (60ºC) is recommended for eluting DNA fragments >5kb.

2.2.7.3.3. Sequencing and *In-silico* analyses by using PCR-RFLP assay**:**

PCR-RFLP assay of this study was carried out according to de Jesús De Haro-Cruz et al., (2011) with some modifications. The main modification is using of *In-silico* PCR-RFLP instead of conventional PCR-RFLP. In brief, Uniplex-PCR by using GoTaq® G2 Green Master Mix was done with a total volume of 25 by using specific primers as show in table and (2-10).

Table (2-10): *In silico* PCR-RFLP

Component	**Volume (µl)**
GoTaq® G2 Green Master Mix **2X**	12.5 µl
Forward primer (2x)	1.5 µl
Reverse primer (2X)	1.5 µl
DNA Template	3 µl
Nuclease- Free Water	6.5µl
Total	25µl

The in-silico PCR-RFLP of the *omp* A sequences were simulated by NEBcutter V2.0 (Vincze et al., 2003), using the *AluI*, restriction enzyme which recognizes and cuts at specific sequence (5′-AG/CT-3) along the *omp* A sequences. Determination of the DNA size was also analyzed by the software (NEBcutter V2.0).

2.8. Statistical Analysis:

Frequency distribution of *Ch. trachomatis* was introduced in the form of percentage and histograms; Genetic analysis was performed by using chi-square (*2) test. P value less than (0.05) was considered. Statistical analysis was performed by using Spss 19 version. Data expressed as mean (ISD).

Chapter Three

Results and Discussion

Results and Discussion:

3.1. Distribution of *Chlamydia trachomatis* among Patients PID:

All 200 collected endocervical swab specimens were diagnosed by the gynecologists as having pelvic inflammatory disease (PID). From all 200 collected endocervical swab specimens, found that only 22/200 (11%) were positive for *Chlamydial trachomatis* in genetic method as shown in **figure (3-1).**

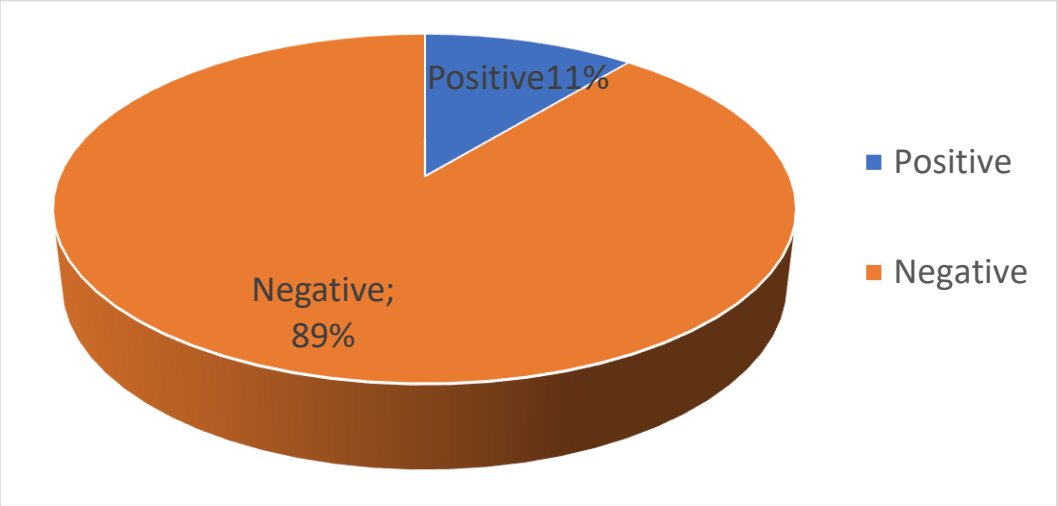


Fig. (3-1): Frequency distribution (%) of *Chlamydia trachomatis* in endocervical swab from women with pelvic inflammatory disease.

Numerous research has attempted to estimate the percentage of *Ch. trachomatis* infections that result in PID, with one study indicating that 8-10% of women with chlamydial infection had PID (Cina et al. 2019).

Other study observed that Chlamydial PID remained the most important preventable cause of infertility and adverse reproductive health outcomes (Low et al.2016).

Tao *et al.*, (2018). observed that the sexually transmitted microorganism was an important cause of pelvic inflammatory disease (PID), and PID was also indicated to increase the risk of infertility, then

PID represents the link between chlamydia infection and infertility. Also, Hillier found that 4.4% sexually active women were reported to have PID (Hillier *et al.*, 2021).

The STIs can cause acute urogenital problems, such as inflammation of the cervix (cervicitis), but their often-asymptomatic presentation (as is often the case for *C. trachomatis* and *N. gonorrhoeae* infections) is the reason why most cases remain undiagnosed, thus contributing to long-term sequelae such as pelvic inflammatory disease (Rowley *et al.*, 2019).

Chlamydia trachomatis is a potentially important cause of PID, and implementing related screening programs would be the most important public health measure for the prevention of PID (Brunham et al. 2015).

Den Heijer *et al.*, (2019). found that the PID risk was higher for women with 2 or more positive *C. trachomatis* tests and observed that women who tested *C. trachomatis*-positive had an increased risk of PID, ectopic pregnancy, and infertility.

In other study the proportions of PID following Chlamydia were founded between 3% and 30%, the difference due to study limitation such as small sample size or limited follow up, unavoidable misclassification of Chlamydia states as it is primarily based on incident nucleic acid tests (Hoenderboom et al. 2019).

The PID was caused by sexually transmitted bacterium, and as PID has been linked to an increased risk of infertility, it serves as a bridge between chlamydia infection and infertility (Anyalechi et al. 2019).

Tang *et al.,* (2020). showed that the risk of adverse outcomes associated with chlamydia is higher in low- and middle-income countries compared to high-income countries.

The occurrence of chlamydial genital infection in women varies according to group and community, as well as exposure to numerous risk factors. *C. trachomatis* has been identified as a significant cause of PID and female infertility worldwide, accounting for around 10-30% of all PID cases (Ahmed and Maisartu 2017).

Other study observed that the risk of developing PID, ectopic pregnancy, or tubal factor infertility was at least 30% higher in women with 1 or more *C. trachomatis*-positive tests compared with women with only *C. trachomatis*-negative tests (Davies ***et al.,*** 2016).

Other study estimated 171 cases of PID, 73 cases of salpingitis, and 2 cases of ectopic pregnancy for every 1000 women with untreated *Ch. trachomatis* (Price ***et al.,*** 2016).

Another study that included 120 000 Western Australian women, a higher PID risk (80%) was observed among women who tested *C. trachomatis*-positive compared with those who tested *C. trachomatis* and gonorrhea negative (Reekie *et al.,* 2018).

3.2. Diagnosis of *Chlamydial trachomatis:*

3.2.1. Immunological Diagnosis of *Chlamydial trachomatis*:

From all 200 collected endocervical swab specimens; only 30/200(15%) give positive results for *Chlamydial trachomatis* with rapid strip serological test and only 22 of all give positive results for *C. trachomatis* by use of PCR and the others 8/30 may give false positive results as show in **table (3-1).**

Table (3-1): The positive results in the immunological and PCR Diagnosis.

Total No.	*Chlamydia trachomatis* positive by immunology %	*Chlamydia trachomatis* positive by PCR
200 (100%)	30 (15%)	22 (11%)

A wide range of frequency variability was seen in certain serological tests conducted in various Iraqi governorates, ranging from 0% in Kirkuk (Kadir *et al.*, 2014) to (26.5% and 39.7%) in Baghdad and Mosul respectively (Ahmed, 2012; Al-Kattan and Mohammed, 2013).

Usually, NAATs (Nucleic Acid Amplification Test) were performed in a central laboratory and require transportation of specimens and transmission of test results to the clinicians. Therefore, NAAT-based diagnostics requires a second visit of patients, potentially leading to delayed treatment or no treatment at all if patients do not re-appear again, which may contribute to the high incidence of infection. rapid diagnostic tests (RDTs) are independent of these demands on logistics, as they allow near-patient (point-of-care) testing and provide results in a few minutes, so that patients may receive antibiotic therapy immediately when they test positive (Meyer, 2016).

Most rapid diagnostic tests (RDTs) are immune chromatographic tests based on lateral-flow-technology and detect chlamydia LPS antigen in genital swabs or urine. Compared to culture and PCR these antigen-based RDTs are significantly less sensitive and less specific (Meyer, 2016).

Due to the problematic, strict criteria; additionally, these serological tests rely on the use of particular antibodies; however, the problem with these antibodies is that the particular anti-chlamydial antibodies may indicate current or past chlamydial infection in sites other than the genitourinary tract. Although PCR has been demonstrated to be cost-effective in chlamydial identification, since the *C. trachomatis* genome could be optimally amplified for visualization, even when it was present in the sample in low concentrations (Dean *et al.,* 2012).

Chlamydia antibody test does not appear to be good screening test for tubal pathology. In view of its high specificity, this test can be used to identify patients with higher chances of tubal pathology requiring operative intervention (Singh *et al.,* 2016).

However, these immunological investigations should not be utilized for screening since prior chlamydial infections frequently create long-lived antibodies that are difficult to identify from antibodies produced in a recent infection. (Klokol *et al.,* 2022).

3.2.2. Molecular Identification of *Chlamydia trachomatis*:

DNA was extracted from all 200 collected endocervical swab specimens; conventional PCR was carried out using these DNA samples for the amplification of specific *omp A* gene; according to the sequences and program listed in **table (2-4-A)**.

After that gel electrophoresis showed that, out of the 200 samples, only 22/200 (11%) produced the specific amplicon with 144bp when compared with DNA ladder; as shown in **figure (3-2)**, while the remaining number (178/200) 89% were negative for this bacterium as shown in **figure (3-1).**

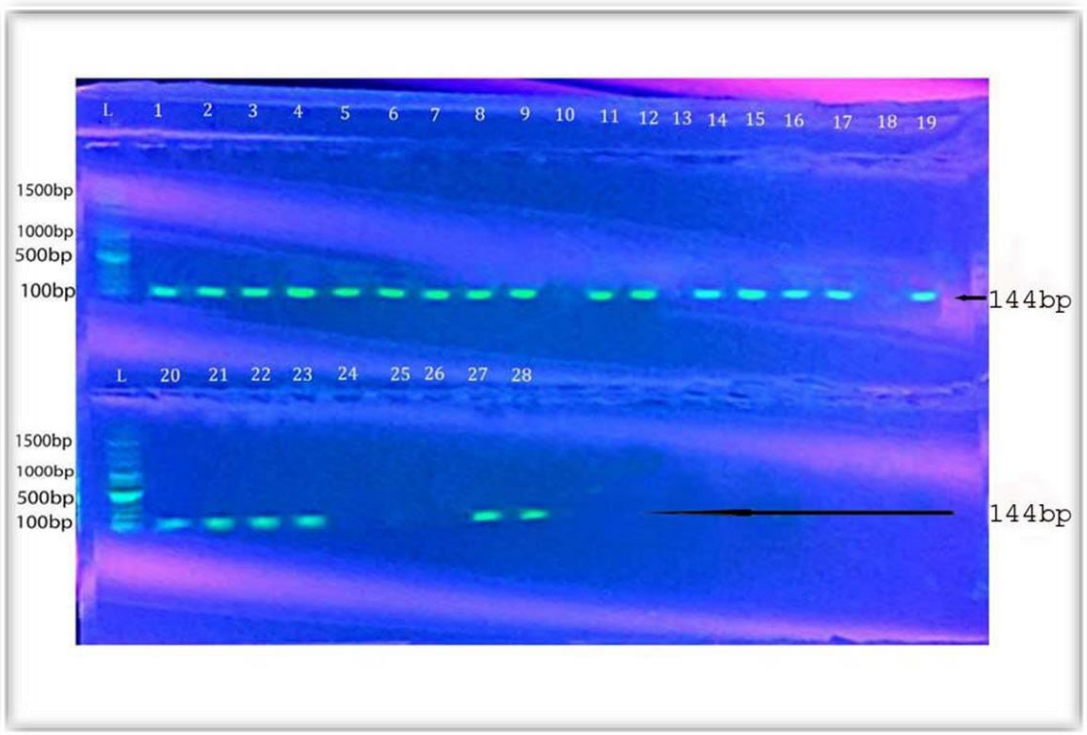


Fig. (3-2): 1.5% Agarose gel electrophoresis at 70 volts for 50min for *OMP* PCR products visualized under U.V light at 280 nm after staining with red safe stain. L: 1500 bp ladder; lane (1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 15, 16, 17, 19, 20, 21, 22, 23, 27 and 28) were positive for *Chlamydia trachomatis* in endocervical swabs among patients with pelvic inflammatory disease. The amplicons size is 144 bp.

