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Molecular and Microbiological Study of *Ureaplasma parvum* in Women with Recurrent Miscarriage

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وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا
أُوتِيتُم مِّنَ الْعِلْمِ إِلاَّ قَلِيلاً

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Dedication

To those who support me all the time…..,
My family……

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***Summary* :**

A total of 130 specimens were collected from married woman with recurrent miscarriage include (72 vaginal bleeding, 41vaginal swab and 17 urine) and 40 specimen were collected from control include (25urine and 15vaginal swab) who admitted to Feminine & children teaching hospital and Gynecology clinic in Al-Muthanna province, through a period (from October 2015 to February 2016) in an attempt to isolate *Ureaplasma parvum* .

Through the study two types of media were used to accelerate the growth of genital *Ureaplasma*. These were referred to as (IH broth medium) and (IH agar medium). These efficient to enhance the growth of genital *Ureaplasma* spp. Among the total 118 / 130 (90.7%) positive culture was obtained from married women with recurrent miscarriage versus 9/40 (22.5%) positive culture from control.

The results revealed that *Ureaplasma spp.* were isolated on IH medium in a rate of (52.3%) from vaginal bleeding of miscarried women, (30.7%) from vaginal swab, (7.6%) from urine. Also vaginal bleeding gave high percentage of isolation (52.3%) when compared with vaginal swab and urine.

The results revealed the frequency of infection by *Ureaplasma spp.* was significantly associated with age , since the age groups (17-36y)(46 %) & (27-36y)(40.7%) showed the highest infection rate, followed by age group (37-46)(13.3%) revealed the lower infection rate.

Conventional PCR identification of *Ureaplasma parvum* was done. Primer UMS-57/UMA222 targeting gene 5' Ends of MBA genes are used for the amplification 326bp. The results revealed the *Ureaplasma parvum* was identified in (29.6%) but in control group *Ureaplasma parvum* was isolated in rate (11.1%).

Ureaplasma parvum isolates were further subtyped into different serovars. (1, 3, 6, 14) based on MBA genes by using Gradient PCR technique. The results revealed the serovar3 most frequent isolate detected in present study in rate (42.8%), while serovar1 (28.5%), serovar6 (14.2%), serovar14 (14.2%) in patient group but in control group only serovar 1 was isolated in rate (11%).

Antibiotic susceptibility was studied, broth dilution method was used for the susceptibility testing of serovar isolates *U.parvum* against eight antibiotics. The results revealed the SV3 isolates fully resistance (100%) to GEN and AZM, and ERY while susceptible to DOX in rate (80%), (60%) to LVX, (60%) to CLR but SV14 isolate revealed fully susceptible (100%) to CLR, CIP, DOX. And fully resistance (100%) to GEN and AZM. While SV1,SV6 showed fully resistance (100%) to AZM , GEN. And SV1 susceptible to DOX in rate (70%),TET (60%), CIP (90), LVX (70), ERY (70), CLR (70%), but SV6 susceptible to DOX in rate (80%),TET (100%), CIP (100), LVX (80), ERY (80), CLR (80%).

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List of Abbreviations

Abbreviation	Meaning
ACL	anticardiolipin
AF	Amniotic Fluid
AMC	Augmentin
AMX	Amoxicillin
APH	Antepartum haemorrhage
ASCUS	Atypical squamous cells of undetermined significance
ATCC	American Type Culture Collection
AZM	Azithromycin
bp	Base pair
BV	bacterial vaginosis
CIP	Ciprofloxacin
CLR	Clarithromycin
CRO	Ceftriaxone
D.W	Distilled Water
DNA	Doxyribo Nucleic Acid
DOX	Doxycycline
EDTA	Ethylene Diamine Tetra Acetic Acid
ERY	Erythromycin
FLC	Fluconazole
g	gram
GEN	Gentamicin
GLOB	Global protein
GSLs	glycosphingolipids
HSIL	High-grade squamous intraepithelial lesion
IgA	Immunoglobulin A
IL	Interleukin
IL-1β	Interleukin-1beta
IUI	intrauterine insemination
LSIL	Low-grade squamous intraepithelial lesion

LVX	Levofloxacin
MBA	Multiple Banded Antigen
Mbp	Mega base pairs
MG	*Mycoplasma genitalium*
MH	*Mycoplasma hominis*
MIC	Minim inhibitory concentration
min	minute
NF-kB	Nuclear factor kappa – light - chain enhancer of activated B cells
NGU	non-gonococcal urethritis
NYT	Nystatine
PAMPs	pathogen-associated molecular patterns
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PPLO	Pleuro pneumonia Like organism
PPROM	Preterm Premature Rupture of Membrane
PTB	Preterm birth
rps	ribosomal proteins
Spp.	Species
STD	sexually transmissible diseases
SV	Serovar
TBE	Tris-Boric acid - EDTA
TET	Tetracycline
TLR	Toll – like receptor
TLRs	Toll-like receptors
TNF	Tumor Necrosis Factor
ul	Microliter
UP	*Ureaplasma Parvum*
USP	*Ureaplasma Species*
UU	*Ureaplasma Urealyticum*
IH medium	Ibtisam Habeeb medium

Chapter One

Introduction and literatures review

1.1. **Introduction**

Recurrent miscarriage is the loss of three or more consecutive pregnancies ending of pregnancy by removing a fetus or embryo before it can survive outside the uterus (Grimes,2010; Clark, *et al*, 2010). An miscarriage which occurs spontaneously and World Health Organization (WHO) explained the around 56 million recurrent miscarriage before the 24th week of gestation occur each year in the world unexplained. (Capoccia, *et al*, 2013; Redelinghuys, *et al*, 2015).

Genital tract infections associated with approximately 50% of preterm labor and miscarriage (Kaandorp, *et al*, 2010). Most of pregnancies result in low infant birth weight and increase risk for miscarriage (14 weeks) also 60% of mortality among infants (with no anatomic/chromosomal defects) is low birth weight (Dhawan & Kokkayil 2015). Any sever infection that leads to bacteraemia or viraemia can cause Sporadic miscarriage. The role of infection in recurrent miscarriage is unclear, the presence of bacterial vaginosis in the first trimester of pregnancy has been reported as a risk factor for second-trimester miscarriage and preterm delivery, (Leitich,2007; Zhang , *et al*, 2014).

Ureaplasma parvum could be important pathogen that may affect pregnancy outcomes and the health of neonates was first given serious consideration when reported of postpartum endometritis with septicemia, chorioamnionitis (Waites, 2015). Since those days, numerous clinical studies have been performed in an attempt to clarify what roles, if any, these organisms play as agents responsible for invasive infections in neonates, premature labor, spontaneous miscarriage, stillbirth, and chronic lung disease of prematurity (Zhang , *et al*, 2014; Huang, *et al*, 2015).

Although more than 30 years of study inside and outside of Iraq, many clinical importance of genital *Ureaplasma parvum* are still incompletely understood for a variety of reasons. These include (1) the high prevalence of these organisms in healthy persons; (2) poor design of many of the earlier research studies, which attempted to relate the more presence of *Ureaplasma parvum* in the lower

urogenital tract to pathology in the upper tract or in offspring; (3) unfamiliarity of clinicians and microbiologists with the complex and fastidious nutritional requirements for *Ureaplasma parvum* and the methods detection (Wetmore *et al*, 2011; Huang *et al* 2015).

Ureaplasma parvum found in the placenta and endometrium is associated with infection, the birth of a dead fetus, spontaneous miscarriage , premature delivery and lower than normal weight of infant. *Ureaplasma parvum* penetrate into amnion in the second trimester it may cause chorioamnionitis (Capoccia *et al*, 2013). *Ureaplasma parvum* was found in the blood of mothers who have had problems with high fever after childbirth this infection can be transmitted to about 40% of babies who were born to a mother with this infection if the mother has it, *Ureaplasma parvum* can infect the lungs of the newborn during childbirth. (Redelinghuys *et al*, 2015)

Ureaplasma parvum are the most prevalent, possibly pathogenic bacteria isolated from the urogenital tract of both men and women, they are also frequently associated with preterm birth and other adverse pregnancy outcomes. (Dhawan, & Kokkayil,2015). Genital *U.parvum* (biovar 2, serotypes 1, 3, 6 and 14) are considered natural inhabitants of the lower urogenital tract of humans as they are often isolated from healthy individuals and involved in a variety of infections in humans the isolation of *U.parvum* from patients with genitourinary tract infections. (Kasprzykowska, *et al* ,2013). Waites, (2015) Found *U.parvum* to be dominant in patients with pelvic inflammatory disease as well as in women who had miscarriages, and it seemed to have more adverse effects on pregnancy outcome regarding birth weight, gestational age, and preterm delivery than *U.urealyticum* and shown that *U.parvum* can be isolated more frequently from patients with a history of recurrent miscarriages than from normal pregnant women .

Ureaplasma parvum are the microorganisms most frequently isolated from amniotic fluid (AF) or placentae in women who deliver preterm between 23 and 32 weeks pregnancy and *U. parvum* has been linked with adverse pregnancy outcomes such as late miscarriage and early preterm birth (Larsen & Hwang ,2010). Also identified *U.parvum* in 57 % of healthy non pregnant women and the organism was far more prevalent than any of the other genital mycoplasma, Chlamydia spp. or viruses. (Kacerovsky, *et al*, 2009). *Ureaplasma parvum* infections require the therapeutic use of antimicrobials. Tetracyclines , macrolides and quinolones are the major antibiotics used in the treatment of genital *Ureaplasma*. However, their therapeutic efficacy may be unpredictable due to increasing resistance. (Dhawan , *et al* , 2012) .

In the light of above, this study included more than one goal :

Investigate the occurrence of *Ureaplasma parvum* in women with recurrent miscarriage in AL-Muthanna. And study the antimicrobial susceptibilities of *Ureaplasma parvum* isolates to determine the most suitable antibiotic for treating these infection.

1.2. Literature Review

Ureaplasma are a group of bacteria mainly characterized by cell wall deficiency, fastidious and mostly be referred to as non-cultivable organisms. Frequent colonization of female genitourinary tract by *Ureaplasma*. Detection of *Ureaplasma* is possible by characteristic growth on appropriate media and urease activity, but species identification of *U.urealyticum* and *U. parvum* must be demonstrated by molecular methods. Differentiation between *U.parvum* and *U.urealyticum* is very important, especially for correct interpretation of laboratory results and evaluation of pathogenicity. (Dhawan, & Kokkayil, 2015). The primary sites of *Ureaplasma* infections are mucosal surfaces, and in humans inflammations is typically seen in the respiratory or urogenital tract. Urogenital *Ureaplasma* of humans is typically associated with *Ureaplasma urealyticum* (UU), *Ureaplasma parvum* (UP), or a complex infection of these species. (Brown, *et al*, 2010).

The clinical states associated with *Ureaplasma* spp. appear to be strain include non-gonococcal urethritis (NGU) (*Ureaplasma parvum*), bacterial vaginosis , spontaneous abortion, or preterm labor (*Ureaplasma parvum*) . In contrast, the causal role of (*Ureaplasma parvum*), pelvic inflammatory disease, spontaneous miscarriage, and infertility is generally accepted, and as such it is now considered an emerging urogenital pathogen (McGowin & Smits, 2011). Infection of pregnant women with *Ureaplasma parvum* has been associated with early rupture of membranes, preterm labor, and the perinatal infants can develop *Ureaplasma parvum* infections of the respiratory tract or central nervous system (Larsen & Hwang, 2010).

1.2.1. Historical Background of *Ureaplasma*

1.2.1.1. Taxonomy and Phylogeny

Ureaplasma belongs to the family *Mycoplasmataceae,* class *Mollicutes* order *Mycoplasmatales.* The genus *Ureaplasma* contains seven host-specific species (*U.parvum , U.urealyticum , U.cati , U.canigenitalium U.diversum , U.felinum , U.gallorale*) (Dando, 2012). The *Ureaplasma* spp. which infect human hosts (*U.parvum , U.urealyticum*) are closely related to *mycoplasma spp.* (Brown, 2010). They were deemed unique among the *Mycoplasma* of human origin in that they metabolized urea and not arginine or glucose. It was thus proposed that there should be a new genus and species designation for these unique organisms within the order *Mycoplasmatales* (Dhawan, & Kokkayil , 2015). *Ureaplasma* belong to the normal commensal flora of the genital tract of human beings, with colonization rates between 60 and 80 percent worldwide (Bayraktar, *et al*, 2010). However, *Ureaplasma* are also implicated in invasive diseases such as urethritis, postpartum endometritis, chorioamnionitis, spontaneous miscarriage and premature birth, as well as low birth weight, pneumonia, bacteremia, meningitis, and chronic lung disease in prematurely born infants (Dhawan, *et al*, 2012).

The frequency with which *Ureaplasma* occur in healthy asymptomatic individuals, it has been suggested that only certain subgroups of the species are pathogenic. The majority of human *Ureaplasma* isolates belong to *Ureaplasma parvum* (biovar2). *Ureaplasma urealyticum* (biovar 1) is isolated less often . We have also reported *U. parvum* (biovar 2) as the predominant biovar in patients with genital tract infections. *Ureaplasma* consists of 14 serovars. *U. parvum* includes serotypes 1, 3, 6 and 14, whereas *U. urealyticum* comprises the remaining 10 serotypes. Some *Ureaplasma* serovars have been found to be more associated with clinical diseases; however, the data are limited because of the difficult diagnostic with traditional genotyping methods (Dhawan, *et al*, 2012).

Ureaplasma Parvum there is emerging evidence that it may play a role in infections of pregnancy or in eliciting conditions associated with prematurity. A recent study indicated that there is a dose related intra-amniotic inflammatory response to *Ureaplasma parvum* and that this is related not only to pPROM, preterm labor, and chorioamnionitis, but also to early onset sepsis in the baby and bronchopulmonary dysplasia. (Larsen & Hwang, 2010). Kataoka's study from 2006 indicated a high prevalence of *Ureaplasma parvum* and a statistical association with late miscarriage and early preterm birth. A modern innovation in microbiology is using the genetic material rather than phenotypic information as a point from which to understand the organism and compared genomes of *Ureaplasma Parvum* identified 280 coding sequences unique to that organism. (Hilton, *et al* , 2010).

A recent study found that *Ureaplasma parvum* was identified in 57% of healthy non pregnant women which was far more prevalent than any of the other genital mycoplasma, a host of viruses or Chlamydia, Trichomonas, or Group B Streptococcus and also *Ureaplasma parvum* was identified in 40% of women post renal transplant genital mycoplasma were more prevalent than in non transplant women 27.5% with *Ureaplasma Parvum* showing a strong dominance (Larsen & Hwang , 2010). A group in Poland recently reported on the prevalence of *Ureaplasma parvum* in women in relation to cervical pathology and found that among 14 women with squamous intraepithelial lesions compared to 39 healthy controls, mycoplasma were found in 34% of states with *U. parvum* predominating (Ekiel, *et al*, 2009).

1.2.1.2.Structural Composition of *Ureaplasma*

Ureaplasma have evolved from Gram-positive bacteria by progressive evolution to lose their peptidoglycan cell wall, they are spherical or coccobacillary-shaped bacteria with diameters between 0.2-0.3μm. The lack of a cell wall renders these organisms insensitive to beta-lactams. This also prevents them from staining by Gram stain and is responsible for their pleomorphic form. they require enriched growth medium with serum supplementation for their growing *in vitro*. *Ureaplasma* has a generation time of approximately 1hour. (Dhawan, & Kokkayil , 2015). *Ureaplasma* spp. may appear as coccobacillary forms in exponential growth phase; filaments are rare. Non motile. Facultative anaerobes. Form exceptionally small colonies on solid media that are "cauliflower head" colonies having a lobed periphery . Unusual pH required for growth (about 6.0–6.5). Optimal incubation temperature for examined species is 35–37°C. Chemo-organotrophic. Like *Mycoplasma*, species of *Ureaplasma* lack oxygen-dependent, NADH oxidase activity. Unlike *Mycoplasma*, species of *Ureaplasma* lack hexokinase or arginine deiminase activities but have a unique and obligate requirement for urea and produce potent ureases that hydrolyze urea to CO_2 and NH_3 for energy generation and growth. (Robertson & Robinson, 2015). *Ureaplasma* was first discovered in1954 by (Shepard , *et al.*, 1954) as a pathogen causing non-gonococcal urethritis in men. Since the organisms produced small colonies (7-15μm diameter), they were originally called T (tiny) strains, T-strain Mycoplasma or T-Mycoplasma. They were deemed unique among the Mycoplasma of human origin in that they metabolized urea and not arginine or glucose. (Dhawan & Kokkayil , 2015). They have no genes involved in amino acid biosynthesis and only a few genes involved in the biosynthesis of cofactors as vitamins. *Ureaplasma* can not synthesize any fatty acids and some even incorporate exogenous phospholipids together with cholesterol in their cell membrane. Their mode of replication is not different from that of prokaryotes dividing by binary fission. *Ureaplasma* are known to consist of just plasma

membrane which makes them good models for membrane studies and due to this reason the availability of these membranes in pure state have enabled in their chemical, enzymatic and antigenic characterization. The membrane mostly consists of 60% to 70% of proteins and the rest 20% to 30% of lipid (Razin & Hayflick 2010).