Most of the NAATs were based on polymerase chain reaction (PCR) which the most sensitive and specific tests to detect chlamydia. These tests also have a high specificity comparable to culture, but in contrast to culture, do not depend on viable pathogens, facilitating specimen transport. Therefore, NAATs are generally considered the test of choice for chlamydia and have replaced culture as the diagnostic gold standard. Antigen tests (EIA, DFA, RDTs) were no longer recommended for chlamydia testing due to insufficient diagnostic accuracy (Nwokolo *et al.,* 2016).

Other study showed that the diagnosis of microorganisms through the culture method was substituted mostly by molecular assays which increased diagnostic sensitivity and decreased the time of infection detection (Yousefi *et al.,* 2019).

According to Chabuck *et al.* (2016) only eight (3.4%) of the 232 females in the study were genetically determined to be *Chlamydia* positive. The remaining 224 women tested negative for the bacterium.

Other study Using PCR technology, showed the prevalence was 84% from swab samples positive for chlamydia, and there was a significant association found between *C. trachomatis* infection and both infertility and miscarriage women (Omer and Hajir, 2018).

As *C. trachomatis* cannot be grown, molecular methods can be used to identify it, comparing tissue-culture, ELISA, or DFA staining to PCR, which is quicker and more sensitive (Caldeira, Ana, 2017).

Another study observed that the ratio of positive results in PCR were (17.5%) higher than in ELISA (14.2%) that may return to the intracellular nature of these pathogens (Al-Nuaimy and Al-Jandeel 2018).

Other study highlighted the importance of early laboratory diagnosis and specific treatment of these agents as these increase the risk of transmission many folds when exist together (Rawre *et al.,* 2019).

The course of chlamydial infections is usually unpredictable, diverse, and asymptomatic and has remained almost unrecognized. Therefore, PCR-based methods can apply successfully to detect *C. trachomatis* in both pregnant and non-pregnant women (Shamkhi *et al.*, 2022).

Numerous studies use PCR to diagnose and estimate the frequency of Chlamydia, with the endocervical specimen serving as the primary and most significant specimen (Rostami *et al.,* 2017).

In this study PCR was carried out by using DNA samples for the amplification of specific *omp* A primer which considered as housekeeping gene.

The intermolecular network at the outer membrane is called chlamydial outer membrane complex (Comc) and is thought to substitute for the very limited amount of chlamydial cell wall peptidoglycan, a component that provides rigidity and structural strength against osmotic pressure to the wall of Gram-negative bacteria (Saka *et al.,* 2011).

Chlamydia Momps are part of a larger family of genetically related outer membrane proteins (the *Omp* A family) that are heat-modifiable, surface exposed porin proteins. *Omp* A proteins have a structurally similar N-terminal domain that is embedded in the bacterial outer membrane. *Omp* A proteins have been targeted as vaccine candidates because of their surface exposure, high immunogenicity, and role in the interaction between the bacteria and their host cells (Confer and Ayalew, 2013).

In other study the endocervical swabs were collected from 324 women. *C. trachomatis* was found in 10.8 % women and the samples were screened for *C. trachomatis* by cryptic plasmid PCR and *omp*A gene (Rawre *et al.,* 2019).

Other study targeting the *omp1* gene, and found 9% of the total 200 samples were positive for *C. trachomatis* (Shamkhi *et al.,* 2022).

Other study disagreed to the results of this study which showed that the molecular prevalence of *C. trachomatis* in women was 22% in Iraq (Ali and Shia, 2018). A review of the literature showed that the molecular prevalence of *C. trachomatis* in women was 13.5% in India (Dhawan *et al.*, 2014).

A total of 73 patients with probable PID or tube-ovarian abscess were included. Only eight patients (11%) with *Chlamydia trachomatis* were identified (Schindlbeck *et al.*, 2014).

This study's frequency (11%) differs from another research conducted in the Arabic World; in Qatar (5.3%) (Al-Thani et al. 2013), whereas in Saudi Arabia (15%). (Kamel, 2013). Furthermore, in Iran, distinct investigations found that the prevalence of *C. trachomatis* was (22%) (Aslanimehr *et al.*, 2015).

Other study found that the rate of *C. trachomatis* infection in women was (10.8%) (Esteghamati *et al.*, 2020). However, some other studies reported a low rate of infection (between 2 and 4%) in this group (Azami *et al.,* 2018).

Other study observed that the prevalence of *C. trachomatis* infection among pregnant women and transmitted the infection to new born at term was reported as 8.8% (Esteghamati *et al.*, 2020).

Furthermore, the molecular tests had to be simple and inexpensive diagnostic assays, because the pathogen's intracellular localization complicates regular diagnosis. Chlamydial infection is far more difficult to diagnose in asymptomatic, chronic or persistent infections with a low pathogen burden (Tagini and Greub, 2018).

Nucleic acid amplification tests, with their high sensitivity and specificity, are currently the first-line tests for the detection of *Chlamydia trachomatis* When replaced by other detection methods, there are more false negative tests, leading to underreported cases and a subsequent underestimation of *C. trachomatis* infection's prevalence (Rodrigues *et al.*, 2022).

3.3. Distribution of PID Patients with Medical Situation:

From all 200 collected endocervical swab specimens**,** this study found that only 22/200 (11%) were positive for *Chlamydial trachomatis*, 8/22(36%) were had bleeding between time with highest percentage according to other grouping, 5/22(23%) were had primary infertility or secondary infertility, and 4/22(18%) were had recurrent miscarriage as shown in **table (3-2)** and **figure (3-3)**.

Table (3-2): Distribution of PID Patients Numbers with medical situation.

Characters	Total Patients Numbers	(No. (%)) Positive to *C. trachomatis*
primary infertility	50/200(25%)	5/22(23%)
secondary infertility	30/200(15%)	5/22(23%)
Bleeding between period	50/200(25%)	8/22(36%)
recurrent miscarriage	70/200(35%)	4/22(18%)
Total	200/200(100%)	22/22(11%)

In this study observed that the bleeding between times had high percentage (36%), follows by primary infertility, secondary infertility, recurrent miscarriage (23%,23%,18%) respectively as show in **figure (3-3)**.

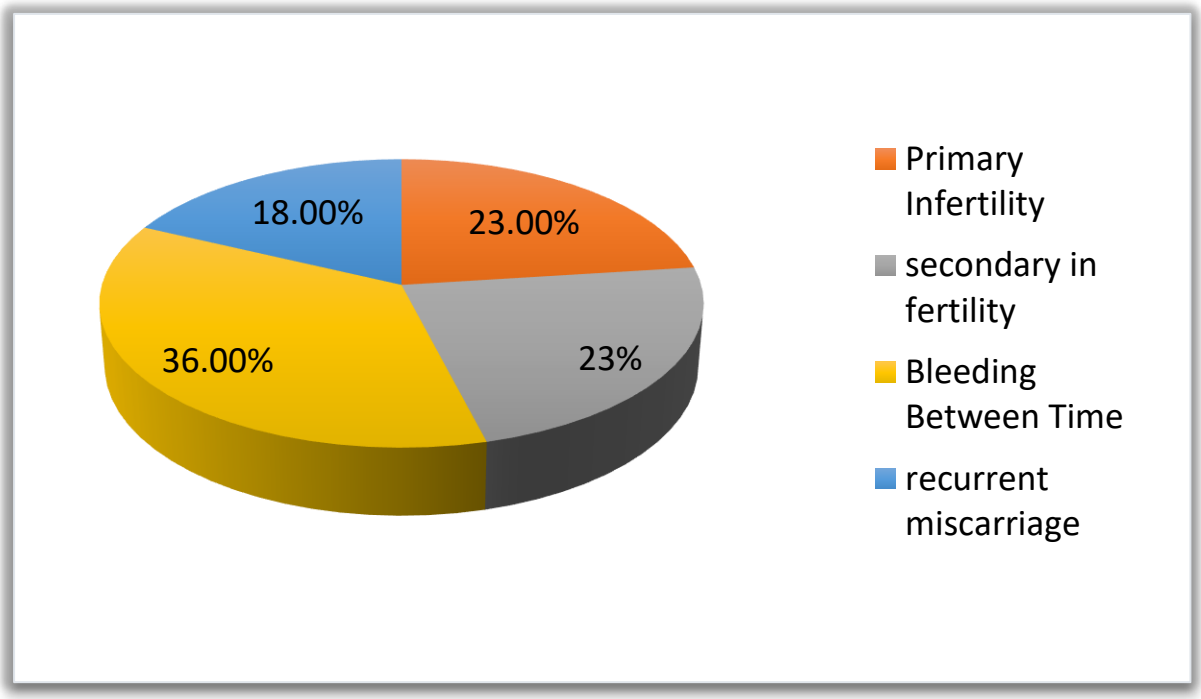


Fig. (3-3): Distribution of Total Patients Numbers with medical situation.

In another investigation, *Chlamydia trachomatis* was present in 21% of infertile women and 3.3% of normally fertile women (Goshayeshi *et al.*, 2015).

Chen *et al.*, (2017). found that contact bleeding (12.50%) were the main clinical symptoms of Chlamydial infection.

In other, high vaginal swab were taken from 215 women, those have been participated in study, 150 were suffering infertility and miscarriage. (Omer and Hajir 2018).

C. trachomatis infection is an important causative agent of miscarriages in women and most abortion cases more than 46 years old in 15.3%, and the abortion in pregnant women in the first period of gestation (Hammadi *et al.*, 2022).

Among bacterial agents, *Chlamydia trachomatis* and *Mycoplasma genitalium*, and among viral agents, human papilloma virus (HPV), have a major role in miscarriage (Rahimkhani *et al.*, 2018).

Ahmadi *et al.*, (2016) showed that *C. trachomatis* infection was associated with spontaneous abortion and found the total prevalence of *C. trachomatis* infection was 38(17.43%) in endocervical swabs of women.

In other study, women with *C. trachomatis* infection were significantly associated with vaginal discharge, abdominal pain, low back pain, burning micturition (Rawre *et al.*, 2019).

Other study observed that pooled prevalence of genital infection was 3·0% in general populations, 2·8% in intermediate-risk populations, 13·2% in female sex workers, 11·3% in infertility clinic attendees, 12·4% in women with miscarriage, 12·4% in symptomatic women, and 17·4% in symptomatic men. Pooled prevalence of rectal infection was 7·7% (Alex *et al.*, 2019).

In women, *Chlamydia trachomatis* could travel from the lower to the upper reproductive tract affecting the uterus, the fallopian tubes and ovaries and severity of inflammation depending on diverse factors (Rodrigues *et al.,* 2022).

Chlamydial infection was examined the relationship between prior chlamydial infection and infertility and showed PID which result from chlamydia significantly increased the chance of infertility after controlling for confounders (Liu *et al.,* 2022).

One of the most common sexually transmitted germs is *Chlamydia trachomatis*. Chlamydial infection, which rises from the lower vaginal canal, can have major reproductive effects, such as infertility. Chlamydia was thought to be responsible for 45% of tubal infertility cases. (Menon *et al.,* 2015).

Many studies had attempted to determine the proportion of untreated infections that result in PID; one study suggested that 8–10% of women with chlamydial infection go on to acquire PID (Herzog *et al.,* 2012).

Cueva *et al.,* (2020) founds regarding clinical manifestations, vaginal discharge was the most prevalent symptom, present in 87 patients (47%) followed by pelvic discomfort, which was experienced by 72 women (39%) and other symptoms, such as lower back pain, malaise, nausea, and vomiting, in 36 subjects (19%) and Risk factors associated with sexual activity were not noted in any medical records.

Rodrigues *et al.,* (2022) showed that *Chlamydia trachomatis* infections were also associated with possible long-term severe injuries. In detail, persistent infection triggers an inflammatory milieu and can be related to severe sequels, such as infertility. This infection could also trigger gynecologic tumors in women (Rodrigues *et al.,* 2022).

According to some researchers, genital *C. trachomatis* is a potential trigger for miscarriage, accounting for about 15% of early miscarriages (<12 weeks of gestation) and up to 66% of late miscarriages (>12 weeks of gestation) (Giakoumelou *et al.*, 2016).

3.4. Distribution of *Chlamydial trachomatis* patients according to Age with medical situations:

From 200/100(50%) women who were diagnosed by the gynecologists as having pelvic inflammatory disease with age group (20-30) found that 11/22(50%) were positive for *Chlamydia trachomatis*. 5/11(45.4%) were positive for Chlamydia had bleeding between time, while 2/11(18.1%) were positive for Chlamydia had primary infertility, secondary infertility, and recurrent miscarriage; which included for endocervical swab sampling as shown in **table (3-3)** and **figure (3-4)**.

Table (3-3): distribution of *Chlamydia trachomatis* in endocervical swabs of PID patients with medical presentation according to age groups (20-30).

Characters (20-30 years old)	**Numbers**	**Chlamydial No. %**
primary infertility	20/200(10%)	2/11(18.1%)
secondary infertility	15/200(7.5%)	2/11(18.1%)
bleeding between period	25/200(12.5%)	5/11(45.4%)
recurrent miscarriage	40/200(20%)	2/11(18.1%)
Total with PID	100/200(50%)	11/11(100%)

This study observed that the positive results for *Chlamydia* in this age group (20-30 years) was 11/22(50%) when compare to other range age and observed that the bleeding between time had the high rate 5/11(45.4%)

in this age group, while other diseases had the same rate 2/11(18.1%) for *chlamydia* positive results as show in **figure (3-4)**.

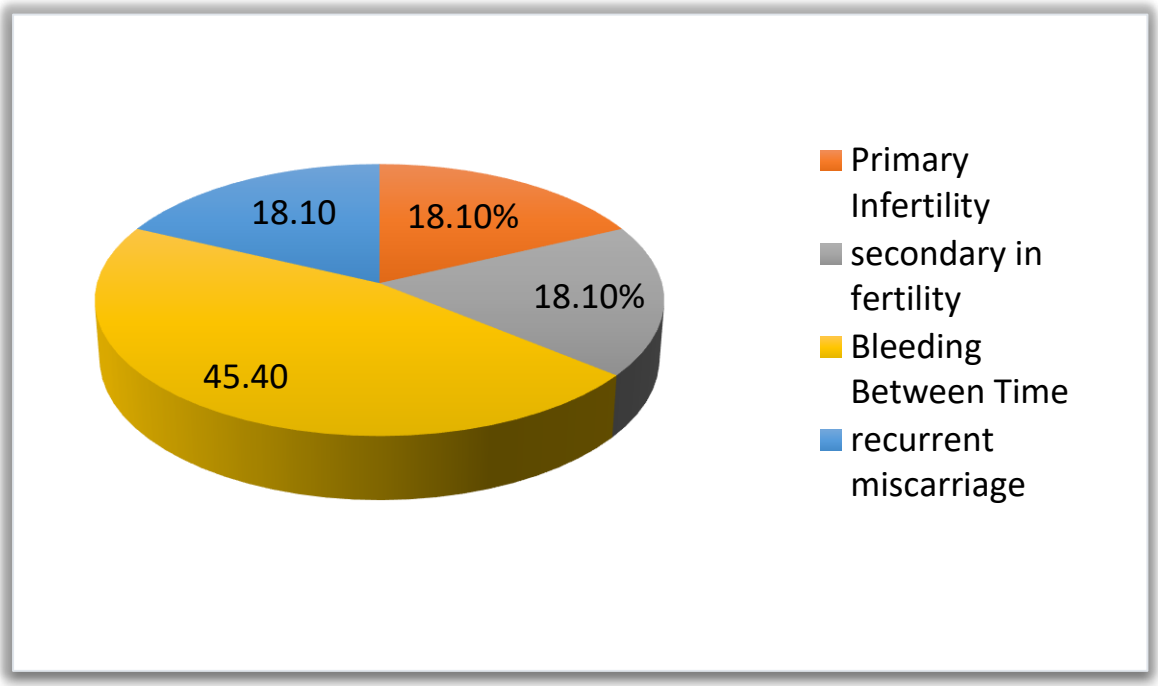


Fig. (3-4): Distribution (%) of Chlamydial patients age between (20-30 years old) with medical presentation.

In the developed countries, STI including chlamydial disease were more common among patients in the age group less than 25 years old, this is due to different cultural setting and the screening programs focus mainly on women at this age group (Fernandes *et al.*, 2014**).** In developed countries, females at the ages of <25 years are considered a high-risk group for chlamydial infection (Newman *et al.*, 2015).

Other study observed that the age group (20- 25years) was the most susceptible to chlamydial infection and the infection was higher in recurrent miscarriages than in single miscarriage (Al-Nuaimy and Al-Jandeel, 2018).

In this study observed that the chlamydial infection percentage in bleeding between time in this age group was higher than other presents (45.4).

Chabuck *et al.,* (2016). analysis the frequency distribution of *C. trachomatis* among age groups of patients with PID revealed that the highest frequency among the age group of 20-40 years was 4/8 (50%).

Conversely in developed countries, females at the ages of <25 years are considered a high-risk group for chlamydial infection (Newman *et al.*, 2015).

Mohammed and AL-Fadhil (2012), in Khartoum, Sudan, found that the majority of Chlamydial positive cases occurred in the age range of 26 to 40 years, with little occurring in the age range of less than 25.

Sexually transmitted diseases (STDs) including chlamydia and gonorrhea, cause pelvic inflammatory disease and infertility. 13.8% of reproductive-age women reported a history of infertility, of whom 40% did not access healthcare. Self-reported PID was associated with infertility, especially in young women (Anyalechi *et al.,* 2019).

The efficacy of a systematic approach for *C. trachomatis* screening in women under 25 is still up for debate. The Prevention of Diseases induced by *Chlamydia trachomatis* trial's primary goal is to ascertain whether early detection and treatment of genital *Chlamydia trachomatis* infection in 18-24 years old women lowers the risk of PID during a 24-month period (Tamarelle *et al.*, 2017).

The C. trachomatis infection is most prevalent in young women (14-25 years), which can be explained by asymptomatic infection, inadequate partner treatment, and delayed development of protective immunity (O'Connell and Ferone 2016).

In women, the most common demographic correlation of chlamydial infection is young age (20 years). This could be explained by anatomic changes in the cervix of younger women, where the squamo-columnar junction, a primary host target for *C. trachomatis*, is everted and hence more exposed. While the elderly is less likely to disseminate sickness due to decreased *C. trachomatis* exposure and physiological changes that limit sensitivity to the rising (Han *et al.*, 2022).

This study observed that the positive results for Chlamydia among this rang age (30- 40 years) were 11/22(50%) when compared to other range age and observed that the primary infertility, secondary infertility, and bleeding between time had the same rate 3/11(27.2%) which had the high rate in this range of age, while recurrent miscarriage had 2/11(18.1%) as shown in **table (3-4)** and **figure (3-5)**.

Table (3-4): distribution of *Chlamydia trachomatis* in endocervical swabs of PID patients with medical presentation according to age groups (30-40).

Characters (30-40 years old)	**Numbers**	**Chlamydial No. %**
primary infertility	30/200(15%)	3/11(27.2%)
secondary infertility	15/200(7.5%)	3/11(27.2%)
bleeding between period	25/200(12.5%)	3/11(27.2%)
recurrent miscarriage	30/200(15%)	2/11(18.1%)
Total with PID	100/200(50%)	11/11(100%)

In this age group of the study observed that the recurrent miscarriage had low percent (18.1%), while other presents had the same present (27.2%) as show in **figure (3-5)**.

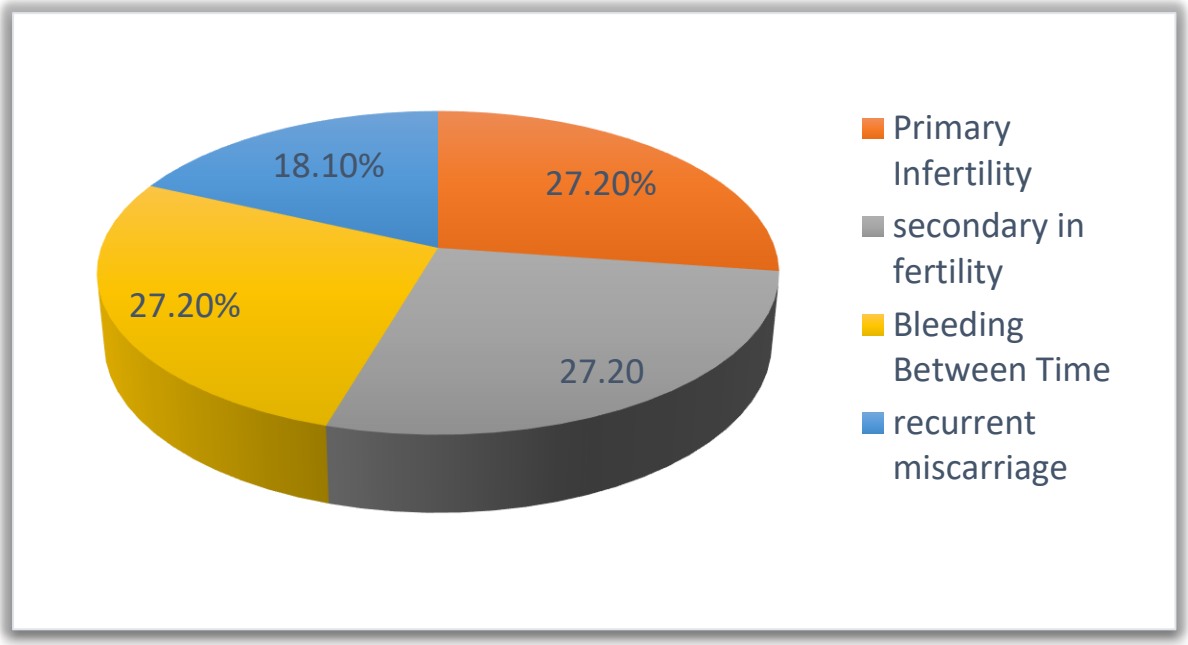


Fig. (3-5): distribution (%) of Chlamydial patients age from thirty to forty (30-40 years old) with medical presentation**.**

Mohammed and AL-Fadhil, (2012) in Khartoum, Sudan, found that the little of positive cases occurred in the age range of above 40 years.

Chabuck *et al.*, (2016) analysis the frequency distribution of *C. trachomatis* among age groups of patients with PID revealed that 2/8 (25%) from the age group more than 40 years.

In this study observed that the distribution of Chlamydial infection in age group (20-30) were equal to that found in age group (30-40) (11%).

Analysis of the age distribution in other study showed that *C. trachomatis*-positivity was highest in patients 20–30 years old (44.53%), followed by those 31–40 years old (33.59%), and was low in patients who were under age 20(5.47%) and those who were over age 40(16.41%) (Chen *et al.*, 2017).

Other study showed that the most abortion cases were more than 46 years old in Chlamydial infection (15.3%), and the abortion in pregnant women in the first period of gestation (Hammadi *et al.,* 2022).

Ali and Shia (2018) showed that women aged <35 years had a higher prevalence of infection, compared to women in the age range of 40-55 years.

Rabiah *et al.,* (2018) reported that the infection was significantly more prevalent among old-aged women.

Other study showed that women with ages ≥30 years old were more likely to have *C. trachomatis* infection (Esteghamati *et al.,* 2022).

In the other study, Women in the age groups (21-25) (26-30) and (30-35) years had the highest prevalence of Chlamydial infection compared with older age category in a study of women according to the pH level, vaginal discharges were varied with age, use of contraceptives, menstrual cycle and with the estrogen level, douching used (Ali *et al.*, 2018).

3.5. Chlamydial infection and association with microRNA:

3.5.1. Gene expression of microRNA-142 level by using real time PCR:

A total of (22) patient endocervical swab samples which give positive results for *Chlamydial trachomatis* were used to study the gene expression of microRNA by using(ΔΔCt) PCR method. The level of expression to microRNA-142 genes in the test sample as well as in control samples normalized with house- keeping gene (GAPDH). The study showed that the expression of the micro-142 gene elevated in *Chlamydia trachomatis* patients when compared to the control group, with the expression of the gene increasing more than 40% when compared to the control group as shown in **table (3-5) and figures (3-6)**.

Table (3-5): microRNA 142- Fold Gene Expression among Control and Patients versus the reference gene (GAPDH).

Groups	**N**	**Expression levels (2^(ΔΔCt))**		
		Mean	**SD**	**SE**
Control	25	1.69	2.45	0.55
Patient	22	42.52	29.77	5.43
P value		<0.0001*		

* Represent a significant difference at p≤ 0.05.

The relative micro-RNA expression levels of target genes as described previously by using PCR efficiencies and mean crossing point deviation between samples and controls, represent a significant difference at p≤ 0.05.

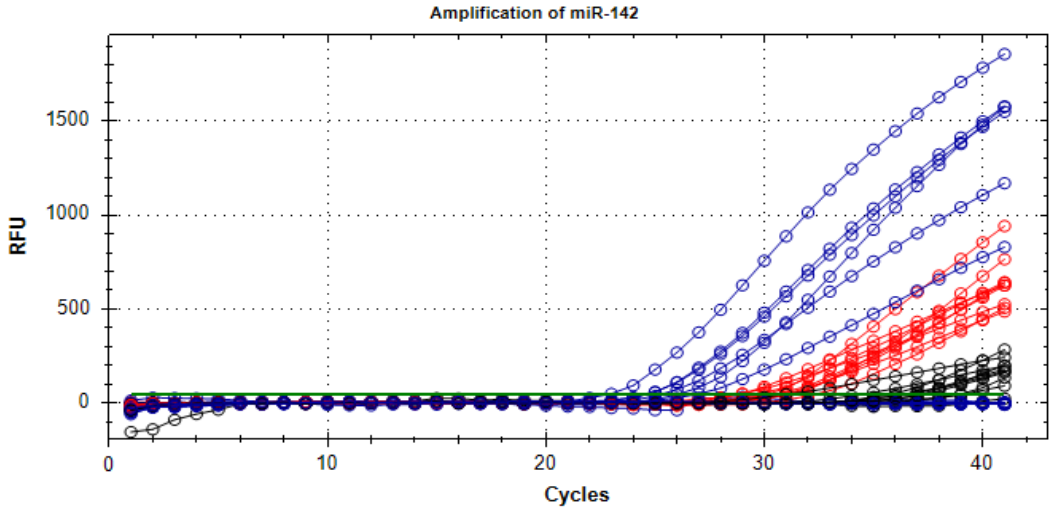


Fig. (3-6): The micro-RNA 142 Gene expression level. This is the first run for 15 samples, Blue lines represent amplification of Reference gene (GAPDH), Red lines represent amplification of samples for Patients, Black lines represent amplification of control samples. RFU=relative fluorescence units.