1.2.2. Genomic Structure of *Ureaplasma parvum*

Ureaplasma parvum has a circular chromosome consisting of 751base pairs. It's chromosome encodes 605 Open Reading Frames and 38 RNA genes. The G+C content is 25% which is relatively low compared to other species. Having a reduced genome it has a fast evolutionary rate. It does not have the LexA repressor which suppresses the SOS response genes. The SOS response genes are used in DNA repair. Its fast evolutionary rate may account for the lack of LexA genes and mutant genes. The tuf DNA sequence is the same within serovars of *Ureaplasma parvum*. It was used to differentiate within the species and at times reflects a better phenotypic relationship than 16S rRNA gene sequencing does. (Kong & Gilbert , 2004). The genome of *Ureaplasma parvum* serovars is between 0.75-0.78 Mbp. The genome of *U. parvum* serovars have on an average 608 genes, of which 201 encode hypothetical proteins. The *Ureaplasma* pan genome contains 1020 protein coding genes of which 515 genes are universally conserved among all serovars performed a whole genome comparison on the nucleotide level using American Type Culture Collection (ATCC) strains of all the 14 serovars. (Paralanov *et al* 2012).

Ureaplasma has several genes coding for surface proteins and lipoproteins. The gene encoding the Multiple Banded Antigen (MBA) has been frequently studied. The 5′ region of the MBA encodes a conserved N-terminal anchor of the lipoprotein whereas the 3′ region of the MBA encodes the C-terminal domain, consisting of multiple tandem repeat units, which are surface-exposed. The C-terminal domain is antigenic and elicits an antibody host response during

Ureaplasma infection. Additions or deletions in the number of repeat units in the downstream region of the MBA are associated with antigenic variation. Furthermore, Multiple banded antigen can phase vary with neighbouring genes, and *Ureaplasma parvum* serovar 3 was recently shown to produce chimeric genes through phase variation. (Paralanov *et al,* 2012*) Ureaplasma* contains one or more integrase recombinase genes. Some serovars contain transposases, or its remnants, and some phage related proteins. (Waites, *et al* , 2005) .

1.2.3. Epidemiology of *Ureaplasma* spp.

The proposed mechanisms for infectious causes of recurrent miscarriage include: (1) direct infection of the uterus, fetus, or placenta, (2) placental insufficiency, (3) chronic endometritis or endocervicitis, (4) amnionitis, or (5) infected intrauterine device. The particular infections speculated to play a role in recurrent miscarriage include *mycoplasma*, *Ureaplasma,* (Ford, and Schust, 2009). *Ureaplasma* can be detected in the cervix or vagina of 40 to 80 % of sexually mature asymptomatic women. *Ureaplasma parvum* there is emerging evidence that it may play a role in infections of pregnancy or in eliciting conditions associated with prematurity. A recent study indicated that there is related intra-amniotic inflammatory response to *Ureaplasma parvum* and that this is related not only to Preterm Prelabour Rupture of Membrane (pPROM), preterm labor, and chorioamnionitis, but also to early onset sepsis in the baby and bronchopulmonary dysplasia (Trembath *et al* 2013) . *Ureaplasma* are considered to be commensal microorganisms of the lower genital tract of women. In study of 162 women, the rate of *Ureaplasma* colonization from urethral and cervical swabs were not different between symptomatic women attending a venereal disease clinic (74%) or women with no urogenital symptoms and normal finding at pelvic examination (71%) (Dando, 2012). Similarly, (Casari *et al* , 2010) reported that there were no differences in the rate of endocervical *Ureaplasma* colonization between women with symptoms of genital tract infection(4.86%)

and asymptomatic women (3.79%). Colonization is more common in females of younger age, , with multiple sex partners, and those using oral contraceptives. Incidence of *Ureaplasmas* in female genital tract is most probably dependent on hormonal status, puerperium, postmenopause, in pregnant women, in sexually inactive women, and in sexually active non pregnant women were 5 %, 24 %, 25 %, 82 %, 40 %, and 67 %, respectively. Studies with supposedly healthy women reported *Ureaplasma* spp. detection rate at approximately 18–87 % for *U.parvum* (Table 1-1).(Marovt *et al* , 2014) .

Table 1-1: Prevalence of *Ureaplasma parvum* in supposedly healthy female population. (Marovt *et al* , 2014) **.**

U. parvum	N	Country	Source
87%	263	Australia	Kong, 2000
52%	877	Japan	Kataoka,2006
53.1%	128	China	Cao, 2007
57%	233	Australia	McIver, 2009
17.9%	39	Poland	Ekiel,2009
41.7%	303	Japan	Yamazaki,2012
3.5 %	3	Iraq	Al-Talqani & l-Musawi,2015

1.2.4. The Role of Genital *Ureaplasma* in Genitourinary Tract Infections

Ureaplasma spp. resides in the urogenital tract and respiratory tract and penetrates the sub mucosa only during immunosuppression . In humans, *Ureaplasma* is transmitted through sexual contact; it can also be transmitted from mother to offspring vertically in utero or through infected body fluids at the time of birth. (Dhawan, & Kokkayil , 2015) . *Ureaplasma* infection causes involuntary infertility. *Ureaplasma* spp. can cause placental inflammation and may invade the amniotic sac early, causing persistent infection and adverse pregnancy outcomes, including premature birth. Experimental infection studies using nonhuman primate models have shown that *Ureaplasma* in amniotic fluid causes regulation of proinflammatory cytokines, leukocytes, and prostaglandins,

potentially contributing to premature delivery and preterm birth . The rate of preterm birth in 2010 was 11.1% , with an estimated 30 million babies born preterm annually. (Blencowe *et al* , 2012). preterm birth is a leading cause of neonatal morbidity and constitutes a significant financial burden on the health care system (Petrou *et al*, 2011). Twenty-five to 30% of preterm birth is preceded by preterm premature rupture of membranes (PPROM). *Ureaplasma spp*. are among the microorganisms most frequently isolated from the placental membranes following preterm labor and PPROM . The isolation of *Ureaplasma spp*. from the placenta or the lower genital tract in pregnancy is associated with both intrauterine inflammation and preterm birth (Breugelmans, *et al* . 2010). The incubation period for *Ureaplasma spp*. an average of 2 days. The disease does not give to know about myself, but people in this period becomes a carrier of infection and can infect other people. After the incubation period of infection in a patient develops symptoms of urethritis- burning and pain in the urethra during urination, and there are mucus, often in the mornings. In most cases, particularly in women *Ureaplasma* asymptomatic. (Huang, *et al*, 2015). Although considered to be commensals of the female lower genital tract, the *Ureaplasma* have been associated with symptomatic vaginitis, urinary tract infection with pyuria and bacterial vaginosis. (Francesco, *et al*, 2009). *Ureaplasma* colonization of the female lower genital tract also has been identified as a risk factor for preterm delivery in pregnant women , in study of 877 women *Ureaplasma parvum* were detected in 52% of vaginal swabs, *U.parvum* colonization was detected in (76.2%) women who delivered preterm, and was identified as a risk factor for late abortion or early preterm birth. (Kataoka's , *et al* , 2006). While (Breugelmans, *et al*, 2010) isolated *Ureaplasma spp*. in (53.6%) from cervical swabs of women who delivered preterm. Thus, *Ureaplasma* spp. are believed to play a causal role in the process of infection-driven PTB and a host of related neonatal morbidities (Viscardi, 2014). Accordingly, there is an

urgent need to develop pharmaceutical regimens for the prevention and treatment of intrauterine *Ureaplasma* spp. infection in pregnancy.

Ureaplasma are reported to be more prevalent than other *mycoplasma* in the female urogenital tract, with *U.parvum* found more often than *U. urealyticum* (Patel & Nyirjesy, 2010). During pregnancy, *Ureaplasma* spp. can cause chorioamnionitis, spontaneous miscarriage, stillbirth and preterm delivery. *U. parvum* might be present in bacterial loads leading to adverse pregnancy outcomes (Kasper, *et al*, 2010) and produce asymptomatic infections of the upper genital tract in women as frequently as *U. urealyticum* (Kasprzykowska, *et al*, 2013). Out of the four *U. parvum* serovars (including serovars 1, 3, 6 and 14), serovars 3 and 14 have been isolated in more cases of genital tract infections than serovars 1 and 6 (Dhawan, *et al*, 2012).

Ureaplasma spp. have been detected in the amniotic fluid of pregnant women as early as the 16th week of pregnancy, in the presence of intact fetal membranes and in the absences of other microorganisms Furthermore it has been demonstrated that *Ureaplasma* can causes clinically silent intra amniotic infections, associated with histological chorioaminonitis which can persist for as long as to month in humans, due to asymptomatic nature of intra amniotic of *Ureaplasma* infection and the fastidious growth requirements of these microorganisms. (Geo, *et al* , 2009). *Ureaplasma* spp. can be transmitted from a colonized woman to her newborn infant in utero either transplacentally from the mother's blood or by an ascending route secondary to colonization of the mother's urogenital tract, or at delivery by passage through a colonized birth canal. The rate of vertical transmission has been reported to range from 18 to 55% among full-term infants and from 29 to 55% among preterm infants The rate of vertical transmission is not affected by method of delivery but is significantly increased when chorioamnionitis is present. The rate of colonization also appears to be higher in very low birth weight infants *Ureaplasma spp.* can be detected in the bloodstream of some women with postpartum or post abortal fever. This

condition is usually self-limited, but some cases of dissemination to joints resulting in arthritis may occur. (Waites *et al*, 2015).

1.2.4.1. *Ureaplasma spp.* and Preterm birth

Intrauterine infection is a frequent and important mechanism leading to preterm birth. Routes of intrauterine infection includes- (1) ascending from the vagina and the cervix,(2) hematogenous dissemination through the placenta, (3) accidental introduction at the time of an invasive procedure, (4) retrograde spread through the fallopian tubes. *Ureaplasma* spp. are generally considered commensal microorganisms and are classified into two species and 14 distinct serovars (SV) . SV1 , SV3 , SV6 , and SV14 belong to *U.parvum* species and the remaining ten SV to *U.urealyticum*. *Ureaplasma* spp. commonly colonize the urogenital tract of both males and females .Vaginal colonization rates can vary greatly in non-pregnant women (upto70%) (Hunjak, *et al*. 2014) and women with uncomplicated pregnancies 70% (Capoccia , *et al* 2013). *Ureaplasma* spp. are some of the most frequently identified microorganisms in placental tissues from preterm deliveries (DiGiulio, 2012). Colonization of the placenta with *Ureaplasma* spp. has been demonstrated to be an independent risk factor for chorioamnionitis (Namba, *et al* , 2010). Detection rates in amniotic fluid (AF) vary from 0% to19 % in early mid-trimester, to 2–80% at preterm labor and 18–100% with preterm premature rupture of membranes (PPROM) (Oh, *et al*, 2010). Analysis of 22 studies found a significant association between the presence of *Ureaplasma* spp.in the vagina and AF with PTB (Capoccia, *et al*, 2013). However, *Ureaplasma* spp. Colonization in the urogenital tract and AF is a relatively common finding in pregnant women and alone it is not sufficiently predictive of PTB to be clinically useful (Combs, *et al* , 2014).

The reasons why commensal *Ureaplasma* spp. cause ascending intrauterine insemination (IUI) leading to PTB in only a subset of women are still unknown but are likely to be complex and multifactorial. . (Ireland & Keelan, 2014). SV-specific virulence has been proposed as an important determinant of risk adverse

outcome. The two *Ureaplasma* species, *U.parvum* is the most commonly isolated species from clinical samples , with two of its SV3 and SV6 associated with adverse pregnancy outcomes (Xiao, *et al* , 2011). Most studies of IUI do not differentiate between *Ureaplasma* SV so data on the relationship between SV prevalence and risk are lacking. Poly microbial interactions may also be significant as diagnosis of abnormal bacterial flora or bacterial vaginosis (BV) is also a risk factor for PTB , independent of *Ureaplasma* spp. colonization status. It is worth noting that poly microbial colonization of the amniotic cavity is common in preterm deliveries , with around half of all infected AF containing two or more microorganisms (DiGiulio,2012). Co-colonization of the vagina with a *Ureaplasma* spp. and another genital *Mycoplasma* spp. has been reported to be associated with more sever adverse pregnancy outcomes compared to colonization with a single organism (Kwak *et al* , 2014). Competence of the cervical barrier to microbial ascension is also likely to play a role in determining risk of PTB. A short cervix in pregnancy has been associated with increased risks of microbial colonization and spontaneous PTB (Romero, 2012); (Guimaraes *et al* ,2013).

Finally, maternal / fetal immunological tolerance or competence is a likely determinant of obstetric outcome associated with colonization by *Ureaplasma* spp. and other microorganisms. Surface-exposed lipoproteins of *Ureaplasma* spp. activate the pro-inflammatory transcription factor NF-kB through TLR ligation (Triantafilou *et al*, 2013), although exposure of intrauterine tissues to *Ureaplasma* spp. Does not generally trigger a robust inflammatory response. Nevertheless, intra-amniotic infection with *U.parvum* has been causally linked to chorioamnionitis, This is consistent with observations from clinical studies showing that chorioamnionitis is more likely in pregnancies infected with *Ureaplasma* spp. (Namba, *et al*, 2010; Combs, *et al* , 2014).

1.2.4.2. Pathogenesis

Ureaplasma attaches to mucosal surfaces with the help of cytadherence proteins. These are expressed on the surface of the bacterial cell. *Ureaplasma* is known to adhere to a variety of human cells including erythrocytes, spermatozoa, and urethral epithelial cells. The key virulence factor of *Ureaplasma* is the MBA, a surface-exposed lipoprotein. TLR1, TLR2 and TLR6 are the pattern recognition receptors through which the MBA activates nuclear factor kappa B. (Zimmerman *et al*.,2011) were demonstrated that the MBA of *U. parvum* underwent alternate phase variation with an adjacent gene, UU376. Furthermore, phase variation of MBA N-terminal (UU171 and UU172) was recently described in both *U. parvum* and *U. urealyticum*. A primary function of antigenic variation is to evade the adaptive immune response (Knox *et al*, 2010). (Dando *et al.,* 2012) have demonstrated using a sheep model that MBA size variability did not prevent recognition by host pattern recognition receptors. However, it may prevent the host immune response from eradicating *Ureaplasma* from the amniotic cavity and thus play a role in the virulence of these microorganisms. *Ureaplasma* expresses Phospholipase A and C. These generate prostaglandins-A known trigger of labor. (Kacerovsky, *et al*., 2013) evaluated the amniotic fluid protein profiles and the intensity of intra amniotic inflammatory response to *Ureaplasma* spp., using the multiplex xMAP technology. The presence of *Ureaplasma spp*. in the amniotic fluid was associated with increased levels of interleukin (IL)-6, IL-8, IL-10, monocyte chemotactic protein-1, macrophage inflammatory protein-1, and matrix metalloproteinase-9. This generalized inflammation of the amnion and the chorio-decidua resulting in the production of pro-inflammatory mediators such as interleukin-1beta (IL-1β), IL-6 and prostaglandins ultimately initiate preterm labor. *Ureaplasma* has also shown an IgA protease activity, which could destroy mucosal IgA. Since IgA is the predominant immunoglobulin secreted at mucosal surfaces, IgA proteases may facilitate colonization by microorganisms by degrading this important component of the mucosal immune system. However,

genome analysis of all serovars has not shown the presence of this gene. (Paralanov, *et al.,2012)*.

The urease activity of *Ureaplasma* generates ammonia from the cleavage of urea, which can cause toxicity to host tissues due to change in pH. Chronic lung disease after exposure to ammonia has been reported in adults. However, (Robinson, *et al.,*2013) showed that an elevated pH in either the amniotic fluid or fetal lung fluid did not correlate with increased inflammatory cell counts in the chorioamnion or fetal lung tissue respectively. But the ammonia liberated by *Ureaplasma* may contribute to the chronic tissue damage and pathology observed within the chorioamnion and the fetal lung in utero. Virulence and persistence of *Ureaplasma* is also influenced by the ability of microorganisms to form biofilms. A study conducted by (Pandelidis *et al.,* 2013) confirmed that most clinical *Ureaplasma* isolates form biofilms *in vitro*. These biofilms may contribute to organism persistence and chronic inflammation but biofilm-formation did not impact MICs for azithromycin or erythromycin.

1.2.4.3. Virulence Factor of *Ureaplasma spp.*

Human pathogenic *Ureaplasma* have been reported to possess many virulence factor each of which was found to play an essential role in pathogenesis of these organisms. In general *Ureaplasmal* proteins have been proposed as virulence factors, which may contribute towards the pathogenesis of *Ureaplasma* infections of the upper genital tract during pregnancy. These include the multiple banded antigen (MBA), urease enzyme, immunoglobulin A (IgA) protease, phospholipase A and phospholipase C proteins (Dando, 2012).

1- Attachment organelles : Cytadherence protein is a necessary first step for infection of mucosal surfaces, these proteins MBA in *Ureaplasma parvum*. The MBA is known to demonstrate size variation (Robinson *et al.*,2013) have been shown that the severity of chorioamnionitis correlated inversely with the number

of MBA size variants that existed within infected amniotic fluid (AF), suggesting that variation of the MBA was associated with *Ureaplasma* pathogenicity.

2-Phospholipase enzymes: These enzyme hydrolyze phospholipids with the release of arachidonic acid. Two types of Phospholipase enzyme are produced by *Ureaplasma parvum*. (A & C) that are localized in the plasma membrane, therefore *Ureaplasma* is associated with amnionitis, spontaneous abortion, prematurity and still birth . It has been postulated that infection of the women genital tract may initiate a sequence of pathologic events related to phospholipase production. (Novy *et al* , 2009).

3-Immunoglobulin A (IgA) protease : This enzyme produced mainly *Ureaplasma parvum.* and it facilities mucosal invasion by hydrolyzing mucosal IgA (Thurman *et al*, 2010).

4-Urease enzyme activity of *Ureaplasma parvum.* generates ammonia from the cleavage of urea, which can cause toxicity to host tissues due to change in pH. (Robinson , *et al.*,2013)

Momynaliev *et al.* (2007) also predicted that *U.parvum* contains a hypervariable plasticity zone, which encodes a putative pathogenicity island.However, there has been limited investigation into the role of these predicted virulence factors of *Ureaplasma* spp.

1.2.5. Laboratory Diagnostic

Diagnosis of urogenital *Ureaplasma* was originally based on direct culture of *Ureaplasma spp*. from the urogenital tract, urine, semen, or cervical mucus, and often requires the services of a specialized laboratory several other diagnostic methods have been developed and employed in recent years including polymerase chain reaction (PCR)-based nucleic acid detection strategies, serological diagnosis, and commercial assays based on detection of ammonia

following the hydrolysis of urea *Ureaplasma spp*. Between the increased sensitivity of PCR and the technical limitations of culturing the organism, nucleic acid detection and PCR is used almost exclusively to diagnose *Ureaplasma spp*. infection . (Brown *et al*, 2010).

1.2.5.1. Culture

Culture is considered the gold standard in the detection of *Ureaplasma* but it is difficult since these fastidious organisms require the presence of serum, metabolic substrate and growth factors like yeast extract for isolation, direct culture is the most common way to reliably detect mollicutes species but there is no single medium formulation is adequate for all mollicutes species due to their different nutritional requirements. (Cheong *et al .,* 2010). Previously most mollicutes species were cultured on media composed in part of cell extracts (Kraybill & Crawford, 1965). (Shepard & Lunceford,1970) used 10 B broth and agar. Since the organisms do not produce any turbidity on growth, pH indicators like phenol red are added. The growth of organisms leads to a change in pH of the media which is visualized as a change in color of the indicator. (Shepard & Lunceford ,1976) used differential agar medium for identification of *Ureaplasma spp*. and the basal medium was first prepared and contained trypton soya broth and urea. (Razin, *et al*., 1977) Putrescine added to medium enable led better growth of *Ureaplasma spp.* (Kundsin *et al.,*1978) used Shepared differential agar medium (A7) containing manganese sulfate. (Fiacoo *et al,* 1984) used modified SP-4 broth and added Ultra-pure Urea at a final concentration of 0.05% (wt/vol) replaced glucose in SP-4U, Ampicillin (1 mg/ml) was used in place of Penicillin in SP-4U contained lincomycin hydrochloride at a final concentration of 15ug/ml. And used Modified MPR broths. MPRU and added ampicillin sodium in a final concentration of 1 mg/ml replaced Penicillin or Erythromycin or both. Also used *Ureaplasma* differential agar medium (A7) with Penicillin was replaced with Ampicillin (1mg/ml) and Amphotericin B (5ug/ml).

Simihairi (1990) used arginine broth and arginine agar for isolation of *Mycoplasma spp*. and *Ureaplasma spp.* from urogenital tract of women. Another study (Tully,1995) used SP-4 medium were modified for isolation and identification of *Ureaplasma spp.* However growth of *Ureaplasma spp*. requires a basic broth medium consisting of peptone supplemented with yeast extract, horse serum and antibacterial, the yeast extract contributes diphosphpyridine nucleotides, and the serum supplies cholesterol or related sterol for growth, since sterol helps stabilize cell membranes and protect the organisms against osmotic destruction, and the antibacterial such as penicillin are used, thallous acetate to prevent growth of contaminants (Razin & Herrmann, 2002). Transport media are developed by added some substrate such as glucose metabolizers. Arginine metabolizers and urea hydrolyzer's to basic medium is supplemented with one or more of these substrates depending on the mollicutes species to be isolated. (Sudolska *et al.,* 2006). While (Waites *et al*., 2012) demonstrated the *Ureaplasma* species grow rapidly in media containing urea, such as 10 B broth and A 8 agar, producing visible colonies within 1 to 3 days. The appearance of brown granular colonies on A 8 agar is sufficient for the diagnosis of *Ureaplasma species,* but culture alone cannot distinguish between the two species *U. urealyticum* and *U. parvum*.

Other study explained the commonly used medium for isolating *Ureaplasma spp*. is the PPLO broth containing urea. The processed samples are serially diluted 10-fold from 1:10 to $1:10^5$ for inoculation. Dilution is essential since it overcomes interference by antibiotics, or other inhibitors, overcomes rapid decline in culture viability and indicates the number of organisms present. The inoculated broths are incubated at 37°C under 5% CO 2 and inspected twice daily. A rise in pH visualized by change in color without any turbidity is indicative of growth. (Dhawan, *et al.*,2012).

In previous study Al-Aubaidi and Fabricant (1968) used horse serum, fetal calf thymus as a source of DNA for cultivation of *Mycoplasma* isolated from animals. And (Al-Aubaidi *et al*, 1972) isolated *Mycoplasma mycoides* from bovine, caprine, and ovine on RYE and basal salts (BS) medium. DNA was used by many workers especially to isolate Mycoplasma from animals giving good results in isolation of genital mycoplasma (Al-Aubaidi and Fabricant,1968; Al-Safar, 1984).While Abdullah (1989) identified *Mycoplasma pneumonia* from respiratory tract infection in Baghdad by serological methods. And Simihairi (1990) used arginine broth and arginine agar for the isolation of the *Mycoplasma hominis* and *Ureaplasma urealyticum* from urogenital tract of women. U9B medium was used for isolation urogenital tract mycoplasmas from women (Al-Bahli, 1993). Moreover, (Hasso and Al-Omran, 1994) used the enzyme linked immunosorbent assay (ELISA) to evaluate humoral immunoresponse to Mycoplasma agalactiae in four groups of goats were inoculated via different routes with the microorganism. While (Kareem, 1997) isolated genital *Mycoplasma* and *Ureaplasma* from infertile couples by used A7, A8, modified Hayflick and AE10 medium. Al-Naimee (2001) isolated *Mycoplasma pneumoniae* on *Mycoplasma agar* from respiratory tract of neonates in Mosul City. Monophsic - diphasic culture setup medium (MDCS) was used for isolation of *Mycoplasma pneumoniae* from respiratory tract (Al-Ghizawi, 2001), Moreover Al-Mossawi (2005) used (MDCS) for isolation genital *Mycoplasma* from synovial . Also Kadhim (2010) used (MDCS) for isolation *Mycoplasma spp*. from synovial fluid. While (Al shammari, *et al*, 2005) isolated *Mycoplasma hominis* in rate (27.6%) from endocervical swabs of women using intrauterine contraceptive device on Hayflick`s agar. And (Abdul-Wahab,2010) used *Mycoplasma* agar to detect *Mycoplasma* upon direct culture method, where *Mycoplasma* broth was used as an enrichment before sub culturing onto the *Mycoplasma* agar. Also (Govender, 2010) isolated genital mycoplasma from endocervical swab on A8 medium.

(Al-Azawi., 2012) used five agar media these are Arginine, modified Hayflick, A7, A8 and modified M5 (IH medium) which supplemented with essential requirements for growth of these microorganisms and also demonstrated the most of *Mycoplasma spp*. and *Ureaplasma spp*. were grown in (IH medium) broth or agar supplemented with horse and other serum. Modified arginine –urea broth (MAU-broth) and Modified arginine – urea agar (MAU-agar) used for isolation *mycoplasma spp.* and *Ureaplasma spp.* (AL-Karawy, 2015). While (Al-Talqani & Al-Musawi, 2015;) used IH medium for isolation *M.hominis* , *U.urealyticum* and *U.parvum* from infertile men. Also Al-Nassri, (2016) used IH medium for isolation *M.pneumoniae* from tonsillitis patients.

1.2.5.2. Molecular Diagnosis

Molecular assays have developed for isolation and specific identification of *Ureaplasma spp.* in different specimen such as genital tract, amniotic fluids, and respiratory tract specimen from newborns (yoon *et al* , 2003). PCR technique has been described as the more sensitive than culture in specimen analyzed after collection if neonates acquired *Ureaplasma spp.* during birth, the organism would be at a low density at day zero and increase over time. Thus rapid PCR assays in which early diagnosis are important can be tremendous benefit in designing antibiotic treatment protocols. (Cultrea *et al*., 2006). Gel based conventional PCR assays for *Ureaplasma spp.* have used specific urease gene, and MBA gene target sequences for detection and identification of this organism (Dhawan, *et al*.,2012). Different target sequences, within specific genes, have been used for *U.parvum and U.urealyticum* PCR detection and including : urease gene and MBA gene . The MBA gene is an appropriate target for PCR assays because of it's repetitive nature within the genome and used targeting the multiple banded antigen (MBA) gene for biotyping. The majority of the *Ureaplasma* isolates belonged to biovar 2 (*U. parvum*). was isolated more frequently from lower genital tract of female than *U.urealyticum* in Australia and the united states (Dando,2012).

Nested PCR were also proposed for detecting *Ureaplasma spp.*(Kong, *et al* 2000) used the MBA gene for identification and subtyping of *U.parvum* and *U.urealyticum* by using specific primer pairs because the MBA gene of these species contain both species and serovar/subtype specific sequence based on whole sequences of the 5'-end of MBA gene of *U.parvum* serovars and partial sequences of the 5'-end of MBA gene of *U.urealyticum* serovars.

Multiplex PCR were also several studies have used this method for detecting *U.parvum*, *U.urealyticum* by screening for (MBA) gene Multiplex PCR was performed with primers specific for highly conserved regions in the multiple band antigen(MBA) gene of *Ureaplasma spp.* (Al-Talqani &Al-Musawi,2015).

Real time PCR assays mainly target the urease genes and their subunits or MBA. real time PCR assays have been shown to be extremely useful for the simultaneous detection and biovar discrimination of *Ureaplasma spp*. in clinical specimens and developed two real time PCR assays for quantitative detection of *U.parvum* and *U.urealyticum*. Moreover, this quantitative PCR allows to confirm the role of *Ureaplasma* spp. in patient with sever genital tract infection.(Cao, *et al* 2007).

1.2.5.3. Serological Assays

Several technique have been proposed for detection of antibodies against bacterial antigens. These technique are : Growth inhibition test (GIT), Indirect haemagglutination test (IHA), Metabolic inhibition test (MIT), Immunofluorescence test (IFT), complement fixation test (CFT), the micro particle agglutination assay and various enzyme linked immune sorbent assay tests (ELISA), (Johnston & Martin, 2005).

Designation of *Ureaplasma* serovar using polyclonal or monoclonal antibodies directed against whole cells or purified antigens have included growth inhibition tests, immunofluorescence of colonies on agar (Iverson-Cabral *et al*., 2006).

The most common test, used for diagnosis of genital *Ureaplasma* species is the complement fixation test, the sensitivity of this assays depends on whether the first serum samples is collected early or late after the onset of illness and on the availability of paired sera collected with an interval of 2-3 weeks. Metabolic inhibition test for *Mycoplasma spp.* and *Ureaplasma spp.,* is based on inhibition of the alkalization of the medium that follows the production of ammonia due to the hydrolysis either of arginine or urea (Razin & Herrmann, 2002).

A major disadvantage associated with serological assays, is that sensitivity is dependent on the precise timing of collection and therefore accurate diagnosis relies on the collection of serum samples at two specific points during the illness (McDonough *et al.*, 2005) . Another disadvantage associated with antibody-based techniques is time-consuming, cumbersome assays that often yield results that are not reproducible, are difficult to interpret, and are inconclusive because of multiple cross-reactions and poor discriminating capacity when used with clinical samples containing two or more serovars. Lack of commercial availability and standardization generally limited serotyping to those laboratories that developed the individual serological reagents (Xiao *et al.*, 2010).

1.2.6. Cytokine Profile and Their Role in *Ureaplasma* Genital Tract Infections

Cytokines generally are glycoprotein they are involved in immunity and inflammation where they regulate the amplitude and duration of the immune response . *Ureaplasma* can activate macrophages and monocytes, leading to the expression and secretion of the major proinflammatory cytokines: tumor necrosis factor (TNF)-α, IL (interleukin)-1β, IL 6, IL-8, IL-12, IL-16, interferon-g10.

Toll-like receptors (TLRs) on immune and epithelial cells recognize pathogen-associated molecular patterns (PAMPs), invariant structures on microorganisms, and activate an innate immune response. At present, 10 distinct members of the TLR family have been identified in man. TLR-2 is involved in the recognition of PAMPs on mycoplasmas (Passos *et al,* 2011) .

Inflammation – mediated preterm birth (associated with intra-amniotic infection) is predicted to occur due to microbial invasion of the choriodecidual space , which stimulates the production of cytokines such as tumour necrosis factor-alpha (TNF-α) , interleukin (IL)-1-α , IL-1β , IL-6 , IL-8 and granulocyte-macrophage colony stimulating factor . These cytokine , in combination with microbial virulence factors and phospholipase , stimulate prostaglandin synthesis , neutrophil infiltration and the release of metalloproteases the up regulation of prostaglandin causes uterine contractions , whereas the metalloproteases weaken the chorioamnion , leading to membrane rupture and ripening of the cervix . A causal relationship was recently demonstrated between intra-amniotic *U.parvum* serovar 1 infection and preterm birth . *Ureaplasma* infection was associated with increased amniotic fluid concentrations of TNF-α , IL-1β , IL-6 , IL-8 , prostaglandin E2 , prostaglandin F2α , matrix metalloproteinase 9 and leukocytes. Therefore *Ureaplasma* considered as a pathogens cause inflammation within amniotic cavity and preterm birth in human of intrauterine infection . (Larsen & Hwang, 2010).