To explore the possibility that a miRNA expression profile from a local site of infection could be used a Batteigers a prognostic indicator of signs and symptoms, the miRNA profile in women infected with *C. trachomatis* with signs and symptoms was evaluated (Batteiger *et al.*, 2020).

Additionally, in a murine model of chlamydial genital infection, had demonstrated that a unique set of miRNAs present in cervical tissue early in infection is associated with progression to upper tract disease (hydrosalpinx). These particular miRNAs were associated with infection by virulent but not a virulent chlamydial strain (Arkatkar *et al.*, 2015).

The present research found that the expression of the micro-142 gene increased in *Chlamydia trachomatis* patients when compared to the control group, with the expression of the gene increasing more than 40%fold when compared to the control group as showed in figure (3-7). MicroRNA-142

was shown to be significantly up regulated in comparison to the control group.

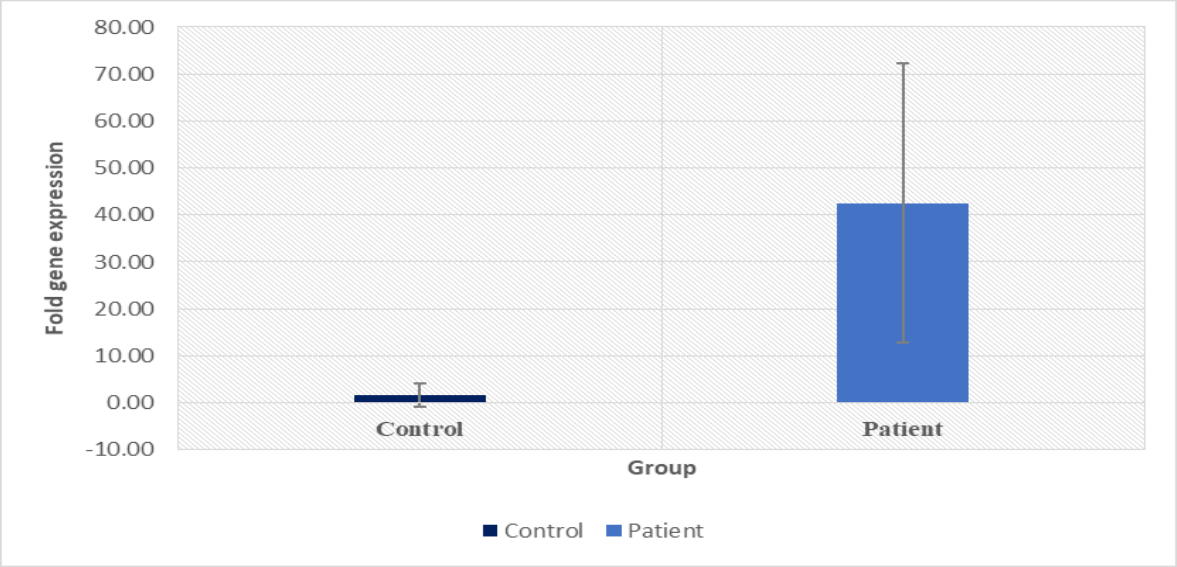


Fig (3-7): microRNA-142-fold Gene Expression among Control and Patients versus the reference gene (GAPDH).

Infertility impacts a considerable number of women worldwide, and it affects different aspects of family life and society. Although female infertility is known as a multifactorial disorder, there are strong genetic and epigenetic bases. Studies revealed that miRNAs play critical roles in initiation and development of female infertility related disorders. Early diagnosis and control of these diseases is an essential key for improving disease prognosis and reducing the possibility of infertility and other side effects. Investigating the possible use of miRNAs as biomarkers and therapeutic options is valuable, and it merits attention (Bahmyari *et al.*, 2021).

Benyeogor *et al.*, (2019) found that the number of miRNAs expressed varied each week after chlamydia infection and reinfection, miRNAs were common in both primary chlamydia infection and reinfection.

Chlamydia trachomatis is important cause of RSA (recurrent spontaneous abortion). However, mechanism leading to RSA in *Ch.*

trachomatis-positive patients is not understood and novel strategies are needed. It was hypothesized that microRNAs play important role in RSA regulation during infection (Ray *et al.*, 2021).

Genital *C. trachomatis* infection may cause pelvic inflammatory disease (PID) that can lead to tubal factor infertility (TFI). Understanding the pathogenesis of chlamydial complications including the pathophysiological processes within the female host genital tract is important in preventing adverse pathology. MicroRNAs regulate several pathophysiological processes of infectious and non-infectious etiologies. the study, tested the hypothesis that the miRNA profile of single and repeat genital chlamydial infections will be different and that these differences will be time dependent (Batteiger *et al.*, 2019).

Batteiger et al. showed significantly higher expression of microRNA-142 in Chlamydia trachomatis group and it was found there is an association of cervical expression of micro-RNA on day 3to pathology on day 35 (Batteiger *et al.*, 2020).

Eledge and Laxmi, (2018) found that the dicer was critical to not only miRNA biogenesis, but also apoptotic signaling, cellular development, and physiological functions of several tissues including the female reproductive tract. Complications of infertility can result from dysregulation of expression or functionality of Dicer in the reproductive tract through embryonic termination, ectopic pregnancies, cyst formation, oviduct malfunction, and many other conditions.

Other findings revealed considerably increased expression of miR-142 and miR-147 in the *C. trachomatis* with signs and symptoms (CTSS) group, while miR-155 expression was seen in both the CTNS and CTSS groups. There is a link between cervical miR-142 and miR-147 expression (Yeruva *et al.*, 2017).

Data from (Batteiger *et al.*, 2020) showed that miR-155 may be a marker of infection, while miR-142 and/or miR-147 may be markers for signs or symptoms. According to study in 2015, miRNA (miR-142 and miR-147) bind to TLR7/TLR8 and cause inflammation in cancer, arthritis, and ischemia models (Yelamanchili *et al.*, 2015).

3.5.2. Gene expression of microRNA-520a level by using real time PCR:

A total of (22) patient endocervical swab samples, RNA was extract to study the gene expression of microRNA-520a by using (ΔΔCt) PCR methods, in this method the level of expression to microRNA-520a gene in the test sample as well as in control samples normalized with house-keeping gene as shown in **table (3-6)** and **figures (3-8)**.

Table (3-6): microRNA 520a Gene Expression among Control and Patients versus the reference gene (GAPDH).

Groups	**N**	**Expression levels (2^(ΔΔCt))**		
		Mean	**SD**	**SE**
Control	25	1.43	1.27	0.28
Patient	22	86.01	23.28	4.25
P value		<0.0001*		

* Represent a significant difference at p≤ 0.05.

The relative mRNA expression levels of target genes as described previously by using PCR efficiencies and mean crossing point deviation between samples and controls, represent a significant difference at p≤ 0.05.

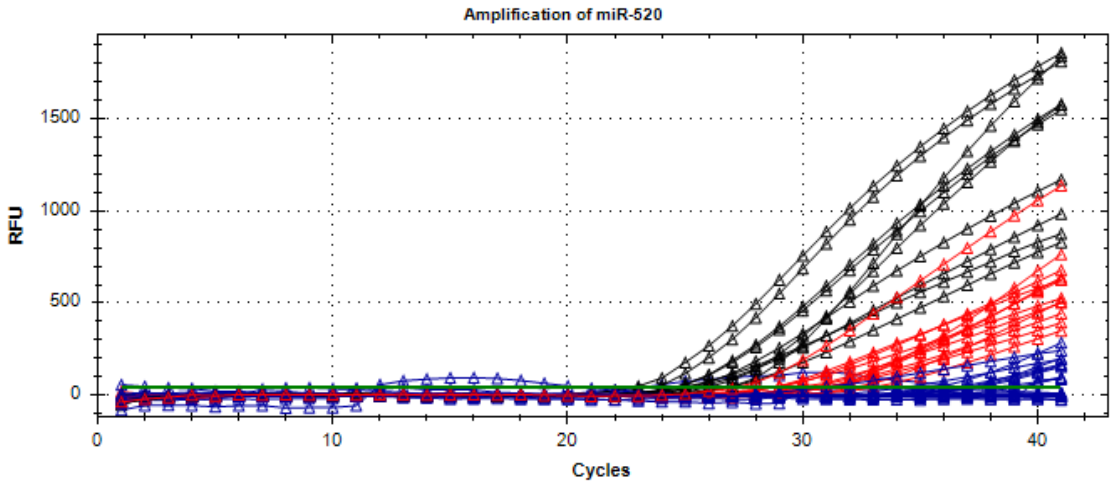


Fig. (3-8): microRNA 520 Gene expression level. This is the first run for 15 samples, Black lines represent amplification of Reference gene (GAPDH), Red lines represent amplification of samples for Patients, Blue lines represent amplification of control samples. RFU=relative fluorescence units.

Chlamydia trachomatis genital infection in women causes serious adverse reproductive complications, and is a strong co-factor for human papilloma virus (HPV)-associated cervical epithelial carcinoma. tested the hypothesis that Chlamydia induces epithelial-mesenchyme transition (EMT) involving T cell-derived TNF-alpha signaling, caspase activation, cleavage inactivation of dicer and dysregulation of micro-RNA (miRNA) in the reproductive epithelium; the pathologic process of EMT causes fibrosis and fertility-related epithelial dysfunction, and also provides the co-factor function for HPV-related cervical epithelial carcinoma. Using a combination of microarrays, immunohistochemistry and proteomics, showed that chlamydia altered the expression of crucial miRNAs that control EMT, fibrosis and tumorigenesis; that maintain epithelial integrity were down-regulated, while other miRNAs that promote EMT, fibrosis and tumorigenesis were up-regulated (Igietseme *et al.*, 2015).

The current study showed that the expression of the micro-520a gene elevated in *Chlamydia trachomatis* patients when compared to the control

group, with the expression of the gene increasing more than 80% when compared to the control group, as shown in the figures (3-9). micro-520a was discovered to be considerably up regulated in comparison to the control group.

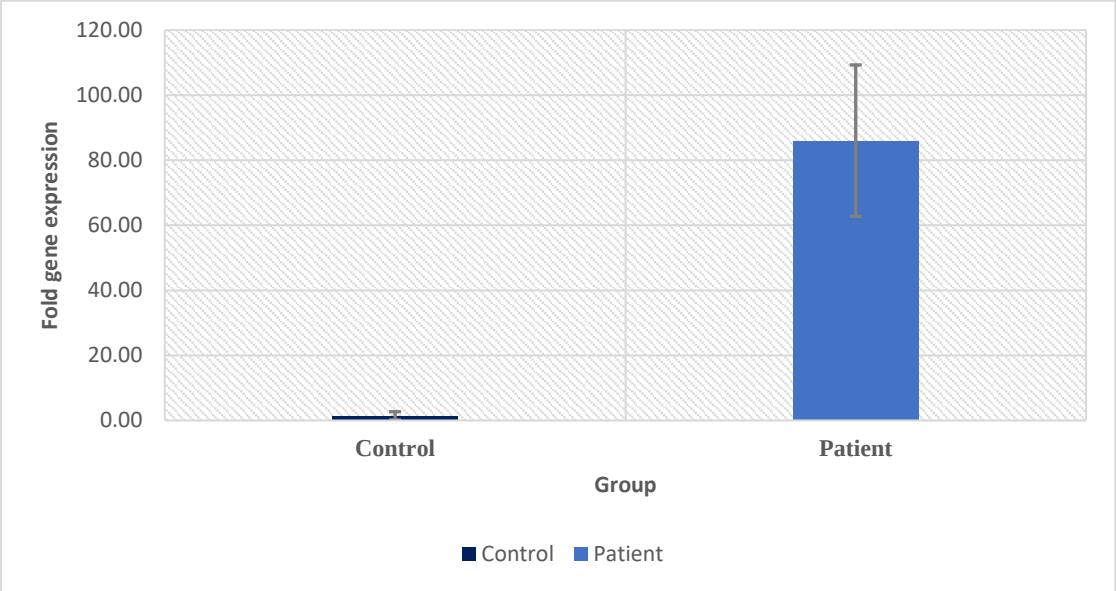


Fig. (3-9): microRNA 520a Fold Gene Expression among Control and CRC Patients versus the reference gene (GAPDH).