1.2.7. Antibiotic Susceptibility Test

The most practical, economic and widely used method is the broth micro dilution test. The method uses a 96 well microtitre plate in which decreasing concentrations of antibiotics are mixed with a standard concentration of organisms in broth medium and the mixtures are incubated. The medium used is 10B broth or Pleuro pneumonia Like organism (PPLO) broth or Shepard or A8 media containing urea, with acidic PH 6.0 to 6.5 is necessary for *Ureaplasma spp.* growth. The multiplying organisms metabolize urea in the medium resulting

in change in pH visible as a color change to red due to the presence of phenol red in the medium. This color change is usually seen 16-18 hours after incubation. If the organism is susceptible to the concentration of antibiotic in the well, growth is inhibited and there is no color change. The MIC is the highest dilution of antibiotic that inhibits the color change at the time when the change in the control without antibiotic has just developed. The method allows several antibiotics to be tested in the same microtitre plate but it requires standardization since time of reading and inoculum size affects the test. It is important to read the MIC endpoint at the first appearance of color change in the growth control well since longer incubation period will shift the MIC and thus give a falsely elevated MIC. (Waites, *et al.* ,2012) . However, broth dilution method is the most widely used, particularly for *Ureaplasma spp*. the method employs *Ureaplasma* media with decreasing antibiotic concentration, inoculated with a standardize number of microorganisms.(Mihai *et al.*, 2011).

Agar dilution methods , A8 agar is the primary agar medium used for the cultivation of *Ureaplasmas,* so it was used for the agar dilution MIC assays. This method is time – consuming, labor –intensive and is not practical for testing small numbers of strains or occasional isolate, however the end point is stable over time, allows detection of mixed cultures readily, and the detection of resistant mutants (Waites, *et al* , 2012).

Direct broth – disk test, utilizing urine sediment as the inoculums and impregnated paper disks as the source of antibiotic, was developed and used to test the activity of minocycline, doxycycline, tetracycline, erythromycin, mepartricin and lincomycin against 35 freshly isolated *Ureaplasma spp*.(Busolo, & Conventi, 1988).

Another study have used of the E-test for susceptibility testing of 68 samples of *Ureaplasma spp*. to ciprofloxacin, ofloxacin, tetracycline, doxycycline, Roxithromycin, erythromycin , clarithromycin and azithromycin . The results

were compared with those results being obtained from agar dilution method, Their results indicated that E-test was more efficient. (Cakan , *et al* , 2003).

Commercially susceptibility testing kits are available. These include SIR mycoplasma kit (Bio rad), mycoplasma IST2 kit (Biomerieux) and Mycofast Revolution assay (ELiTech Diagnostic, France) is a commercially available assay that allows for detection, identification and antimicrobial-susceptibility testing of genital mycoplasmas and the *Ureaplasma* spp. both kits comprise micro wells containing different concentration of an antibiotic. Antibiotics used for detection *Mycoplasma* and *Ureaplasma* include : doxycycline, tetracycline, azithromycin, josamycin, erythromycin, ofloxacin, clindamycin. If *Mycoplasma* and *Ureaplasma* inoculated into the antibiotics containing wells are susceptible to an antibiotic present, growth will be inhibited and the broth will remain yellow. (Redelinghuys, *et al*, 2014; AL-Karawy, 2015; Al-Nassri, 2016).

1.2.8. Susceptibility and Antimicrobial Resistance

The administration of antimicrobial agents to pregnant women with preterm rupture of the membranes (PROM) may extend the gestation period and decrease the risks of associated complications and neonatal infections (Greenow *et al* 2011). The antimicrobial agent of choice should be considered carefully, as some agents are teratogens - i.e. the agent can cause malformation or functional damage to an embryo or fetus or may have toxic effects on the neonate (Santos, *et al* 2012). Macrolides are often used empirically because of tetracyclines and fluoroquinolones being contraindicated in pregnancy (Greenow *et al* 2011). However, the amniotic sac is not effectively penetrated by erythromycin and *Ureaplasma* are not eradicated from the vagina or cervix by this agent. Newer macrolides (e.g. azithromycin and clarithromycin) allow for better tolerability and the once daily dosing benefit can increase compliance Treatment with azithromycin is equally successful compared to erythromycin but with fewer side effects (Redelinghuys *et al*, 2013). *Ureaplasma spp*. contains one or more

integrase recombinase genes. Some serovars contain transposases, or its remnants, and some phage related proteins. The *tetM* gene was identified as part of a Tn916 transposon, in serovar 9 which has acquired tetracycline resistance. A report covering the years 2000-2004 from several states in the USA showed that 45% of unique clinical isolates of *Ureaplasma* spp. contain *tetM* and are tetracycline-resistant. (Waites, *et al* , 2005). The possibility of exchange of DNA between Ureaplasma and other pathogen within urogenital tract may contribute to gene transfer, which may promote antibiotic resistance in such pathogens.(Govender,2010).

1.2.9. Resistance of *Ureaplasma parvum* to Antibiotics

There is only limited information on resistance development of *Ureaplasma parvum*. *Ureaplasma parvum* is innately resistant to antibiotics which act on cell wall components (the beta lactams). *Ureaplasma* species have natural resistance to lincosamides (e.g. clindamycin) (Kokkayil, & Dhawan, 2015). Xiao *et al* (2011) Observed resistance to macrolides is associated with mutations in the 23S rRNA gene, while resistance to tetracycline is associated with the presence of the moveable tet M transposon. The tet M gene encodes a protein which binds to ribosomes and in the case of *Ureaplasma spp.* it has been demonstrated to be associated, on the chromosome, with Tn916, a conjugative transposon (Beeton *et al*, 2016). Previous study suggested that *Ureaplasma* resistance to quinolone is mainly due to the mutants of target enzyme-DNA helicase the residues 68-107 areas are the quinolone-resistant areas (quinolones regions of drug-resistance, QRDR) (Xiao *et al*, 2012).

Besides beta lactams, *Ureaplasma* also show resistance to sulphonamides, trimethoprim and rifampicin. Resistance to rifampicin is attributed to the presence of a single amino acid at position 526 of the beta sub unit of RNA polymerase. (Paralanov *et al*, 2012)

In a recent study conducted by (Dhawan, *et al*.,2015) involving patients with infertility and genital discharge, all isolates of *Ureaplasma spp*. isolates were

susceptible to doxycycline and josamycin. 77% of the isolates were susceptible to ofloxacin and 71% to azithromycin. (Dhawan, *et al*, 2012). Though most studies report lower resistance rates for tetracyclines (<5%), a recent study by (Redelinghuyhs, *et al.*,2014) demonstrated only 27% susceptibility of *Ureaplasma* isolates to tetracycline. In a study by Chiang-tai, *et al*.,(2011) in Shanghai, biovar 1 (*U. urealyticum*) showed high sensitivity rates (above 90%) to all antimicrobial agents; but biovar 2 (*U.parvum*) maintained higher sensitivities (above 95%), only to doxycycline and minocycline. In fact, only a small number of biovar 2 strains were sensitive to roxithromycin and quinolones. And *U. Parvum* was exhibited resistance to tetracycline and doxycycline because apparently is the acquisition of streptococcal tetM gene transposon carrying tetracycline resistance genes (tetM). *U. parvum* all strains were susceptible to josamycin & miocamycin.(Mardassi *et al*, 2012).

Ureaplasma parvum was showed resistance to trimethoprim, sulfonamides and rifampicin because they do not synthesize folic acid . *Ureaplasma* have higher mutation rate than conventional bacteria which mean that can rapidly develop resistance to drugs including quinolone, erythromycin and azithromycin resistance development in *U. parvum* by A point mutation in parC (Pro-57Leu).and Two novel mutations in parE (Ile-73Thr and a methionine insertion at codon 86) were found in an ofloxacin-resistant strain. (Kawai *et al*, 2015; Beeton *et al*, 2016).

Chapter two

Materials and Methods

Material and Methods

2.1. Material

2.1.1. Laboratory Equipments and Instruments : The laboratory equipments and instruments used in this work are shown in table (2-1)

Table (2-1): Laboratory equipments and instruments used in this study.

Equipments	Manufacturer (origin)
Autoclave	Sturdy (Taiwan)
Centrifuge	Hettich (Germany)
Compound light microscope	Olympus (Japan)
Digital Camera	Sony (Japan)
Distillator	Lab Tech (Korea)
Electric oven	Memmert (Germany)
Electrophoresis	Consort (Beligium)
Eppendrof tubes	Bioneer (south Korea)
Hot plate with magnetic stirrer	Heidolph (Germany)
Incubator	Memmert
Laminair-flow cabinet	GallenKamp (UK)
Micropipette (automatic)	Eppendorf(Germany)
pH- meter	Jeway (UK)
Refrigerator	Concord (Lebanon)
Sensitive balance	Sartorius (Germany)
Thermal cycler	Primeus (Germany)
UV- transilluminator	ECX – 15.m. (European)
Vortex	Stuart (UK)
Water bath	Kottermann (Germany)
Millipore filter	Bioneer (south Korea**)**

2.1.2. Chemicals

The chemical materials used in this work are listed in table (2-2) below :

Table : (2-2) : biological material used in this study .

Material	Company / origin
Agar - agar	Difco / USA
DNA (calf thymus)	BDH / England
Horse serum	OXOID / UK
L- cysteine -Hcl	BDH / England
Yeast extract	Difco / USA

Table : (2-3) Chemical materials used in this study.

Material	Company / origin
Agarose	BDH/England
DNA Loading Buffer (6X)	MBI Fermentas/UK
Ethidium bromide	BDH/UK
Glycerol (C3H8O3)	Fluka
MgSO4. H_2O	BDH / England
Phenol red	
Sodium hydroxide (NaOH)	
Sucrose	Difco
TBE buffer 10X	Promega(USA)
Urea	Sigma / USA

Table : (2- 4) Antibiotics used in this study .

Antibiotics	Company / Origin
Amoxicillin	ACAI \ Baghdad-IRAQ
Augmentin	Atabay Kimya SAN.VE Tic A.S. \ GEBZE-KoCAELi-TURKEY
Azithromycin	KONTAM/China
Ceftriaxone	Werne / Germany
Ciprofloxacin	Ajanta House,Charkop/india
Clarithromycin	Ajanta / India
Doxycycline	Ajanta House,Charkop/india
Erythromycin	Ajanta / India
Fluconazole	MICRO LABS LIMITED / India
Gentamicin	MENARINI/Italy
Levofloxacin	Ajanta House,Charkop/india
Nystatine	Squib / England
Tetracycline	Ajanta House,Charkop/india

2.1.3. Culture media

The Culture media were used in this study are shown in table (2-5)

Table : (2-5) Culture media used in this study .

Culture media	Company / country
PPLO agar	OXIOD / England
PPLO broth	
Soya bean casein digest broth	Himedia / India

2.1.4. PCR Materials

The PCR materials used are shown in Tables and its appendices as follow

Table : (2-6) Commercial Kit used in this study

NO	Type of Kits	Company/country
1	DNA extraction kits (Reagent Genomic DNA Kit)	Geneaid /USA
2	2x PCR Master mix solution (Maxime PCR Premix Kit (i-Taq))	Intron Biotechnology/Korea
3	DNA ladder 100 bp	

Table : (2-7) Content of DNA extraction kit

Material
Type : Reagent Genomic DNA Kit 1- Cell lysis buffer 2- Protein removal buffer 3- Isopropanol buffer 4- Ethanol 70% 5- Elution buffer (TE)

Table : (2-8) 2x PCR Master mix solution Kit

Materials	
i-Taq TM DNA polymerase	2.5 U
dNTPs	2.5 mM each
PCR Reaction Buffer (10x)	1x
Gel loading buffer	1x

Table : (2-9) DNA ladder

Materials
Ladder consist of Double-stranded DNA with size 100bp .

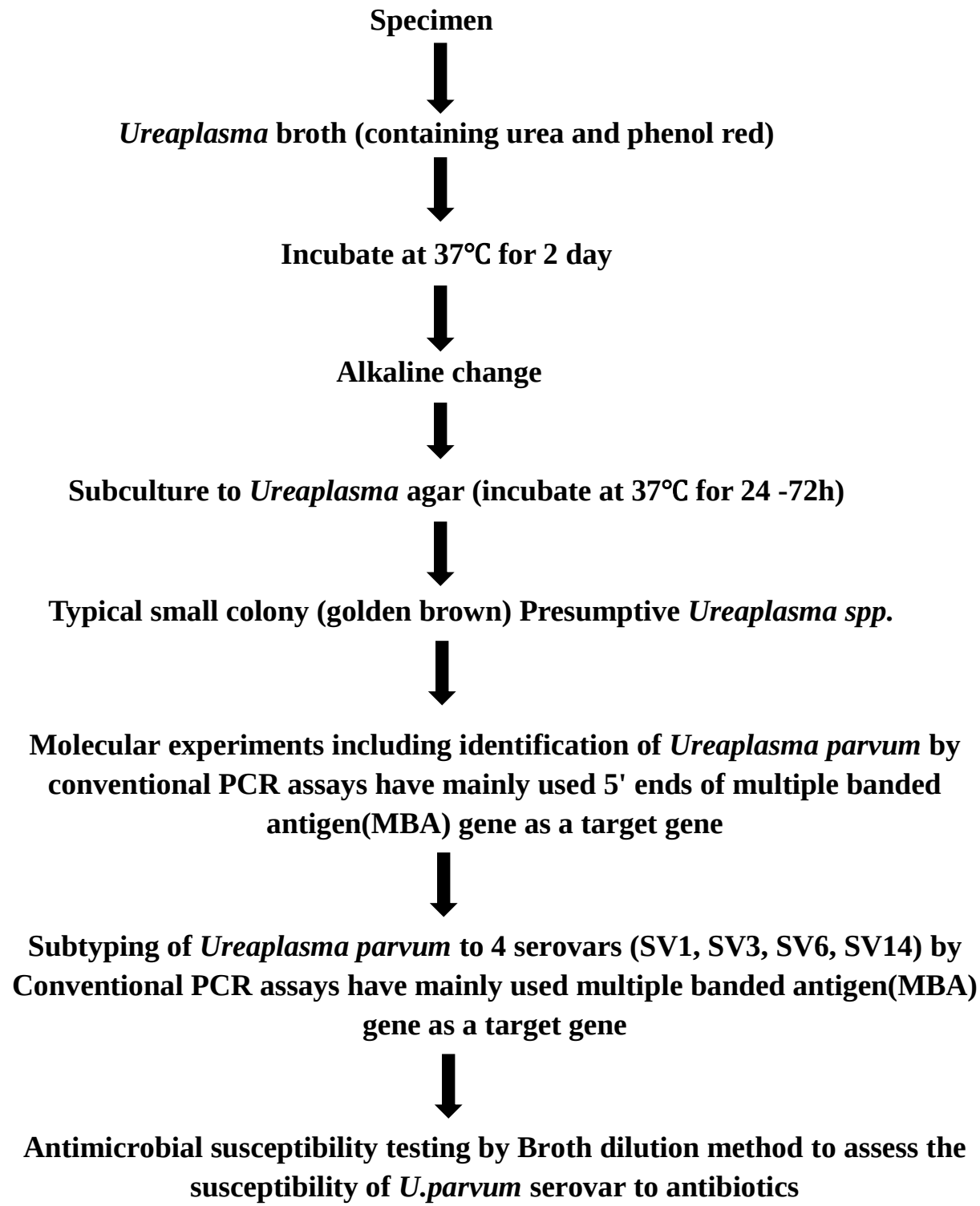


Figure (2-1) : Flow chart for isolation of *Ureaplasma parvum* from genital specimens .

2.2. Methods

2.2.1. Specimens Collection

Specimens obtained from women were admitted to Feminine & children teaching hospital and Gynecology Clinic in Muthanna province: A total of 170 specimens include 130 specimens obtained from married women with recurrent miscarriage including (vaginal bleeding, vaginal swabs and urine) while 40 specimens included urine & vaginal swab were collected from married women without miscarriage (control) during period of October 2015 to February 2016. Information about patients were taken according to formula show in appendix. Some specimens from each woman were collected by vaginal swabs was inserted into the posterior fornix, in the upper part of the vagina and rotated with drawing them carefully to avoid any possible contamination uterus while bleeding specimens were collected from miscarriage women who undergo curettage surgery. All specimens and swabs were placed in tubes containing *ureaplasma* broth (IH). All tubes were incubated at 37C for 2 days then loopful were transferred to plates containing *ureaplasma* agar (IH). All plates were incubated aerobically at 37℃ for 24 – 72 hours then examination the bacterial growth (Kareem,1997) .

2.2.2. Preparation of Growth Supplements and Solutions

2.2.2.1- Horse serum : provided from HiMedia – India and GIBCO-New Zealand in 100 ml vial . It was added for enrichment the medium as recommended by AL- Aubaidi and Fabiieecant (1968).

2.2.2.2- Yeast extract : Yeast extract was prepared by adding 125 grams of active bakers yeast were added to 500 ml of distilled water (D.W) at 45℃ in large beaker . After mixing well , it was boiled for 30 minutes and allowed the yeast cells to settle and clarified by centrifugation for 30 minutes at 1000 rpm . The supernatant was filtered by using Millipore filter . The mixture then was sterilized by autoclaved for 15 minutes . The sterile yeast extract was dispensed

aseptically in 10 ml sterile screw cappe bottle and stored at -20℃ (Marimion and Harris 1996) .

2.2.2.3 - Cysteine solution (2%) : This solution was ready by dissolving 2gm of (L-cysteine) in small volume of D.W, and completed up to 100 ml D.W, then sterilized by Millipore filter paper and stored at 4℃. It was used for supplement bacterial growth as recommended by (Razin and Vincent , 1983) .

2.2.2.4 - Urea solution (10 %) : It was prepared by adding 10 gm of urea in small volume of D.W , and completed up to 100 ml D.W , then sterilized by Millipore filter paper and stored at 4℃. It was used for supplement bacterial growth as recommended by Razin and Vincent (1983).

2.2.2.5 - DNA solution (0.2%) : It was prepared by dissolving 0.2gm of deoxyribonucleate (calf thymus) in small volume of D.W , and completed up to 100 ml D.W, sterilized by Millipore filter paper and stored at 4℃ (Al – Aubaidy and Fabricant 1968) .

2.2.2.6 – Putrescine – dihydrochloride solution (0.2 %) : It was prepared by dissolving 0.2 gm of putrescine – dihydrochloride in small volume of D.W , and completed up to 100 ml D.W , sterilize by Millipore filter paper and stored at 4℃ (Kareem , 1997) .

2.2.2.7 – Phenol red solution (0.4%) : It was prepared by dissolving 0.4 gm of phenol red in small volume of D.W and completed up to 100 ml D.W, sterilized by Millipore filter paper and stored at 4℃ (Marimion and Harris , 1996) .

2.2.2.8-Tris Borate-EDTA-buffer solution (TBE) : This solution was prepared by mixing 10ml of stock TBE- 10x buffer with 90ml of distilled water, then stored at 4℃ until used in electrophoresis.

2.2.2.9-Ethidium Bromide solution (0.5%) : It was prepared by dissolving 0.05mg of Ethidium Bromide in 10 ml distilled water , final concentration 0.5 mg /ml . It was used in electrophoresis as specific DNA stain and stored in a dark reagent bottle according to the recommended of (sambrook and Rusell , 2001) .

2.2.3. Bacterial Inhibitors

2.2.3.1. Crystalline Ceftriaxone : It was prepared by adding 0.5 gm of crystalline ceftriaxone to 5 ml of D.W , sterilized by Millipore filter paper and used soon after preparation . It was Used for inhibition the growth of other bacteria as recommended by Razin and Vincent (1983) .

2.2.3.2. Augmentin (Klavunat): It was prepared as crystalline ceftriaxone .

2.2.3.3. ACAMOXIL(Amoxicillin): It was prepared as crystalline ceftriaxone.

2.2.3.4. Nystatine solution : It was prepared by dissolving 1 gm of nystatine (after crashed) to 10 ml of D.W, sterile by Millipore filter paper and used for inhibition of fungi growth as recommended by Razin and Vincent (1983) .

2.2.3.5. Fluconazole solution : prepared by dissolving 0.5 gm of fluconazole in 5 ml of D.W , sterile by Millipore filter paper (0.22)*um***.**

2.2.4. Preparation of Culture Media

2.2.4.1.*Ureaplasma* broth – Urea broth (IH broth) :- This medium was Comprising of the following components **:**

PPLO broth	3 gm
Yeast extract powder	2 gm
Trypton soya broth	2 gm
D.W	70 ml

These medium were mixed well and heated in water bath at 100℃ for 1 hour , Then autoclaved , cooled to 57℃ , (PH = 5.5 -6.0) then the following supplements were added :

Horse serum	10 ml
Yeast extract	5 ml
Urea solution	2 ml
DNA	2 ml
Putrescin – dihydrochloride	1 ml
Cysteine	1 ml
Ceftriaxone	3 ml
Nystatine + Fluconazole	3 ml
Phenol red	0.5 ml

Mixed well and distributed into tubes each tube contains 2 ml (Al-Azawiy, 2012).

2.2.4.2.*Ureaplasma* Agar – Urea agar (IH Agar) :- This medium was Comprising of the following components :

PPLO agar	5.8 gm
BBL Trypton soya broth	2 gm
$MgSO4.H_2O$	0.031 gm
Yeast extract	2 gm
Agar – agar	3 gm
D.W.	165 ml

These materials were mixed well and heated to 100℃ in water bath for 5 hours then autoclaved , cooled to 57℃ , (PH = 5.5 - 6.0) then supplemented with the following supplements :

Horse serum (unheated)	20 ml
Yeast extract (25%)	4 ml
Urea stock solution (10%)	4 ml
DNA (0.2%)	3 ml
L- Cysteine (2%)	3 ml
Putrecine - dihydrochloride	3 ml
Ceftriaxone	4 ml
Nystatine + Fluconazole	4 ml
Phenol red	1 ml

These components were mixed well then poured in petridish (Al-Azawiy, 2012).

2.2.5. Colonial Morphology and Microscopic Examination

The specimens under test were inoculated in *Ureaplasma* broth (IH) at 37℃ aerobically for 2 days , with daily checking the growth for *Ureaplasma* when the broth became an alkaline (urea change) a small inoculums have been spreading on IH agar and incubated at 37℃ micro aerobically for 24hours in candle jar with small wet cotton to provide a little moisture. While (Razin and Vincent, 1983) incubation anaerobically on agar for 1-5 days , because *Ureaplasma* colonies grow within the agar , they cannot be removed with the loop, so a block of agar was cut and inverted on the surface of a fresh *Ureaplasma* agar plate (this step was repeated for 3 times) to provide a pure colonies . A single colony was picked from each primary positive culture , and forward for it is identification depend on their diagnostic characteristics represented by morphological properties (colony size , shape and color). Colonies were investigated directly by Light microscope (low power) since the colonies of *Ureaplasma parvum* as granular appearance and dark brown color due to accumulation of magnesium oxide inside and outside the colony . and then confirmed by using molecular diagnosis technique.

2.2.6. Sub Culturing

2.2.6.1-Broth to Broth Transferal :- 0.1 ml of 2-3 days ancient broth culture was transported into 2 ml new medium. All inoculated liquefied media incubated aerobically at 37℃ . (vellecea *et al* .., 1979).

2.2.6.2-Broth to Agar Transferal :- 0.1 ml of 2 days ureaplasma broth (IH) culture was streaked by using sterile loop on ureaplasma agar (IH) media , incubated an aerobically in candle jar at 37℃ . (vellecea *et al* .., 1979).

2.2.7. Maintenance of Bacterial Isolate

Bacterial isolate were maintenance as stock in (IH) broth medium supplemented with 20% glycerol then stored at -20℃ for 12 months .

2.2.8. Molecular Experiments

Molecular experiments included the extraction of *Ureaplasma* DNA .

2.2.8.1. DNA Extraction

2.2.8.1.1 Thermal Method (Biernat, 2006) :

1- Transfer cells 2ml of the samples to microcentrifuge tube and centrifugation for 20 minute at 12000rpm.

2-Discard the supernatant to wash the pellet in P.B.S (phosphate buffer solution) with centrifugation for 20 minute at 12000 rpm and Re-suspend in 50ul of D.W.

3- Boiling for 10 minutes and outcome was used directly in PCR .

2.2.8.1.2 Reagent Genomic DNA Kit Method :

This method was done according to additional technique to confirm the diagnostic by routine work . The genomic DNA purification Kit supplemented by the manufacturing company (Geneaid , USA) .

1-Single colony was inoculated in 4ml of (IH) broth , then Incubated for 48 hours at 37℃.

2-Transfer cells (up to 1×10^7) to a 2 ml microcentrifuge tube and harvest with centrifugation for 20 seconds at 6,000 x g .

3-Remove the supernatant , retaining approximately 50ul of residual buffer to re-suspend the white cell pellet by vortex .

4-Add 300ul of cell lysis buffer to the sample and mix by vortex .

5-Incubate at 60℃ for at least 10 minutes to ensure the sample lysate is clear . During incubation , invert the tube every 3 minutes.

6-Add 100ul of protein Removal Buffer to the Sample lysate and vortex immediately for 10 seconds .

7-Incubate on ice for 5 minutes then Centrifuge at 16,ooo x g for 3 minute .

8-Transfer the supernatant from step 7 to clear 2 ml microcentrifuge tube.

9-Add 300ul of isopropanol and mix well by inversion. then Centrifuge at 16,000 x g for 5 minutes.

10-Discard the supernatant and add 300ul of 70% ethanol to wash the pellet then Centrifuge at 16,000 x g for 3 minutes

11-Discard the supernatant and air dry the pellet for 10 minutes.

12-Add 50ul of TE and incubate at 60℃ for 30 minutes to dissolve DNA pellet. During incubation , tap the bottom of the tube to promote DNA re-hydration. Then DNA was stored of -20℃ .

2.2.9. Primer Preparation and PCR Identification

All primers in this study were prepared according to manufacturer's instruction by dissolving the lyophilized primers with deionized distal water to form stock solution with concentration of 100 pmol / µl, primers working solution with deionized water, using the equation C1V1 = C2V2 to get final working solution (10 pmol / µl) for both primers. PCR identification of *Ureaplasma parvum* was done according to (Kong et al ,2000). Primer for diagnosis *Ureaplasma parvum* UMS-57/UMA222 (table 2-10) are used for the amplification 326bp. Primers for detection *Ureaplasma parvum* serovars UMS-83/UMA1A, UMS3S/UMA269 , UMS-54/UMA269 and UMS14S/UMA314A , (Table 2-11) used for amplification 578 bp , 400 bp, 370 bp, 572 bp respectively. They were used for identification and subtyping of *U. parvum* to amplify the repetitive of the Multiple banded Antigen (MBA) genes of *U. parvum* serovars.

Table (2-10) PCR primer employed in the detection of *U.Parvum* .

Organism	Primer (F) (R)	Sequence (5'- 3')	Size of amplified product (bp)	Target gene
U.parvum	**(F) UMS-57**	**-82(T/C)AA ATC TTA GTG TTC ATA TTT TTT AC -57**	**326**	**5 Ends of MBA genes and upstream regions**
	(R) UMA222	**245 GTA AGT GGA TTA AAT TCA ATG 222**		

Table (2-11) PCR Primers employed for subtyping of *Ureaplasma parvum* in to serovars .

Organism	Primer (F) (R)	Sequence (5'-3')	Size of amplified product (bp)	Target gene
U.parvum Serovar 1	UMS-83 UMA1A	**F**(TTACT GTA GAA ATT ATG TAA GAT TGC) **R**(TTT CTT TTG GTT CTT CAG TTT TTG AAG)	578	MBA
U.parvum Serovar 3	UMS3S UMA269	**F** (TTA CTG TAG AAA TTA TGT AAG ATT ACC) **R**(AA CTA AAT GAC CTT TTT CAA GTG TAC)	400	MBA
U.parvum Serovar 6	UMS-54 UMA269	**F**(AAT CTT AGT GTT CAT ATT TTT TAC TAG) **R**(ACCA AAT GAC CTT TTG TAA CTA GAT)	370	MBA
U.parvum Serovar 14	UMS14S UMA314 A	**F**(AAT TAC TGT AGA AAT TAT GTA AGA TTA AT) **R**(GTT GTT CTT TAC CTG GTT GTG TAG)	572	MBA

2.2.9.1. The Reaction Mixture

Amplification reaction mixture for each gene was supported in a final volume of 20ul containing the following in table (2-12). And the total volume of mixture (20ul) was added to PCR tube master mix which content all PCR regent mentioned in table (2-8) :

Table (2-12) Contents of the PCR reaction mixture

NO	PCR reaction mixture	Volume
1	Primer (F : 10 pmol/ul)	2 ul
2	Primer (R : 10 pmol/ul)	2 ul
3	DNA template	5 ul
4	PCR water	11 ul
Total volume		20 ul

2.2.9.2. PCR Condition for Detection UMS57/UMA222

Amplification of specific gene 5' Ends of MBA genes and upstream regions for *U.parvum* made according to Kong *et al* (2000) .

Table (2-13) PCR condition for detection UMS57/UMA222

PCR Cycles	Steps	Temp	Time
	Initial denaturation	95℃	5 min
40 cycle	Denaturation Annealing Extension	95℃ 58℃ 72℃	30 sec 30 sec 1 min
	Final extension	72℃	5 min

2.2.9.3 Technique for Subtyping the *Ureaplasma parvum* in to all 4 Serovars by PCR :

Amplification of specific gene (MBA) for subtyped the *U.parvum* in to 4 serovars made according to Kong *et al* (2000) . Used in this protocol gradient PCR to get on optimum temperature for step annealing to exhibit as sharp band.

Table (2-14) PCR condition for detection UMS-83/UMA1A, UMS3S/UMA269 , UMS14S/UMA314A and UMS-54/UMA269

PCR Cycles	Steps	Temp	Time
	Initial denaturation	95℃	5 min
40 cycle	Denaturation Annealing Extension	95℃ 55-62℃ gradient 72℃	30 sec 30 sec 1 min
	Final extension	72℃	5 min

2.2.9.4.Detection of Amplified Harvests by Agarose Gel Electrophoresis

Successful PCR amplification was confirmed by agarose gel electerophoresis as suggested by sambrook and Russell (2001) . Agarose gel was prepared by dissolving 1gm of agarose powder in 50 ml of TBE(PH8) buffer Con. (2%) to detect bands of PCR and (0.5g) 1% to assess the presence of the DNA , in boiling water bath at 100℃ , allowed to cool to 50℃ and ethidium bromide at the concentration of 0.5 mg / ml was added. The combs was fixed at one end of the tray for making wells used for loading DNA specimen . the agarose was poured gradually into the tray and allowed to solidify at room temperature for 30 min . The comb was then removed gradually from the tray . The tray was fixed in an electerophoresis chamber filled TBE buffer that covered the surface of the gel , and 8 ul of each DNA sample was transferred into the wells in agarose gel after mix with 2 ul of loading dye to assess the presence of the DNA (DNA extract) while 10 ul of PCR product (amplicone) don't mix with loading dye was transferred into the wells in agarose gel, and in one well we put the 5 ul DNA ladder The electric current was allowed at 100 volt for 60 min. UV transilluminater was used for the remark of DNA bands , and gel was photographed using a digital camera .

2.2.10. Antimicrobial Agents

Antimicrobial susceptibility testing is performed against eight antimicrobial agents that include Fluoroquinolones group, Macrolides group Tetracycline's group and Aminoglycosides group as shown in table (2-15).(Kawai, *et al*, 2015), (Miura, *et al*, 2014),(Redelinghuys, *et al* , 2013). Each antimicrobial agent was prepared according to the manufactures instruction at a stock concentration of 250mg/5 ml.

Table: (2- 15) Antibiotics used in Antibiotic susceptibility testing.