The role of miRNA 520a as mitogen- activated protein Kinase 2 (MAP3K2) which is a kinase belong to the serine / threonine protein Kinase family (Yu *et al.*, 2015).

The micro-RNA 520 a has been characterized as a tumor suppressor in several human cancer, so the over expression of miR 520 a can inhibit cancer cell invasion and proliferation (Park *et al.*, 2013).

Recent studies indicated that microRNAs (miRNAs), including immune cell type-specific miRNAs modulate inflammation and immune response to *Chlamydia trachomatis* genital infection (Francis et al. 2022).

Change in miRNA expression after Chlamydia infection and predicted the role of miRNA might play in disease outcome (Chowd hurry *et al.*, 2017).

Conducted Real time PCR to confirm some of unique miRNA a herd in the Chlamydia infected patients like mir-142, miR 520 at which found significant up regulation in comparison to control group (Batteiger *et al.*, 2020).

When compared to asymptomatic infected women, infected women with signs and/or symptoms (CTSS) and (CTNS), had different miRNA profiles and found that MiR-520 showed 3.9- to 9.0-fold increases in expression in the CTNS group. (Batteiger et al. 2020). Micro-RNAs are also abundant in human embryonic stem (hES) cells, and miR-520 has been linked to hES cell function (Galliano and Pellicer, 2014).

Previous research has shown that dysregulated microRNA had a role in hepatocarcinogenesis. miRNA 520a were shown to be down regulated in human hepatocellular carcinoma (HCC) cells compared to normal hepatic cells in the current investigation. Overexpression of miRNA 520a suppressed cell growth and caused cell cycle arrest at the G0/G1 checkpoint. (Dong *et al.*, 2015).

Batteiger *et al.*, (2020) found that miRNA are important factors in host cell changes and response during genital Chlamydial infection.

The microRNAs play crucial roles in tumor formation and progression by targeting several genes. MiRNA 520a 3p has been identified as a tumor suppressor gene in lung and breast malignancies. However, the expression and functional importance of miRNA 520a in gastric cancer are not fully characterized. Furthermore, the study found that the expression of miRNA 520a 3p was considerably downregulated in GC tissues and cells. (Su *et al.*, 2019).

3.6. Sequencing of *Chlamydia trachomatis*:

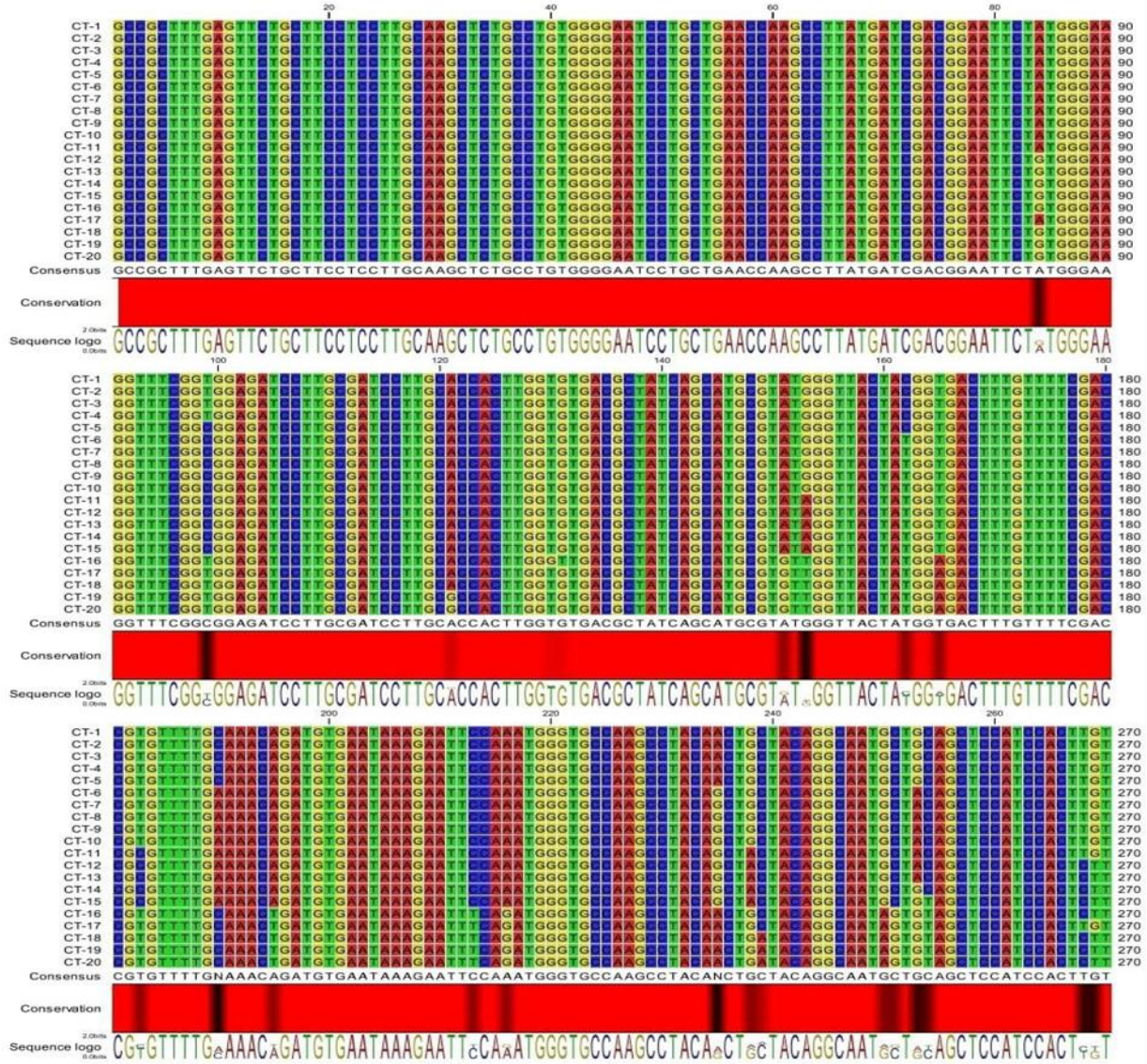
3.6.1. Phylogeny Analysis by *omp-1* Sequencing in *Chlamydia trachomatis*:

Only 20 tested isolates of *C. trachomatis* were successfully amplified in the *omp1* PCR with amplicon size 1134pb when compared with allelic ladder as shown in **figure (3-10).**

Fig. (3-10): 1.5% Agarose gel electrophoresis at 70 volts for 50min for *OMP* PCR products visualized under U.V light at 280 nm after staining with ethidium bromide stain. L: 1500 bp ladder; lane (1, 2, 3, 4, 6, 7, 8, 9, 11, 12, 13, 14, 16, 17, 18, 21, 22, 24, 25 and 26) were positive for *Chlamydia trachomatis* in endocervical swabs among patients with pelvic inflammatory disease. The amplicons size is 1134 bp.

Sequence analysis of the *omp1* gene from amplified DNA clinical isolate of *C. trachomatis* found that all nucleotide sequence were easy to read and interpret when compared by BLAST similarity search and the evolutionary relationships between clinical isolates were detect as show in figure (3-11).