Antibiotics	Concentration	Company/origin
Ciprofloxacin	250mg	Ajanta / India
Levofloxacin	250mg	Ajanta / India
Azithromycin	250mg	Kontam/China
Erythromycin	250mg	Ajanta / India
Clarithromycin	250mg	Ajanta / India
Doxycycline	100mg	Ajanta / India
Tetracycline	250mg	Ajanta / India
Gentamicin	120mg	Menarini/Italy

2.2.10.1. Antibiotic Susceptibility Testing

Antibiotics susceptibility was determined by the standard broth dilution method by using IH broth medium as previously described IH broth containing serial dilution of antibiotics and antibiotics – free controls broth were inoculated with bacterial suspension inoculation in IH broth medium . The inoculated tube were incubated 2 days aerobically at 37℃ **,** then spreading on IH agar medium and incubated anaerobically at 37℃ and read after 24 hours under compound light microscope (Olympus Japan) at X10 magnification. The MIC was defined as the lowest antibiotics concentration that totally inhibited the development the visible growth on the agar plate the tentative breakpoints used for susceptible (S), resistant (R) .

2.2.10.2. Procedure of Antimicrobial Susceptibility Testing by Broth Dilution Method

1-Culture a fixed amount of bacteria in IH broth medium in sterile test tubes number concentrations required preparation of the antibiotics .

2-Add the antibiotics are asending concentrations so that the first tube (NO.1) contains a zero concentration of antibiotics (control) then followed the second tube (NO.2) that has lower concentration of the antibiotic and followed the third tube (No.3) which contains double the concentration found in the tube (No.2) .

3- Incubate all tubes in optimum temperature (37℃) then investigate tubes after incubation to identify on tubes containing the growth in term of turbidity, The clear tubes indicate lack of growth occurring as a results of the effectiveness of the antibiotics. While in this study bacteria *Ureaplasma parvum* during growth do not appear any turbidity in broth therefor streaking on IH agar medium and incubate anaerobically for 24 hour at 37℃then investigate plates to identify on plates containing the growth in term of colonies , the clear plates indicate lack of growth occurring as a results of the effectiveness of the antibiotics.

Calculation :To preparation the concentration gradient of the antibiotics 0 , 4 , 8 , 16 , 32 , 64 , 128 , 256,… ug/ml

250mg of ciprofloxacin dissolve in 5 ml D.W , 250mg/5ml , 50mg/1ml Turn units to microgram 50 × 1000 = 50000 ug/ml = Stock 1.Suppose stock 2 = 200ug/100 ml prepare from stock 1

$C1V1=C2V \longrightarrow 50000\times V1=200\times 100 \longrightarrow V1=0.4$ ml

0.4 ml from stock 1+ 99.6 D.W Become Stock 2 = 200ug/100 ml prepare him all the concentration required and with final volume of 2 ml

1. 0 = C1V1= C2V2 = 0 ⟶ Controls
2. 4 = 200×V1= 4×2 ⟶ V1= 0.04ml
3. 8 = 200×V1= 8×2 ⟶ V1= 0.08 ml
4. 16 = 200×V1= 16×2 ⟶ V1 = 0.16 ml
5. 32 = 200×V1= 32×2 ⟶ V1 = 0.32 ml
6. 64 = 200×V1= 64×2 ⟶ V1 = 0.64 ml
7. 128 = 200×V1= 128×2 ⟶ V1 = 1.28 ml
8. 256 = 200×V1= 256×2 ⟶ V1 = 2.56 ml

2.2.11. Statistical analysis

The data was analyzed using SPSS statistic software version 20. For comparison of qualitative variables. Using T test, Chi-square test, association between *U. parvum* infection and recurrent abortion was statistically significant (P-value <0.05) & odd ratio. (Dhawan, *et al* 2012).

1-Descriptive statistics :

A-Table creation included (Number, percentage, Mean, Standard deviation)

B-Graphical presentation(Figure histogram, Bar chart).

2- Inferential statistics (Chi-square, T test, one way Anova)

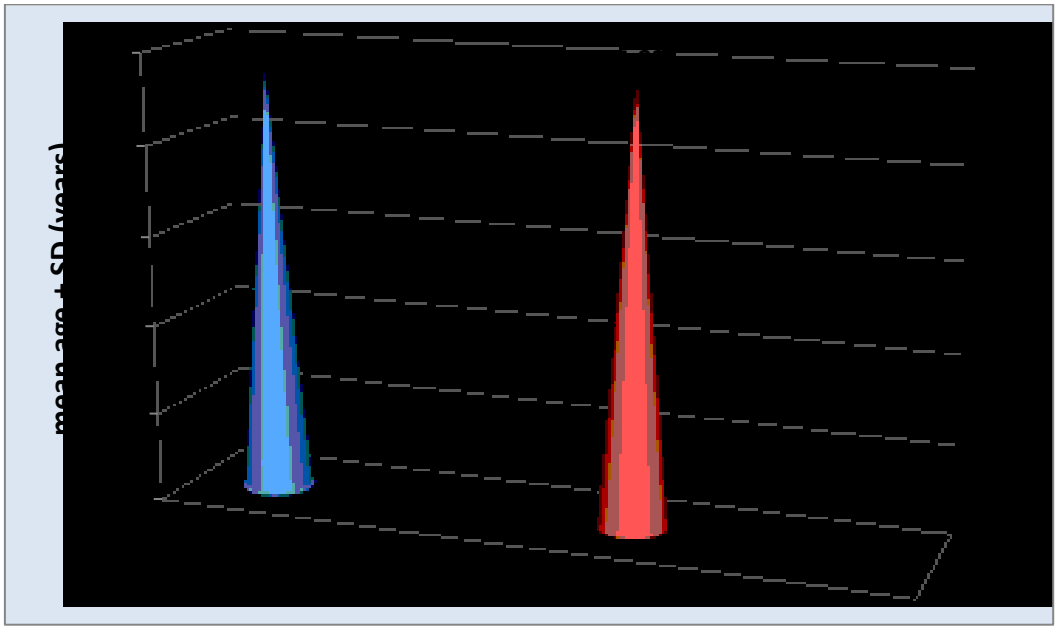
Chapter three

Results and Discussion

Results and Discussion

3.1. The Specimens

A total of 170 specimen were collected,130 specimens were collected from married woman with recurrent miscarriage include (72 vaginal bleeding, 41 vaginal swab and 17 urine) and 40 specimen were collected from control include (25urine and 15 vaginal swab) and check up for colonization with *Ureaplasma spp*. Mean age was 27.93+6.8 (34.73) years of patient group with a range of 17-45 years, while the mean age was 29 +5.83 (34.83) years of control group with a range of 19-45 years, as shown in figure (3-1). There was no statistical difference in mean age between patient and control group (P > 0.05).This finding is required to prove age matching between control group and patients group for such a case control study.



p- value = 0.342, non statistical difference (P > 0.05)

Figure (3-1) Difference in mean age of patient and control group.

3.2. Comparison Between Age Groups of Miscarried Women and Percentage of Isolation of *Ureaplasma spp*.

The results of this study showed that the age patients range 17-26 &27- 36 years old represented a high rate (46%), (40.7%) respectively compared with group (37-46) represented (13.3%) as shown in figure (3-2) included in this study. The incidence of *Ureaplasma* spp. infection was also reported by some other studies as higher in age 26-30 years .(Gupta *et al*, 2009). The high isolation detection in miscarried women of age group 17-26 & 27-36 years. This may be attributed to the sexual activity of females together with hormonal change, so the genital tract is more susceptible for infection (Domingues *et al*., 2003; Demba *et al*., 2005). The high rate of *Ureaplasma spp.* infection detected in this study suggests that this agent is widespread among miscarried women.

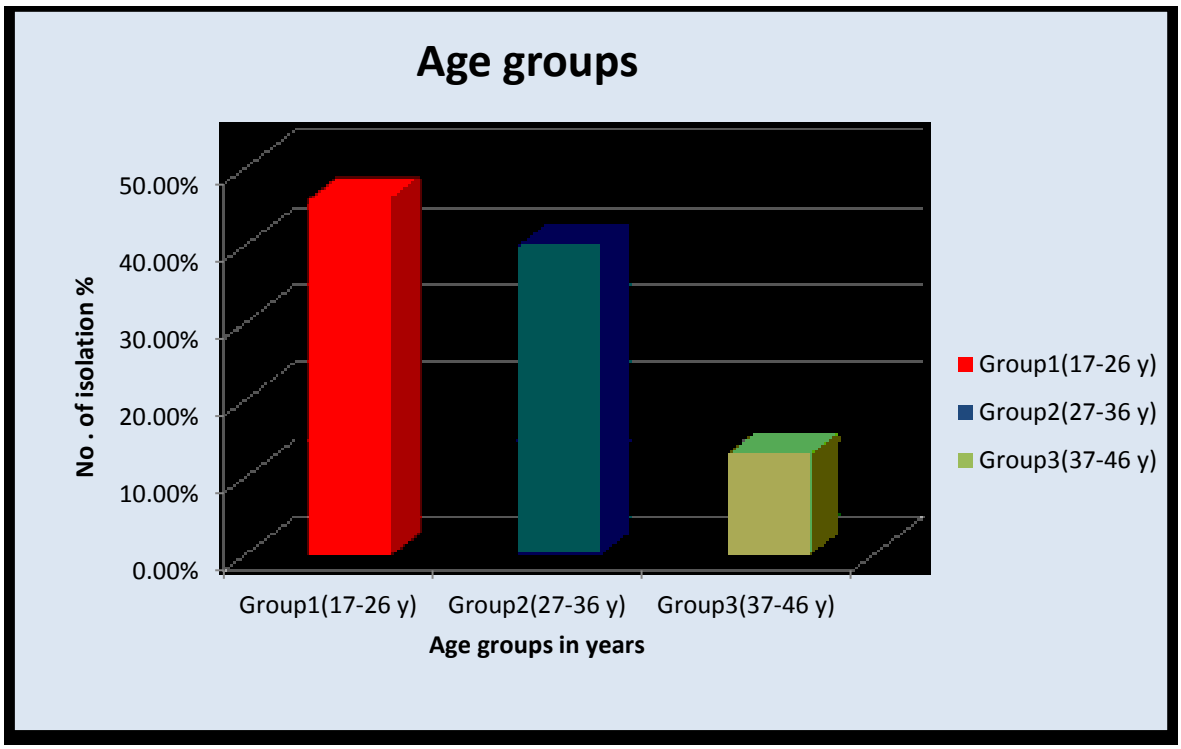


Figure (3-2) Histogram showing the distribution of *Ureaplasma spp.* according to 10 years age intervals of patients with recurrent Miscarriage.

This can be attributed to the sexual activity among this groups since there is an increased in estrogen hormone produced from female genital tract leading to change the vaginal environment which is regarded as a factor for infection (Quan, 2000 and Zeighami *et al*., 2009) because the estrogen is important hormone during pregnancy , it makes to adjust the level of the hormone progesterone is essential in the formation and development of the fetus, so pregnancy is one of the reason leading to the rise in the hormone estrogen (Lee *et al*., 2013). For this reason, when the increased of estrogen, it affects the hormone progesterone is necessary in pregnancy, which affects the thickness of the lining of the uterus and the difficulty of adapt fetus with her. However it is generally difficult to determine whether these agents cause colonization or infection. Since the incidence of infection is affected by some factors, such as: menstrual cycle, bacterial and protozoan infection (co-infections), and socio-economic conditions like poverty. (Cordova *et al*., 2000). Also the age group 17-26 & 27-36 years are the most widely accepted for marriage and reproduction in our society for this reason the proportion of isolation the *Ureaplasma spp*. be high . It has been shown in this study that the percent of isolation of *Ureaplasma spp*. was directly associated with age .The isolation rate decrease in 37-46 years this may be due to the changes that associated with a decrease in the incidence of genital *Ureaplasma*. also due to vagina multilayer lining are atrophic in ages 37-46 years old. All of these reasons associated with decrease in the incidence of genital *Ureaplasma* spp. From the results of this study, can conclude that the genital *Ureaplasma* were significant correlation with age. Statistical analysis: (P-value = 0.001) appeared highly significant between patients and controls according to age interval with ($P< 0.05$).

3.3. Laboratory Identification of *Ureaplasma spp.* (Colonial Morphology)

In this study *Ureaplasma spp.* isolates was identified by examination of colonial morphology on IH agar media used in this study as dark golden-brown or rich deep brown and granular appearance . All isolates revealed positive urea analysis . These results were in accordance with (Miles & Nicholas , 2010 ; Agbakba *et al*., 2008) .

3.4. Isolation of *Ureaplasma spp.* on Culture Media

Detection of *Ureaplasma* spp. is possible by characteristic growth on appropriate culture media but species identification of *U.parvum* along with serovar identification by molecular method is important especially for correct interpretation of laboratory results and evaluation of pathogenicity. In present study, *Ureaplasma* broth media (IH broth) & *Ureaplasma* agar media (IH agar) were used to isolate *Ureaplasma spp.* the rate of isolate are (52.3%) from vaginal bleeding, (30.7%) from vaginal swab and (7.6%) from urine .The reason for the high isolation of *Ureaplasma spp.* on IH medium can be attributed to supplementation with DNA , Putrecine – dihydrochloride, cysteine which enhance the microorganisms with lipid materials essential for growing of these bacteria . Also a mixture of antibiotics was used (Ceftriaxone , Amoxicillin , Augmentin , Nystatine , & Fluconazole) to prevent contamination may occur in the conventional broth media. The amount of horse serum reduced from 20 ml to 10 ml to reduce the cost and it is sufficient for growth of these microorganisms . *Ureaplasma* agar media (IH medium) is supplemented with essential requirements for growing of these microorganisms. These are horse serum , yeast extract , urea , cysteine , antibacterial and antifungal that inhibited the growth of other bacteria and fungi and the optimal PH (6.0) (Al-Azawi, 2012).

The medium contains urea and sensitive indicators of ammonia , Manganous sulfate which is firstly used by (shepard & Lunceford, 1976; Sudolska, 2006) sulfate salt of manganese was described to support the growth . Manganous sulfate was added in a final concentration of 0.03% was therefore , selected as the ammonia – detecting reagent of choice . *Ureaplasma spp.* colonies appeared within 2 days , identified as dark golden-brown owing to accumulation of manganese oxide in the colony.

Moreover, putrecine – dihydrochloride was added to enhance the *Ureaplasma spp.* growth and development of the precipitate in the colonies (Phillips *et al* , 1986). in addition the size of *Ureaplasma spp.* were seen to be larger when add putrecine – dihydrochloride. The originality of this media can be attributed to horse serum (10%) instead of (20%), yeast extract as a powder , BBL trypton soya broth together with PPLO, also used DNA and putrecine in same medium . Moreover the mixture of antibiotics prevent contamination of medium by bacteria and fungi that easily contaminate conventional media . Moreover, agar – agar was added to the medium was more than 3% which believed it inhibited the bacterial growth (Al-Azawi, 2012). Therefore, IH media considered as enrichment, differential and selective for *Ureaplasma spp.* because it resolved the important problem of culturing these bacteria.

Other study used IH medium for isolate *Mycoplasma spp.* and *Ureaplasma spp.* (Al-Talqani & Al-Musawi, 2015) isolated *Mycoplasma hominis*, *Ureaplasma Urealyticum* and *Ureaplasma Parvum* on IH medium from infertile male in rate (5.8%),(5.8%) and (3.5%) respectively. Also (Al-Nassri, 2016) isolated *Mycoplasma Pneumonia* in rate (18%) on IH media from tonsillitis patients. Another study was isolated *Ureaplasma Urealyticum* in rate (16.8%) from infertile female, (20%) from infertile male and isolated *Mycoplasma hominis* in rate (27.7%), (1.6%) from infertile female and male respectively on MAU-medium similar to IH medium. (Al-Karawy, 2015). In the present study

the results on IH medium *Ureaplasma spp*. was identified as dark golden brown or rich deep brown colonies Figure (3-3). Moreover , *Ureaplasma spp.* was identified by its granular appearance Figure (3-4) .

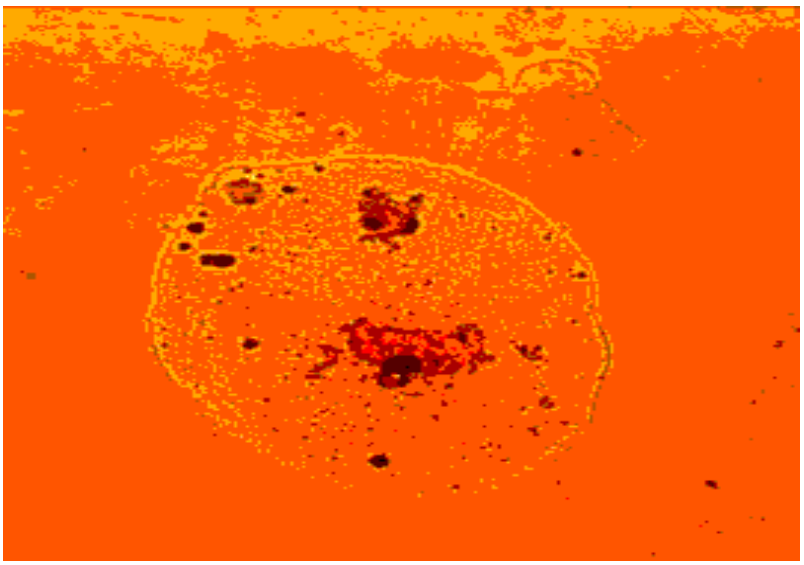


Figure (3-3) *Ureaplasma spp.* as dark golden- brown colonies in IH medium under light microscope 10X

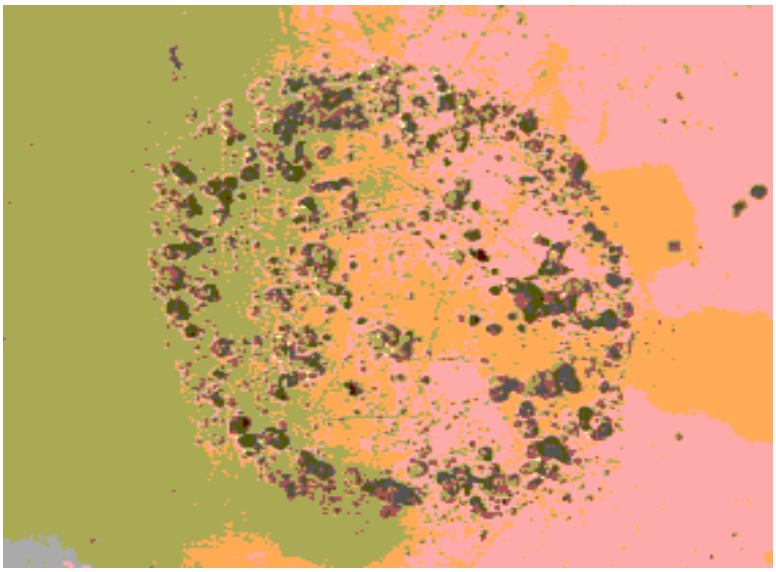


Figure (3-4) *Ureaplasma spp.* as granular appearance in IH medium under light microscope 10X

The dark golden-brown or rich deep-brown colonies are magnesium oxide which accumulate due to hydrolysis of urea by urease produced by *Ureaplasma spp.* , liberates hydroxyl group from water , and these hydroxyl moieties oxide magnesium sulfate to magnesium oxide , causing the deposition of golden brown precipitate in the colonies themselves and near it Figure (3-5), (Shaperd, *et al* 1972; Muralidhar, *et al*, 2014).The dark golden-brown or rich deep-brown colonies are manganese reaction product (manganese dioxide) within and directly on the surface of *Ureaplasma* colonies.

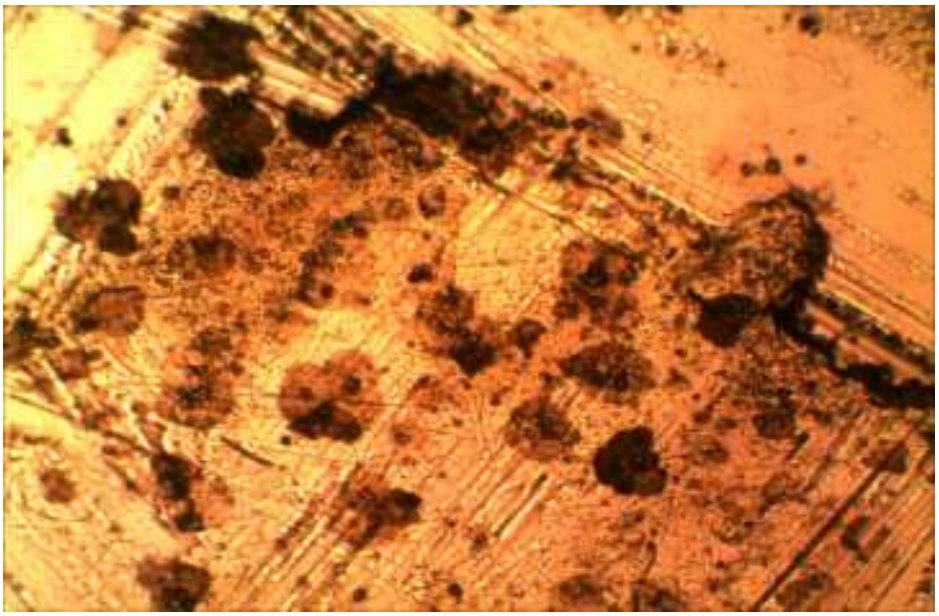


Figure (3-5) Accumulation of manganous oxide due to growth of *Ureaplasma spp.* in IH medium under light microscope 10X

The results of culturing revealed that the size of the colonies are large and dark golden-brown or rich deep-brown colonies after two days incubation in IH broth compared to the size of the colonies as a small light golden brown colonies after one day incubation in IH broth, because the colonies growth is not complete yet fully. and the size of the colonies as a not clear light brown colonies after three days incubation in IH broth, because the colonies begin to unravel after a nutrient deficiency, figure (3-6).

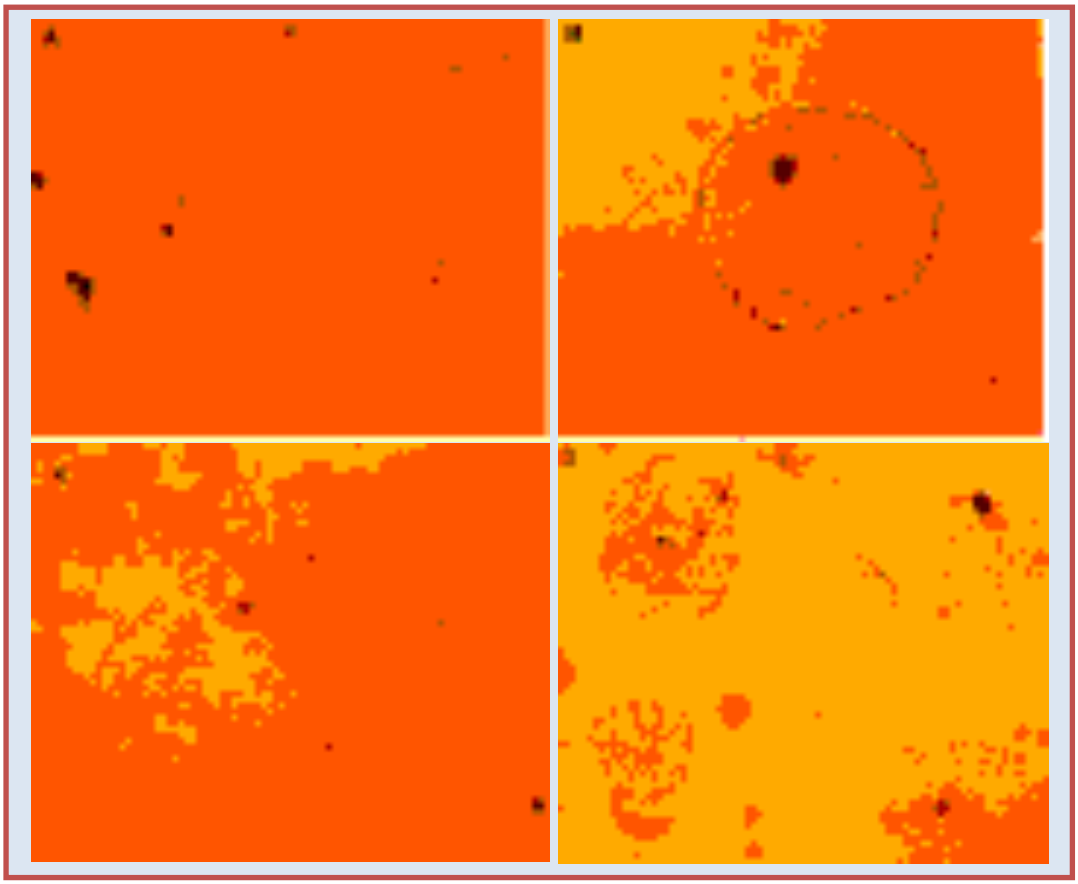


Figure (3-6) A: *Ureaplasma spp.* in IH agar was identified as a small light golden – brown colonies after one day incubation in IH broth under light microscope 10X.

B & C : *Ureaplasma spp.* in IH agar was identified as a dark golden – brown colonies after two days incubation in IH broth under light microscope 10X.

D: *Ureaplasma spp.* in IH agar was identified as a not clear light brown colonies after three days incubation in IH broth under light microscope 10X.

The stages of colony morphogenesis were reconstructed after examining several hundred colonies during various periods of growth. The time necessary for the complete development of a colony varied with the species, but was approximately 2 days for *Ureaplasma spp.* Therefore, to obtained optimum growth and clear colonies for *Ureaplasma spp.* preferably specimens incubation two days in IH broth then subculture in IH agar. The present study findings indicate that growth of *Ureaplasma spp.* grows poorly at the surface and most of the colony appears to be embedded in the agar due to the rapid development and

the small size of these colonies, it was difficult to characterize the initial stage of growth of *Ureaplasma spp.* (Giovanni, *et al*, 1980 ; shaperd, *et al*, 1972).

3.5. Relationship Between The Isolation of Genital *Ureaplasma spp.* and Type of Specimen .

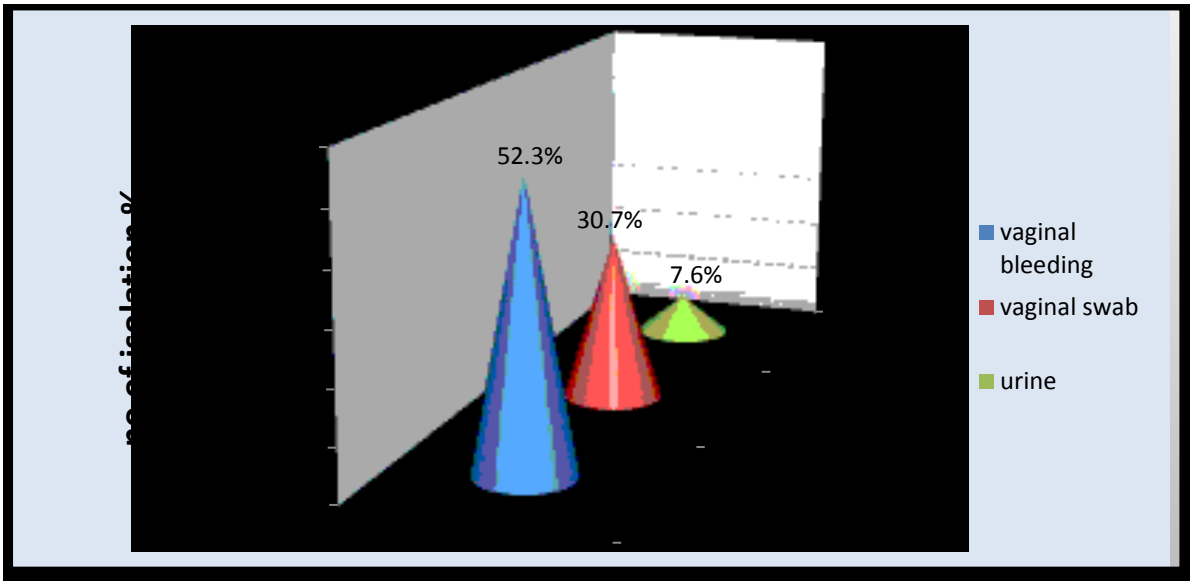


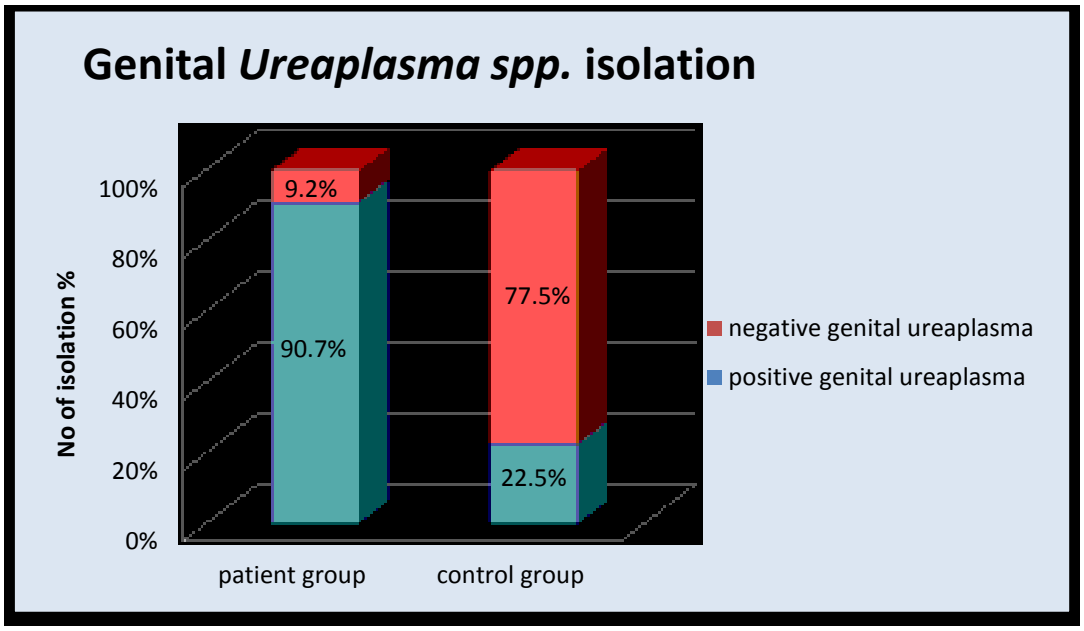
Figure (3-7) Distribution of genital *Ureaplasma spp.* isolate among clinical specimen.

Figure (3-7) shows the distribution of bacterial isolate of genital *Ureaplasma* according to the clinical specimen . The results exhibited that vaginal bleeding from miscarried women given high percentage of isolation (52.3%) followed by vaginal swab (30.7%) and urine (7.6%) . According to the available knowledge genital *Ureaplasma spp.* are not screened by routine examination of vaginal bleeding , urine , vaginal swab from miscarried woman in health laboratories in Iraq. Little studies that working culture examine for detection of these organisms in specimens taken from miscarried woman. The results obtained by this study explain isolated genital *Ureaplasma* in percentage more than 38% from vaginal bleeding samples, this may be due to dysfunction of placenta and the vaginal bleeding is a marker for placental dysfunction, vaginal bleeding is most likely to be seen around the time of the luteal-placental shift (Yang, *et al*, 2005). There has been little investigation of first trimester bleeding . It is interesting that the peak

in bleeding episodes coincides with the development of a hormonally functional placenta. In very early pregnancy, the corpus luteum produces progesterone. The shift from luteal production to placental production of progesterone occurs by the seventh week of pregnancy and can result in a temporary reduction in progesterone levels if the placenta is not producing sufficiently.(Gomez, *et al*, 2005). Decreasing levels of progesterone are associated with the onset of menses outside of pregnancy; similarly, during pregnancy, decreasing levels may trigger an episode of vaginal bleeding and limit successful maintenance of the pregnancy. Thus, bleeding at this time in pregnancy may signal that the early placenta has not developed optimally (Harville, *et al,* 2003). One of the routes of intrauterine infection with *Ureaplasma spp.* by hematogenous dissemination through the placenta this mechanism occur by which microorganisms are able to pass through the cervix, infect the maternal and fetal (chorioamnion) layers of the placenta and often access the amniotic fluid and outcome common intramniotic infection lead to abortion. (Goldenberg *et al,* 2000).

An explanation for these variations may be related to the type of specimens investigated for isolation, the methods used for transport and storage , and media used for primary isolation of *Ureaplasma* spp. In light of the observation and experience during this study, centrifuged urine sediments yield more positive cultures than the urine specimen without centrifuged .