Detailed analysis revealed that there were limited sequence differences within genotypes, however some of these sequence variants were observed in a single sample, whereas others were observed in several samples as shown in alignment figures (3-11).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Consensus
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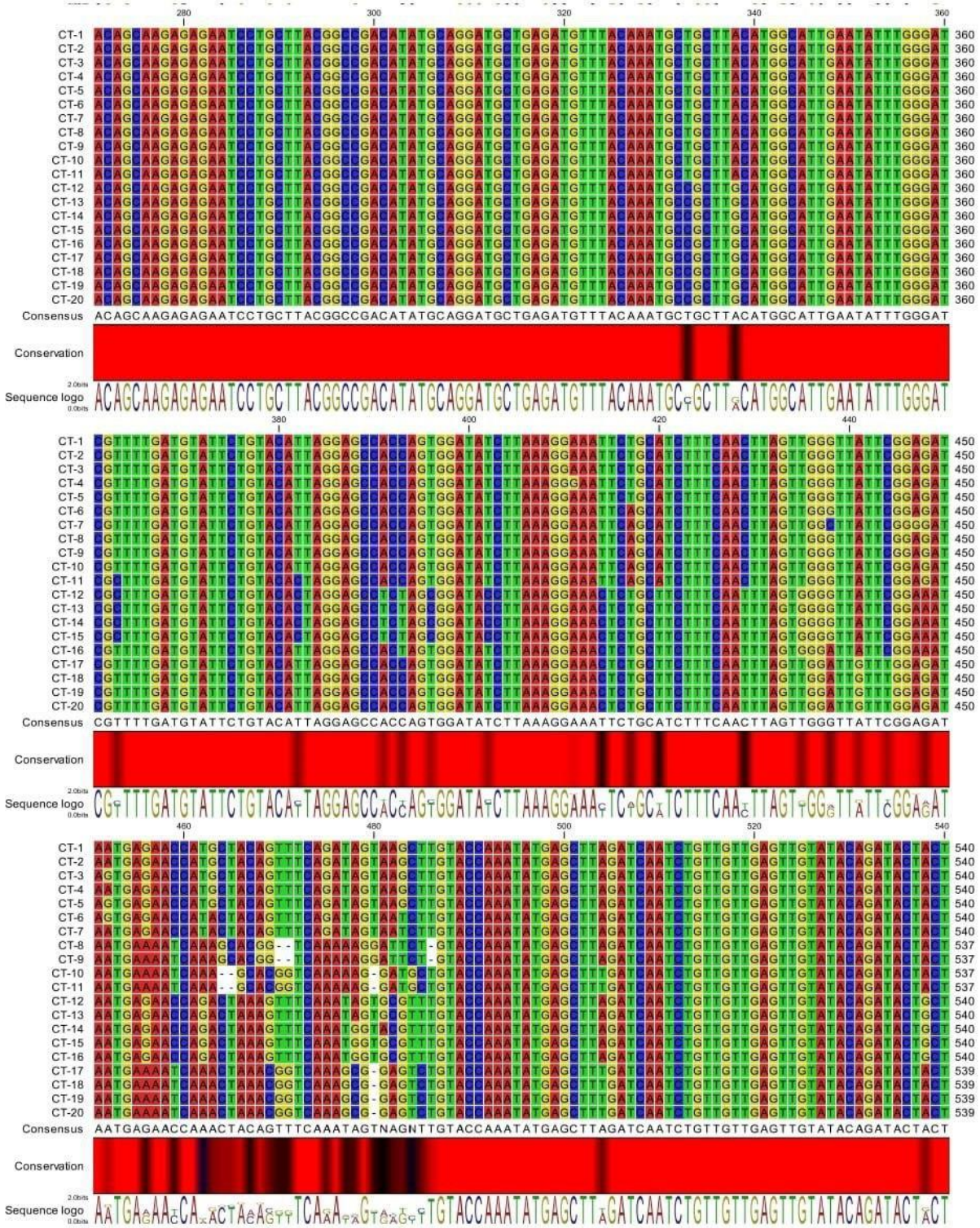
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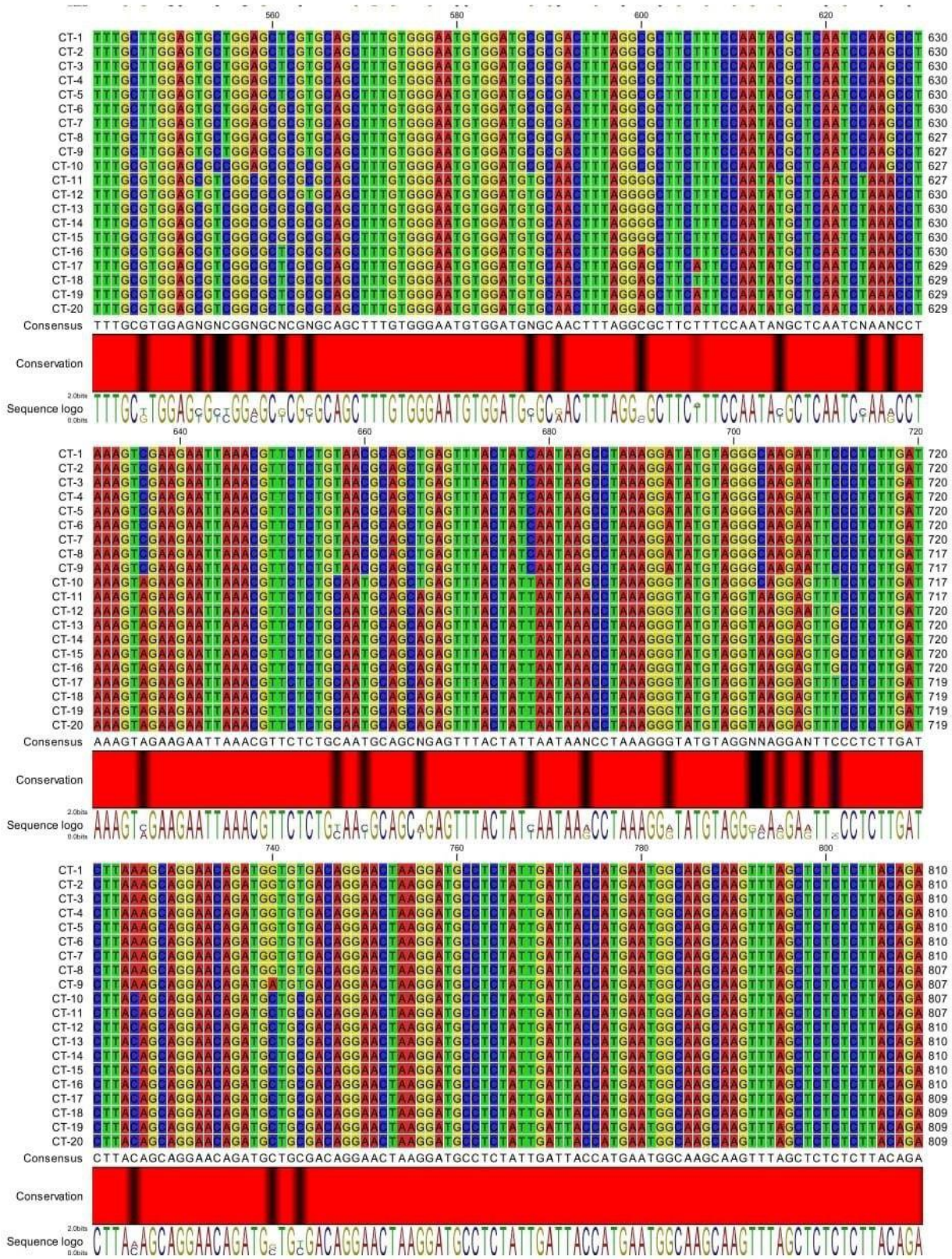
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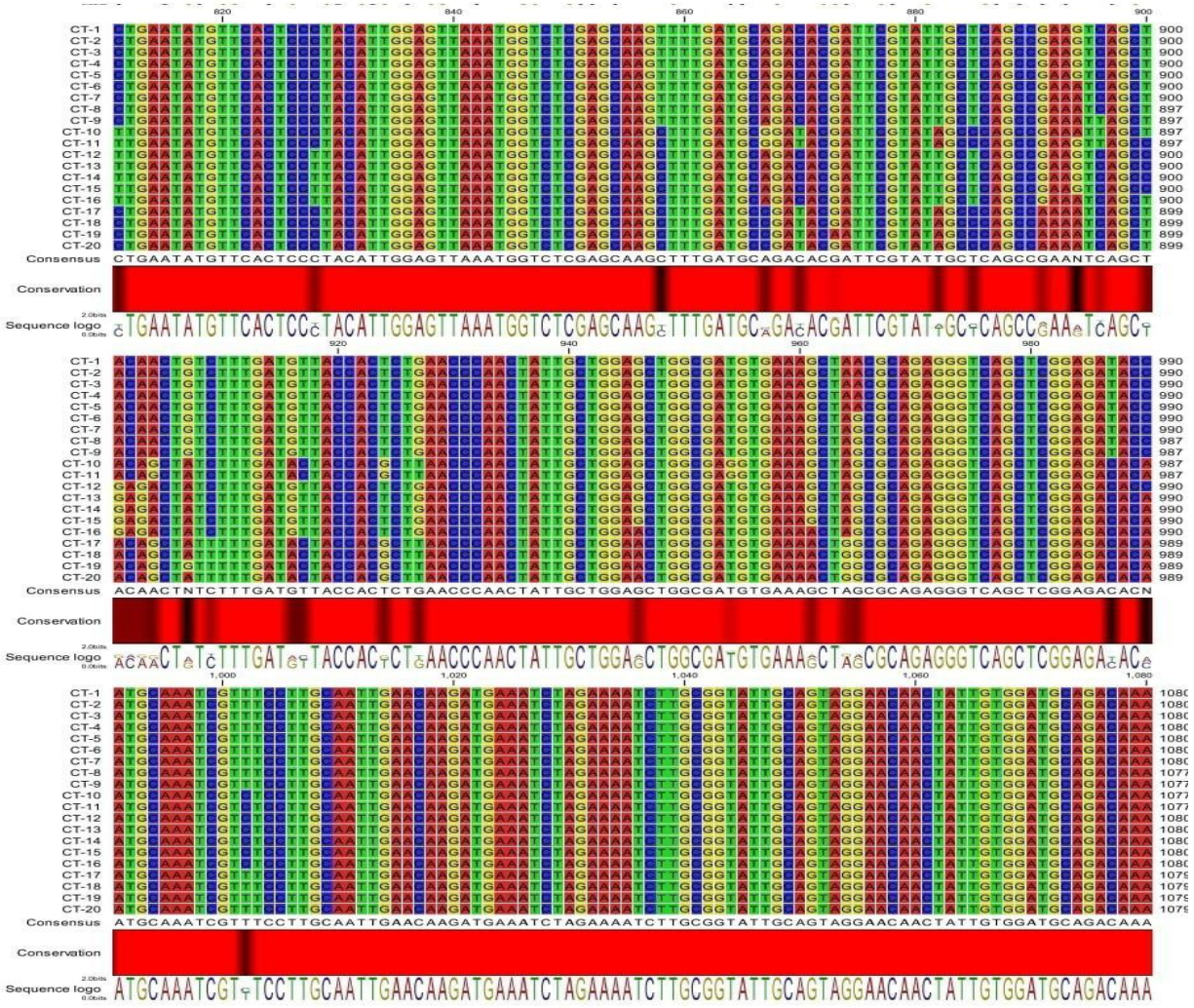
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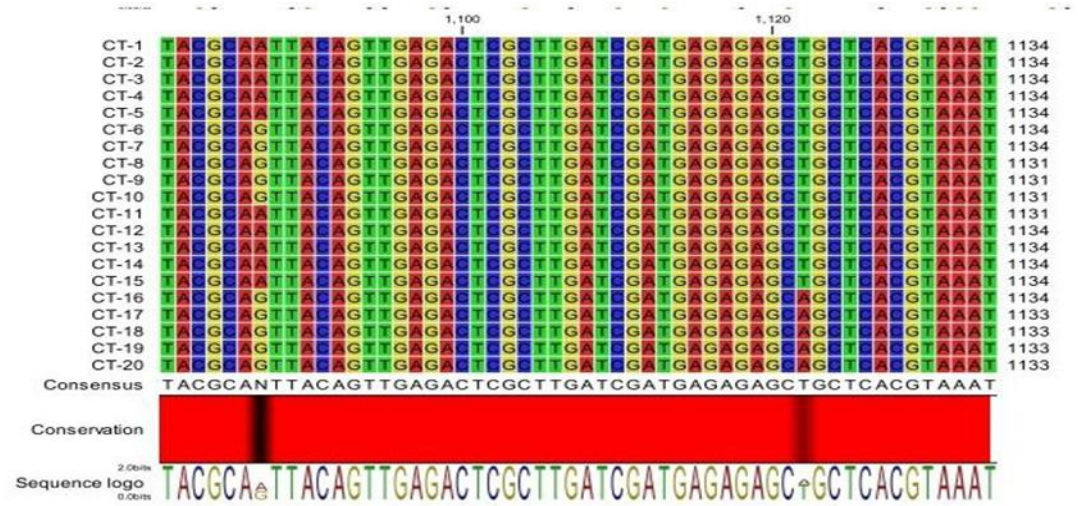

Fig. (3-11): Multiple Sequencing Alignment *Chlamydia trachomatis*.

The phylogenetic analysis of *omp1* gene showed that *Ch. trachomatis* were segregated in to two main clusters (A and B) as shown in **figure (3-12)** and **(3-13)**.

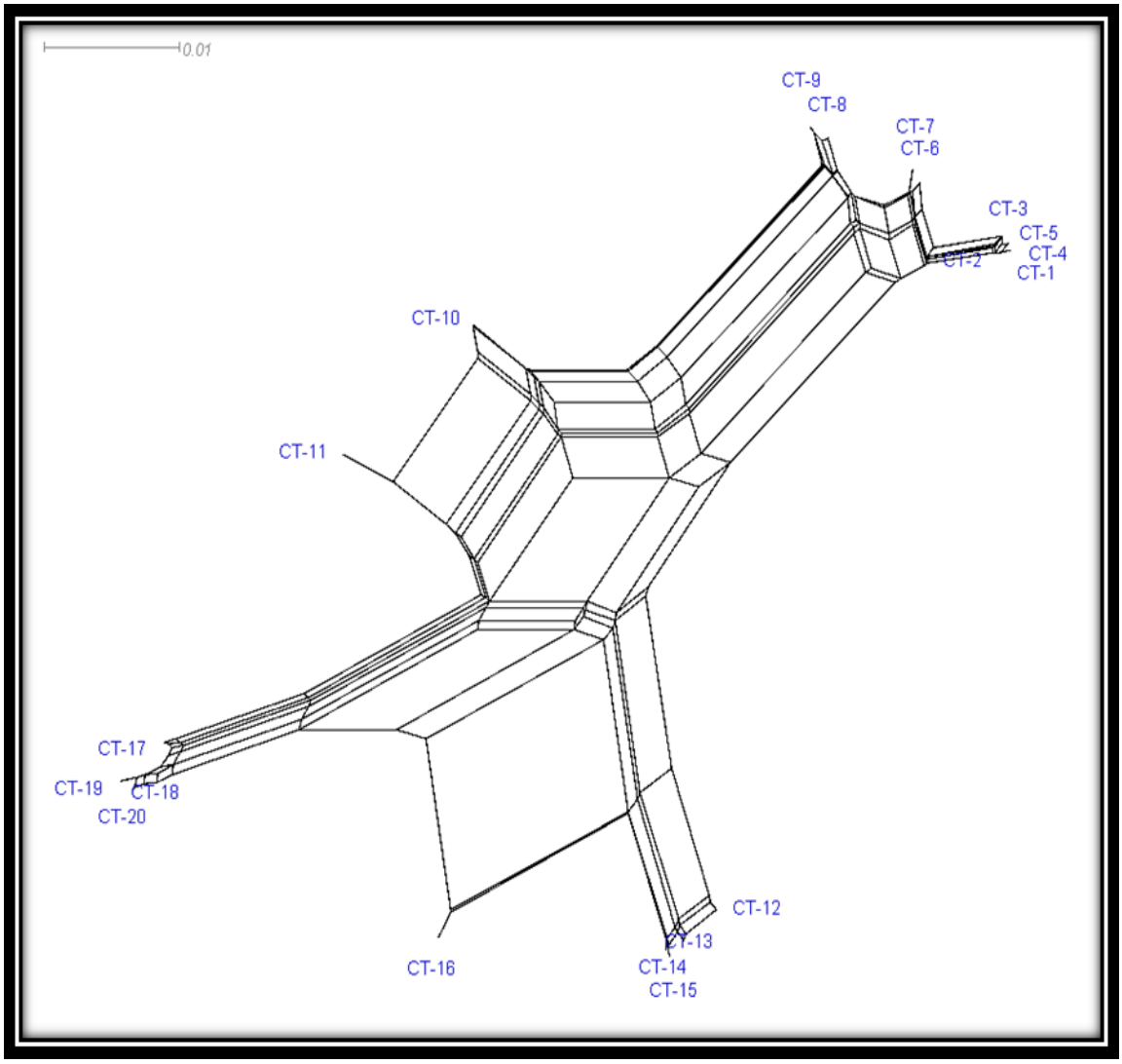

Fig. (3-12): Split tree-decomposition analysis based on *omp1* gene sequences of 20 *Chlamydia trachomatis* isolates. Note: multi parallelogram formations indicate recombination events.

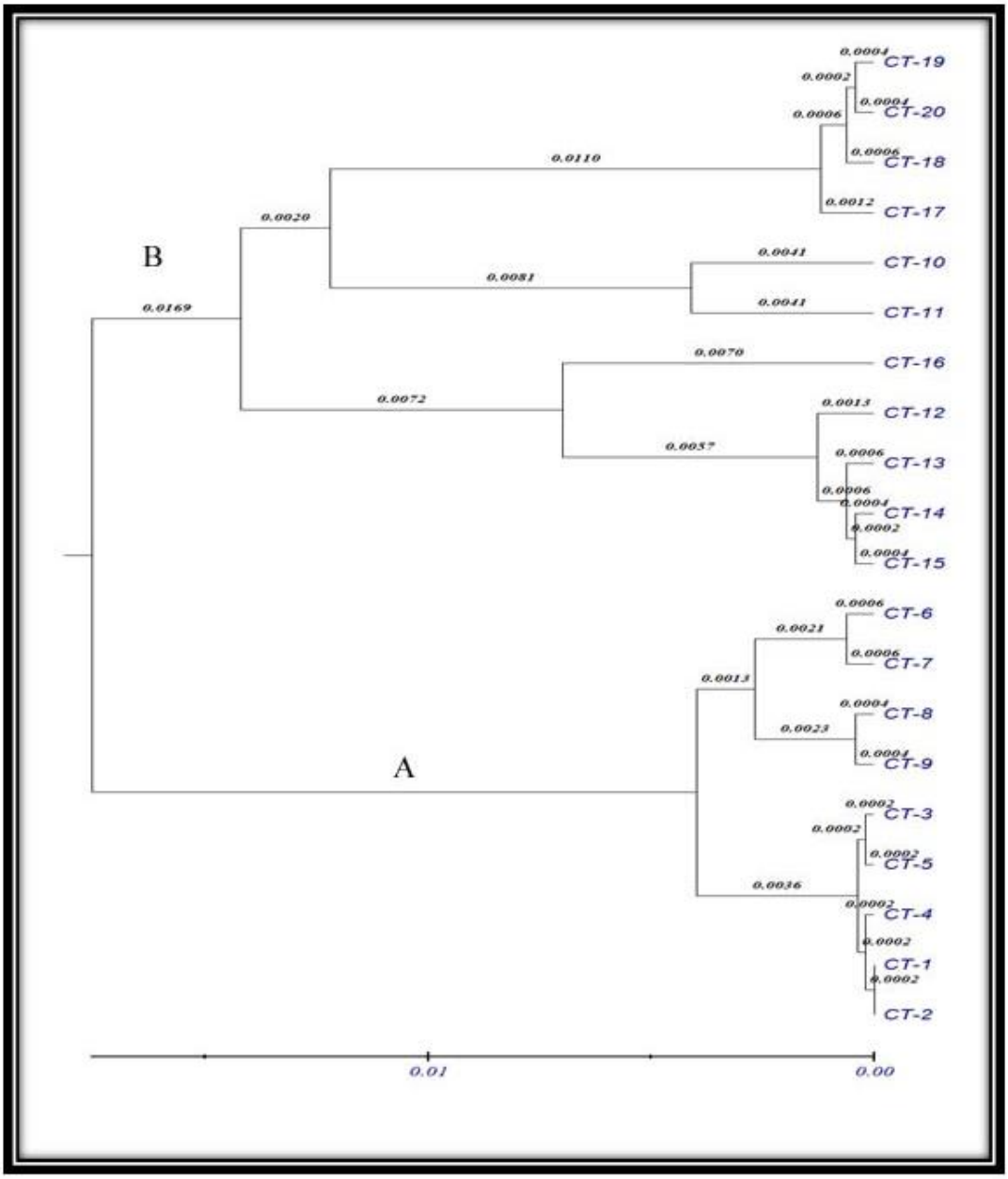

Fig. (3-13) Phylogenetic analysis based on *omp*1 gene sequences of 20 *Chlamydia trachomatis* isolates using UPGMA (unweighted pair group method with arithmetic mean) method.

The A cluster divided in to two sub cluster and contain 4 group and these groups contain 9 isolates and only two isolates (CT2-CT1) showed 100% identical or similarity while other isolates characterized by small genetic distance with each Cluster. The cluster B contain two sub cluster

which 4 group and these groups contain 11 isolates which have small genetic distance as show in **figure (3-13)**.

Some of nucleotide substitutions in amino acid replacements and could thus potentially alter the function and antigenicity of the Momp.

Sequencing of the *omp*A gene provide discrimination of *C. trachomatis* strain and provided additional information when applied to contact tracing (lysen *et al.,* 2004).

Examination of the *omp*A gene of *C. trachomatis* and its surrounding loci demonstrate an increase in nucleotide substitutions and differing phylogenetic histories (Brunella *et al.,* 2012).

Mira and Ochman, (2002) referred that is it not known what's mechanisms promote the mutational increase in *omp*A and its neighboring loci relative to the rest of genome.

The major outer membrane protein of Chlamydia, which encoded gene (*ompA*) contains four symmetrically spaced variable domains (VDs-IV), maintains the structural rigidity of the outer membrane and facilities porin formation permit diffusion of solutes through the intracellular reticulate body membrane (Shamkhi *et al.,* 2022).

Sequencing data analysis show variability in *ompA* sequence as shown in **table (3-7)**.

It was found that the most substitution mutation displayed C > T in 18.9612, then G > A in 17.3018 follow by A > G in 14.9856 and T > C in 13.5256.

Table (3-7): Sequencing data analysis in *ompA* sequence**.**

From\To	**A**	**T**	**C**	**G**
A	-	5.0124	3.5755	14.9859
T	4.8360	-	13.5256	4.1887
C	4.8360	18.9612	-	4.1887
G	17.3018	5.0124	3.5755	-

It was suggesting that the genetic variability observed in this study and the most sequence variation was observed in single nucleotide variation or two nucleotide substitution.

Jurstrand *et al.,* (2001) found it has been speculated that the *omp 1* genovariant occur as a result of point mutation and recombination events selected by immune pressure, however several of the nucleotide substitution that detected were synonymous, which suggest that they were evolutionarily neutral.

Rawre *et al.,* (2018) found that the sequencing of 22 *C. trachomatis ompA* performed and mutation were observed and the occurrence of point mutation and nucleotide variation was observed, however two to three nucleotide substitutions were also observed.

The genomic mutation observed with the *omp* sequence genotypes in *C. trachomatis* may be natural consequence of the microbial evolution combined with the high diagnostic selective pressure on *C. trachomatis*.

Brasiliense *et al.,* (2016) showed that the phylogenetic analysis of *ompA* sequence produced a tree that grouped the samples in to five clades and the similarity values ranging from 99.7 to 100%, nine nucleotide substitution were observed, eight which resulted in amino acid replacement.

3.7. Genotyping of *Chlamydia trachomatis* by in silico PCR- RFLP:

The nucleotide sequence analysis of the *omp*-1gene of *Chlamydia trachomatis* revealed a high diversity in the number and nucleotides sequence of this gene. It is possible to apply the nucleotide sequence of *omp-1* gene as an epidemiological marker.

This result of in silico PCR-RFLP using AluI enzymes and simulation separating on gel electrophoresis demonstrated that the enzyme was able to generate several DNA banding patterns as show in **figure (3-14).**

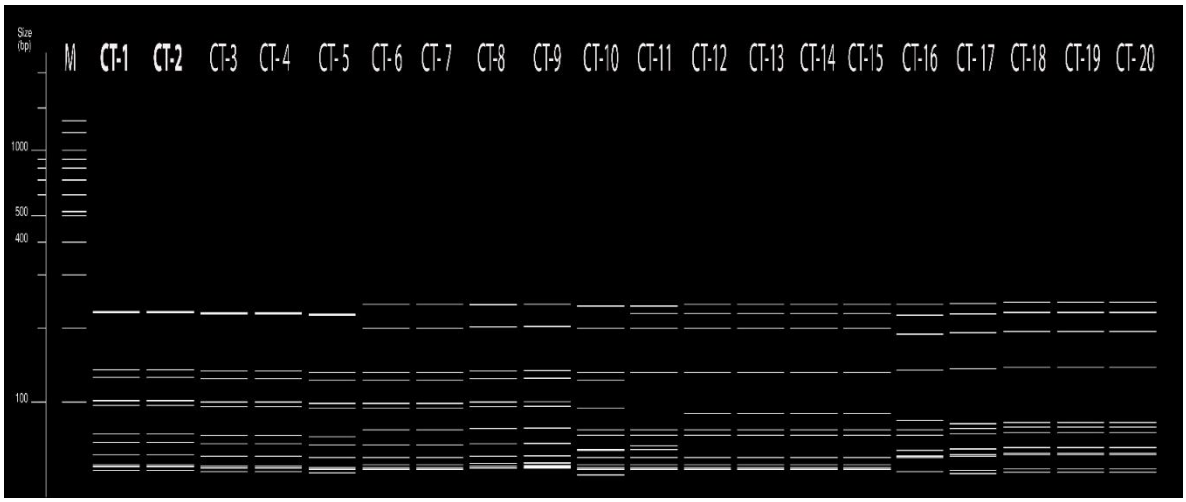

Fig. (3-14): *Insilco* RFLP-gel PCR products of 20 *Chlamydia trachomatis* isolates according to *omp1* gene.

These different patterns correspond to *C. trachomatis* isolated from different sources. While the isolates showed closely related DNA banding patterns indicted that probably originated from the same source as shown in table (3-8).

Table (3-8): Distribution of Bands that generated by RFLP method for all studied isolates.

N	Size bp	ct1	ct2	ct3	ct4	ct5	ct6	ct7	ct8	ct9	ct10	ct11	ct12	ct13	ct14	ct15	ct16	ct17	ct18	ct19	ct20
1.	244	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-
2.	243	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
3.	241	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-
4.	228	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
5.	227	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6.	225	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
7.	204	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
8.	195	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
9.	145	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
10.	142	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
11.	132	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
12.	102	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
13.	96	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
14.	89	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-
15.	80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
16.	74	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
17.	68	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18.	61	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
19.	59	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20.	48	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
21.	47	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
22.	42	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
23.	41	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+
24.	33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
25.	31	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
26.	21	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
27.	17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
28.	15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
29.	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
30.	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
31.	9	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32.	6	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+

According to the phylogenic analysis based on the PCR-RFLP results showed that all isolates grouped in to two cluster A and B as shown in figure (3-15).

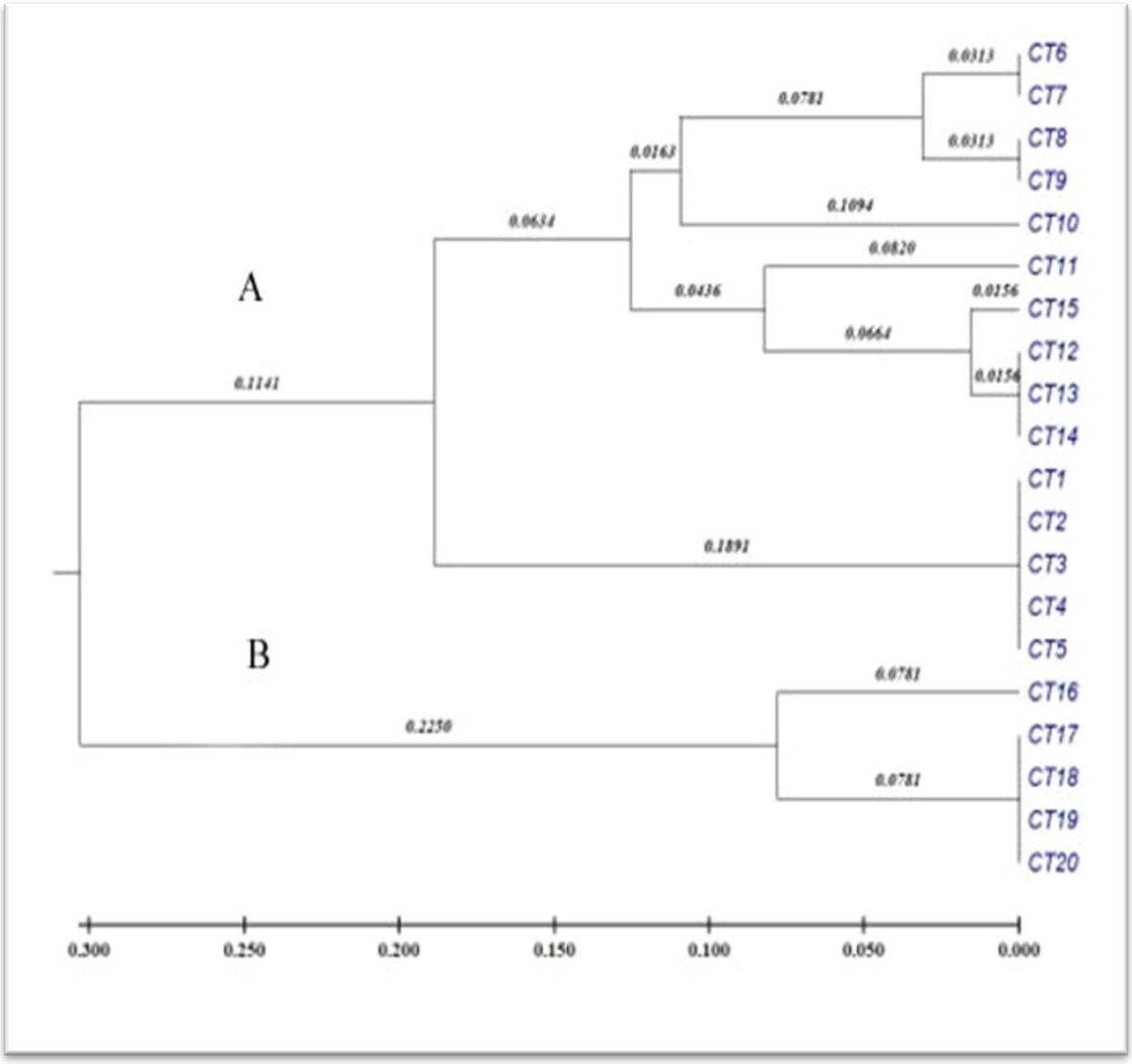

Fig. (3-15): Phylogenetic analysis based on RFLP-generating bands of 20 *Chlamydia trachomatis* isolates using UPGMA (unweighted pair group method with arithmetic mean) method targeting *omp1* gene.

The cluster (A) contained two subcluster and contained (15) isolates in which number (1,2,3,4 and 5) were identical 100% and occurred in same subcluster, also isolates number (6 and 7) gave similarity 100% and isolates number (8 and 9) identical 100% and isolates (12, 13, 14) gave the same similarity as shown in figure (3-15).

While the subcluster (B) contain (5) isolates which isolates (17, 18, 19, and 20) identical 100% on the bases of PCR- RFLP similarity of omp-1 gene phylogenetic analysis was performed and found the evolutionary relationship among (20) *C. trachomatis* isolates the genetic variability observed in this study within the genotypes in clinical isolates may be due to the occurrence of point mutation.

PCR- based RFLP analysis or sequencing of the amplified ompA gene are considered to be more sensitive and more specific than serotyping, also these methods may be important for a thorough understanding of the pathogenesis and epidemiology of genital Chlamydial infection (De Haro-cruz *et al.*, 2011).

According to this study (9) genotype was found from (20) clinical isolates which due to high similarity occur between the isolates this may be that all isolates detect from the same site of infection.

Ramnarain *et al.*, (2023) found that from 42 samples five different serovars were detected with the ALUI enzyme, and the PCR- RFLP technique based on *omp*-1 gene amplification provide a sensitive and reliable method for typing *C. trachomatis* isolates.

Other study included 128 *C. trachomatis*-positive women. DNA was extracted from cervical swabs. *omp*1 gene PCR-RFLP and sequencing were used to confirm the subtypes of *C. trachomatis*, 4 different serovars were detected with the Alu I enzyme (Chen *et al.*, 2017).

PCR-RFLP analysis is a rapid, simple, and powerful tool for differentiating serovars of *C. trachomatis*, this study revealed that 7 serotypes were identify after digested with *Alu* I enzyme (Seo *et al.*, 2000).

Ngandjio *et al.*, (2004) found that from eighteen reference strains of *Chlamydia trachomatis* only 6 different serovars were differentiated by *omp1* PCR- and nested PCR-based RFLP analysis, using restriction digestion *Alu* I enzyme.

Other study search about sequence analysis using BLAST similarity search of the *omp*A gene, from the 106 clinical isolates of *Chlamydia trachomatis* revealed that there only 9 genotypes (Chung et al. 2020).

Conclusions

&

Recommendations

Conclusions:

1- Technological evolution in clinical laboratory diagnostics has advanced considerably by allowing for the direct molecular detection of pathogen in a clinical specimen rather than relying on isolation and cultivation.

2- The importance of uses of a sensitive and specific method for the identification of *C. trachomatis* in women by using house-keeping gene *(omp*) outer membrane protein.

3- The presence of Momp genes in *C. trachomatis* examined in present study suggests, that these genes essential to the survival of these bacteria and linked with chlamydial genital tract infection.

4- Disease outcomes are dependent upon the complex interactions between virulence factors and evasion strategies used by *Ch. trachomatis* and host immune response including variance in genetic markers associated with infection and manifestations.

5- Several potential biomarkers such as microRNA 142 and 520a may be associated with susceptibility to *C. trachomatis* and severity and progressive of diseases caused by *C. trachomatis*.

6- Genotyping of *C. trachomatis* was important to study the pathogenesis and epidemiology of genital chlamydia infection.

Recommendations:

1- The interaction of *C. trachomatis* co-infections with other sexually transmitted pathogens should be understand which may affect on prevalence of sexually transmitted microbes in the management of infections.
2- Further studies are needed to assess changes in miRNA expression following treatment and to determine whether biomarker development is useful or feasible.
3- more research is needed to look for more point mutations in the genome which will enable us to explain why these genovariants different in severity of disease.
4- Women remain asymptomatic after *C. trachomatis* infection, so routine chlamydial screening of young women should be recommended to prevent consequences of untreated chlamydial infections.
5- Typing of *C. trachomatis* remains important in epidemiological surveillance, revealing transmission networks and distinguishing reinfection from treatment.
6- Uses other molecular method for genotyping of *Chlamydia trachomatis* like whole gene sequence and pulsed field gel electrophoresis (PFGE).

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Appendix

Appendix (1): A questionnaire including information and risk factors needed to select and diagnose patients with pelvic inflammatory disease.

1. Number: Date of sampling:

2. Name:

3. Age:

4. Parity: G P A

5. Pregnant: No Yes Gestational age:

6. Symptoms: pelvic Pain Discharge Bleeding fever

7. History of PID:

8. History of contraception:

9. History of infertility:

10. Medical history:

11. Drug history:

الخلاصة:

كانت *Chlamydia trachomatis* تنتمي إلى عائلة Chlamydiaceae، وهي البكتيريا المنقولة جنسيا الأكثر شيوعا والتي تؤدي إلى التهابات الجهاز التناسلي البشري. تصيب *Chlamydia trachomatis* الخلايا الظهارية العمودية وتسبب عدوى باطن عنق الرحم، والتي تكون بدون أعراض بشكل أساسي.

كان مرض التهاب الحوض سببا رئيسيا لكل من العقم البوقي والحمل خارج الرحم. *Chlamydia trachomatis* هي عامل خطر مهم لمرض التهاب الحوض.

تضمن هذا البحث (دراسة حالة - سيطرة) دراسة وتحليل *Chlamydia trachomatis* وراثيا في المرضى الذين يعانون من مرض التهاب الحوض، والذي تضمن 200 عينة مسحة باطن عنق الرحم تم جمعها من مريضات يعانين من علامات و / أو أعراض مرض التهاب الحوض؛ تم تشخيصهم على أنهم مصابون بمرض التهاب الحوض من قبل طبيب أمراض النساء وكان لديهم عوامل خطر لهذه العدوى. بالإضافة إلى ذلك، تم استخدام 25 عينة مسحة باطن عنق الرحم من الإناث الاصحاء تستخدم كمجموعة سيطرة، والتي استخدمت في الجزء الثاني من هذا العمل. المرضى الذين تم نقلهم إلى العيادات الخارجية لأمراض النساء والتوليد، في مستشفيين في محافظة بابل: مستشفى بابل للولادة والأطفال، ومستشفى الهاشمية التعليمي العام. وكذلك العينات المأخوذة من العيادات الخاصة، خلال الفترة من مارس إلى أغسطس 2022.

تم إخضاع جميع العينات البالغ عددها 200 عينة للتشخيص المناعي لداء *Chlamydia trachomatis* *وأعطت 200/30 (15٪) فقط نتائج إيجابية* لداء *Chlamydia trachomatis* *مع اختبار مصلي شريطي سريع.*

خضعت العينات لاستخراج الحمض النووي. تم استخدام الحمض النووي المستخرج من المسحات للكشف الجزيئي عن *Chlamydia trachomatis*، بين مرضى مرض التهاب الحوض، باستخدام جينات *omp* التدبير المنزلي (144bp) كعلامات جينية عبر تفاعل البوليميراز المتسلسل التقليدي؛ حيث أعطت نتائج تقريبية 200/22 (11٪).

من بين جميع عينات مسحة باطن عنق الرحم التي تم جمعها والبالغ عددها 200 عينة، وجدت هذه الدراسة أن 22/8 (36٪) فقط كانوا يعانون من نزيف بين الوقت مع أعلى نسبة وفقا للمجموعة الأخرى، وكان 22/5 (23٪) يعانون من العقم الأولي والعقم الثانوي، و22/4 (18٪) تعرضوا للإجهاض المتكرر.

فقط 200/100 (50٪) من النساء اللواتي تم تشخيصهن من قبل أطباء أمراض النساء على أنهن مصابات بمرض التهاب الحوض مع الفئة العمرية (20-30) وجدن أن 22/11 (50٪) كن إيجابيات *Chlamydia trachomatis*. 11/5 (45.4٪) كانت إيجابية لل *Chlamydia* التي كانت تنزف بين الوقت، في حين أن 22/2 (18.1٪) كانت إيجابية لل *Chlamydia* التي تعاني من العقم الأولي والعقم الثانوي والإجهاض المتكرر. بينما وجدت 200/100 (50٪) من النساء ذوات الفئة العمرية (30-40 سنة) أن 22/11 (50٪) كانت إيجابية لل *Chlamydia trachomatis*. لوحظ أن العقم الأولي والعقم الثانوي والنزيف بين الوقت كان له نفس المعدل 11/3 (27.2٪) والذي كان له معدل مرتفع في هذا النطاق من العمر، في حين أن الإجهاض المتكرر كان 11/2 (18.1٪).

تم استخلاص (22) عينة مسحة باطن عنق الرحم للمريض والتي تعطي نتائج إيجابية لداء *Chlamydia trachomatis* واستخدامها لدراسة التعبير الجيني للحمض النووي الريبي الميكروي باستخدام طريقة تفاعل البلمرة المتسلسل (CtΔΔ). مستوى التعبير عن جينات microRNA - 142 و520-a في عينة الاختبار وكذلك في عينات السيطرة مع الجينات التأسيسية. تظهر الدراسة أن التعبير عن الجين micro-142 و520-a كان مرتفعا في مرضى *Chlamydia trachomatis* عند مقارنته بالمجموعة الضابطة، مع زيادة التعبير عن الجين بأكثر من 40٪ و80٪ على التوالي عند مقارنته بالمجموعة الضابطة.

أيضا، تم تضخيم 20 عزلة مختبرة فقط *من Chlamydia trachomatis* بنجاح كعلامات جينية عبر تفاعل البوليميراز المتسلسل التقليدي *بحجم1134pb حيث أظهر تحليل تسلسل* جين omp1 من العزلات السريرية لل *Chlamydia trachomatis* والتحليل التطوري لجين *omp1* أنه تم *فصل Chlamydia trachomatis* إلى مجموعتين رئيسيتين (أ وب).

انقسمت العنقود (أ) إلى مجموعتين فرعيتين واحتوت على 4 واحتوت هذه المجموعات على 9 عزلات وعزلتين فقط (CT2-CT1) أظهرت تطابقا أو تشابها بنسبة 100٪ بينما تميزت العوازل الأخرى بمسافة وراثية صغيرة مع كل مجموعة. بينما تحتوي المجموعة (ب) على مجموعتين فرعيتين هما 4 مجموعات وتحتوي هذه المجموعات على 11 عزلة لها مسافة وراثية صغيرة أظهر تحليل بيانات التسلسل تباينا في *تسلسل ompA*

وجد أن الطفرة الأكثر استبدالا أظهرت C > T في 18.9612، ثم G > A في 17.3018 تليها A > G في 14.9856 وT > C في 13.5256.

اظهرت النتائج في السيليكو لتفاعل البلمرة مع الرفلب لجين omp1 باستخدام إنزيمات Alu1 أنه تم العثور على 9 أنماط وراثية من 20 عزلة سريرية ووفقا لتحليل النشوء والتطور، أظهرت النتائج أن جميع العزلات مجمعة في مجموعتين. احتوت المجموعة (أ) على مجموعتين فرعيتين واحتوت على (15) عزلة. بينما احتوت المجموعة (ب) على (5) عزلات.

وفقا للبيانات الواردة في هذا البحث لاحظت الدراسة ان العديد من المؤشرات الحيوية مثل 142microRNA و520-أ قد تترافق مع القابلية للإصابة بال*Chlamydia trachomatis* وشدة الامراض التي تسببها *Chlamydia trachomatis* وتقدمها. كان التنميط الجيني لداء *Chlamydia trachomatis* مهما لدراسة التسبب في عدوى ال *Chlamydia* التناسلية ووبئتيها.

وزارة التعليم العالي
والبحث العلمي
جامعة بابل
كلية الطب

ملامح التعبير عن ال micro RNA في باطن عنق الرحم والتنميط الجيني لل *Chlamydia trachomatis* في النساء المصابات.

أطروحة مقدمة إلى

مجلس كلية الطب / جامعة بابل

وهي جزء من متطلبات نيل درجة دكتوراه فلسفه في

العلوم / الأحياء المجهرية الطبية

من قبل

علياء زرع الله حسين عبود

بكالوريوس تحليلات مرضية
كلية التقنيات الصحية والطبية/ بغداد (2007)
ماجستير أحياء مجهرية طبية
كلية الطب / جامعة بابل (2014)

تحت إشراف

الاستاذ الدكتور
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