3.6. Frequency of Isolation and Identification



(OR)=33.8, P-value=0.001,Statistical analysis under P-vaule < 0.05

Figure (3-8) percentage of isolation Genital *Ureaplasma* from women with recurrent miscarriage and control.

The positive results of isolate constructed, a total of 130 specimen were collected from married woman with recurrent miscarriage , while 40 specimen were collected from controls. The result of culturing revealed that 118/130 (90.7%) were positive from married woman with recurrent miscarriage versus 9/40 (22.5%) were positive from controls while 12/130 (9.2%) were negative from married woman with recurrent miscarriage versus 31/40 (77.5%) were negative from controls. as shown in Figure (3-8).

In this study the prevalence of *Ureaplasma spp*. was 118 out of 130 (90.7%) in women with recurrent miscarriage and 9 out of 40 (22.5%) in control group and the association between *Ureaplasma spp*. infection and recurrent miscarriage was statistically highly significant (P<0.05) between woman with recurrent miscarriage and controls. Considering the relatively similar sample size and study method, analysis the role of *Ureaplasma spp*. in recurrent miscarriage.

The results of the study demonstrated that colonization of the lower genital tract with *Ureaplasma spp.* can produce asymptomatic infection of the upper reproductive system in women and microorganisms can colonize the endometrium of non-pregnant women and therefore may infect the embryo at the time of implantation. *Ureaplasma* have been isolated from the endometrium of non-pregnant women undergoing diagnostic hysteroscopy for infertility. In these women, *Ureaplasma* colonization of the endometrium was not associated with inflammation or clinical signs of endometritis, indicating that *Ureaplasma* were present as asymptomatic colonizers. (Onderdonk, *et al*, 2008).

These findings also imply that *Ureaplasma spp.* may be present in the upper genital tract at the time of conception and might be involved in adverse pregnancy outcomes by Intrauterine infection the mechanisms by which intrauterine infections lead to preterm labor and recurrent miscarriage are related to activation of the innate immune system. Microbial products bind to TLRs and activate proinflammatory cytokine production resulting in the subsequent stimulation of prostaglandins, other inflammatory mediators and matrix-degrading enzymes. Prostaglandins stimulate uterine contractility, whereas degradation of the extracellular matrix in the fetal membranes leads to PPROM (Passosl, *et al* 2011).

Ascending of *Ureaplasma* from the vagina and the cervix is a frequent route of Intrauterine infection and important mechanism leading to recurrent miscarriage and many investigators believe that ascent of microorganisms from the lower to upper genital tract occurs during the second trimester, but the precise timing remains undetermined (Larsen, & Hwang , 2010). The female upper genital tract is traditionally considered to be a sterile anatomical site, microorganisms causing infections of the upper genital tract during pregnancy are predicted to gain access to the chorioamnion, amniotic fluid and fetus by numerous mechanisms (Romero *et al*., 2007).

The recovery of *Ureaplasma* from the chorioamnion increased with the duration of rupture of fetal membranes, which suggested that ascension from the lower genital tract may be a primary source of infection. And that bacteria are able to invade the female upper genital tract during pregnancy by migration from the abdominal cavity through the Fallopian tubes, or by an invasive ascending infection. Of these routes, an ascending infection from the vagina is predicted to be the most common mechanism resulting in intra-amniotic infection. (Zervomanolakis *et al.* 2007). Other studies demonstrated that both woman with recurrent miscarriage and controls are encountered for *Ureaplasma* infection (Nigro , *et al* , 2011) isolate *Ureaplasma Spp.* from woman with recurrent miscarriage and healthy asymptomatic pregnant women. (Ahmadi, 2014) isolated genital *Ureaplasma* in 26 out of 218 cases (11.92%). The prevalence of *Ureaplasma spp.* infection was 18 out of 109 (16.5%) in case (spontaneous miscarriage) and 8 out of 109 (7.3%) in case control groups, in Iran . However , some other studies detected these organisms in high rate approximately (26%) of pregnant women and (15.3%) of the controls in Turkey (Aydin *et al,* 2010*)* . Another study detected *U.parvum* In total, 19 (47.5 %) of the 40 samples were positive for *Ureaplasma* spp. by culture (Kasprzykowska , 2014) . In a previous study was investigated using culture of endocervical swabs it found the spontaneous miscarriage group, the rate of *Ureaplasma* infection, was 74.1% (43/58), but in the normal group, the rate was 48.0% (24/50) and their difference was significant ($P<0.01$) (Ye LL, *et al*, 2004) .

In conclusion , routine bacterial cultures are not sufficient to recover genital *Ureaplasma* . Furthermore , *Ureaplasma spp.* accretion colonies were difficult to identify , often appearing small and lacking in precipitate formation. Long time of cultivation may lead to contaminate of the growth by fungi and bacteria which grow rapidly on conventional media . These observations were observed by many workers (Vincent,2012). IH media were modified to contain more growth

enhancers to facilitate cultivation of genital *Ureaplasma* , reduced the time consumed for growth , inhibit the contaminant bacterial and fungal types.

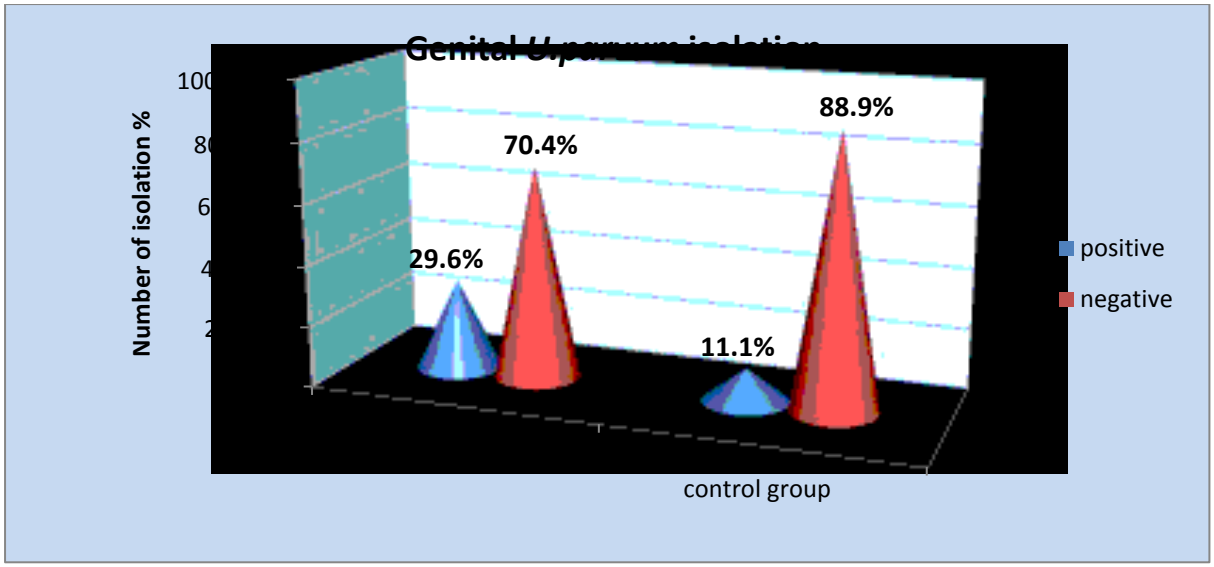
3.7. Relationship Between Women with Recurrent Miscarriage and Percentage of Isolation of *Ureaplasma spp.*

In this study frequency of *Ureaplasma spp.* was high in miscarried women (90%). Dhawan *et al*, (2012) isolated *Ureaplasma spp.* from miscarried women in India in rate up to our results (95%).Other study in Poland, (Alicjia. *et al* , 2009) isolated *Ureaplasma spp.* in (74.5%) from women with Atypical squamous cells of undetermined significance (ASCUS) , Low-grade squamous intraepithelial lesion (LSIL), & High-grade squamous intraepithelial lesion (HSIL) . Moreover , Kong *et al* , (2000) in Australia were isolated *Ureaplasma spp.* in rate (79.5%) from vaginal swab of pregnant women with sexually transmitted disease and abortion. *Ureaplasma spp.* responsible for triggering autoimmune response. (Bayoumi *et al*, 2006) determined that the antiphospholipid antibody syndrome is considered as autoimmune disease which causes recurrent miscarriage characterized by elevated titer of phospholipid antibodies : Lupus anticardiolipin antibodies (ACL) . These antibodies are believed to cause thrombosis in maternal circulation, leading to the events that lead to fetal losses. A possible pathophysiologic explanation is that the microorganisms are able to ascend from the cervix through the internal ostium and colonize the fetal membranes . They as well as neutrophils and lymphocytes produce an inflammatory response that may destroy or weaken the fetal membrane . It is demonstrated that microorganisms produce mucinase that may hydrolysis protective cervical mucine as well as immunoglobulin A (IgA) protease, which can destroy mucosal membrane IgA, an important element of the reproductive tract immune system . Bacteria may also an increase of arachidonic acid and prostaglandins , increasing the frequency of preterm non-labor uterine contraction. Vaginal , cervical and fetal membrane infection is associated with maternal sepsis , uterine contractility disorders (Choi *et al* ., 2012). Compared to other microorganisms that cause disease in humans,

very little is known about the host immune response generated during in utero *Ureaplasma* infection. Throughout pregnancy the immune system integrates the maternal immune response and the fetal-placental immune response, and is associated with both pro-inflammatory and anti-inflammatory stages (Mor *et al.*, 2011). The developing fetus is generally considered to be immune naïve; however, the expression of fetal innate immune factors increases over gestational age and maternal IgG antibodies are transported across the placenta and reach 10% of circulating maternal levels by 17-22 weeks of gestation and 50% by 28-32 weeks of gestation (van den Berg *et al,* 2011). IgM is also detectable within umbilical cord blood at 22 weeks of gestation (Marconi, *et al,* 2011); therefore, the fetus is capable of mounting an immune response in utero.

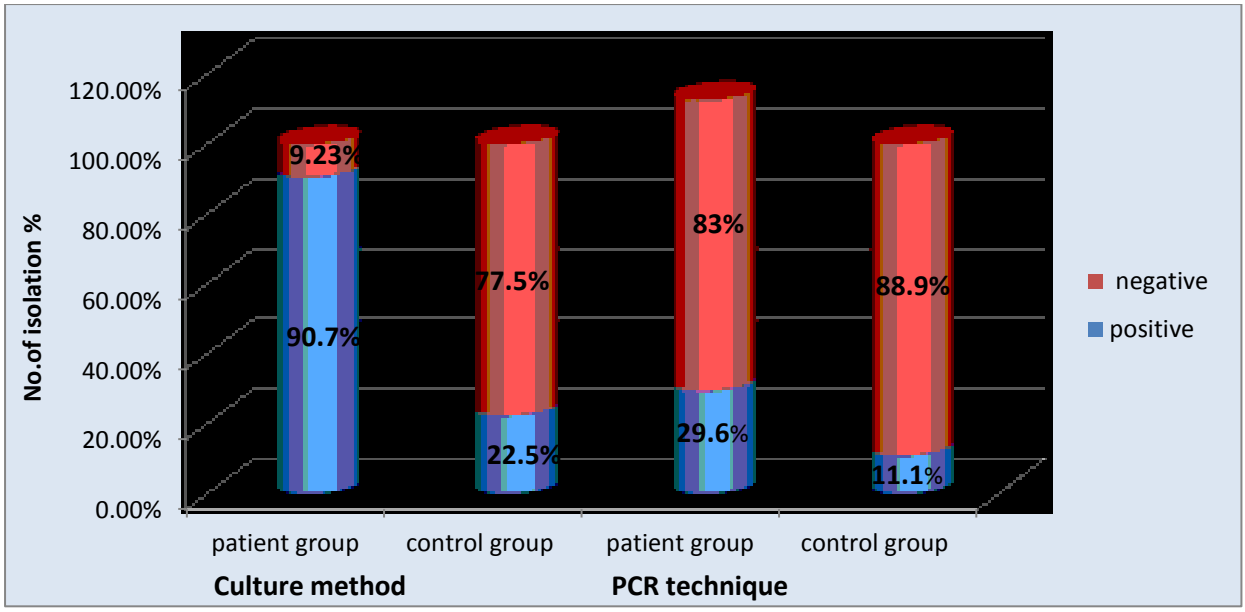
3.8. Molecular Detection for Diagnostic of *Ureaplasma parvum* by Polymerase Chain Reaction (PCR)

Accordingly PCR technique was also suggested to be included in this study particularly it is little previous study according to our knowledge in Iraq employed this technique for diagnostic of *Ureaplasma parvum*. The DNA was extracted from positive culture, amplification by PCR . The primer targets used in this study were based on our pervious observation that the heterogeneity of the intergenic spacer regions is greater than that within the genes (Kong *et al* , 2000). The Multiple banded antigen(MBA) contain both species and serovar-defining regions. To improve sensitivity and specificity and to provide more choice we used species specific primers based on (Kong *et al* , 2000). The primer pair UMS57-UMA222 was specific for *Ureaplasma parvum*. (Agbakoba, *et al*, 2008; Fouad, *et al,* 2014). The selected isolates were taken from clinical cases (miscarriage). *U.parvum* can be detected rapidly and accurately by PCR such that treatment may be established in the early stages of infection , also this method avoids the problems associated with culturing (Nassar, 2007; Cultrera, 2006). *U.parvum* was identified in UMS57/UMA222 primer 35/118 (29.6%) in patient group, 1/9 (11%) in control group by using PCR , as shown in figure (3-9).



P-value = 0.001, OR = 3.37, Statistical analysis under p-vaule < 0.05

Figure (3-9) Occurrence of *Ureaplasma parvum* among patient group and control group.



P-value = 0.001 Statistical analysis under p-vaule < 0.05

Figure (3-10): Occurrence of *Ureaplasma Spp.* in patients groups and control group by using two methods.

The results showed in Figure (3-10) the Efficiency of different laboratory investigations in *Ureaplasma parvum* between patient group and control group. To access the validity of tested techniques (Culture, and PCR) in isolation and identification of *Ureaplasma parvum* obtained from women with recurrent miscarriage. Result showed that out of the 130 patient cases, 118 (90.7 %) were positive versus 12 cases (9.2%) of negative culture. While in the control 9 cases (22.5%) gave positive culture and 31(77.5%) were negative culture. By using polymerase chain reaction technique, out of the 118 patient cases, 35 patient cases (29.6 %) gave positive results, and 83 patient cases (83 %) gave negative results. In the control 1 cases (11.1%) were positive and 8 cases (88.9%) gave negative. In this study all the this results might be revered to the fact that *Ureaplasma* are normal inhabitant in asymptomatic individuals. The RCR assay was more accurate in detection of *Ureaplasma parvum* than culture methods. The sensitivity of detection *U.parvum* by PCR assay is connected with the nature of the target gene (MBA) by the efficiency of primers binding which determines the efficiency of amplification. (Knox, *et al* , 2003; Agbakoba, *et al*, 2008; Al-Talqani & Al-Musawi, 2015). The nucleic acid based technique have several advantages than culture methods, including rapid results and specific organism detection. This is critical in a hospital setting, since rapid pathogen detection is important for faster and improve patient treatment. (Qasema, *et al*, 2002; Mothershed & Whitney, 2011).

Size variation of the MBA has also been associated with different severities of histological chorioamnionitis in a sheep model of intra-amniotic *Ureaplasma* infection, clinical *U. parvum* serovar 6 strain produced MBA size variants within the amniotic fluid of pregnant sheep. (Knox *et al.* 2010). Therefore, it was suggested that the number of MBA size variants produced within the amniotic fluid may contribute to the pathogenesis of intra-uterine *Ureaplasma* infection. It has also been suggested that size/phase variation of the MBA may be a mechanism by which *Ureaplasma* are able to avoid recognition by the host

immune response (Zimmerman *et al*, 2009). As the MBA is predicted to be the major virulence factor of *U. parvum*, further investigation is required to determine the role of this surface-exposed antigen.(Dando, 2012). The results of this study revealed positive *Ureaplasma parvum* isolates 35(29.6%) by using primer UMS-57/ UMA222 target gene 5' end of MBA gene as shown in figure (3-11).

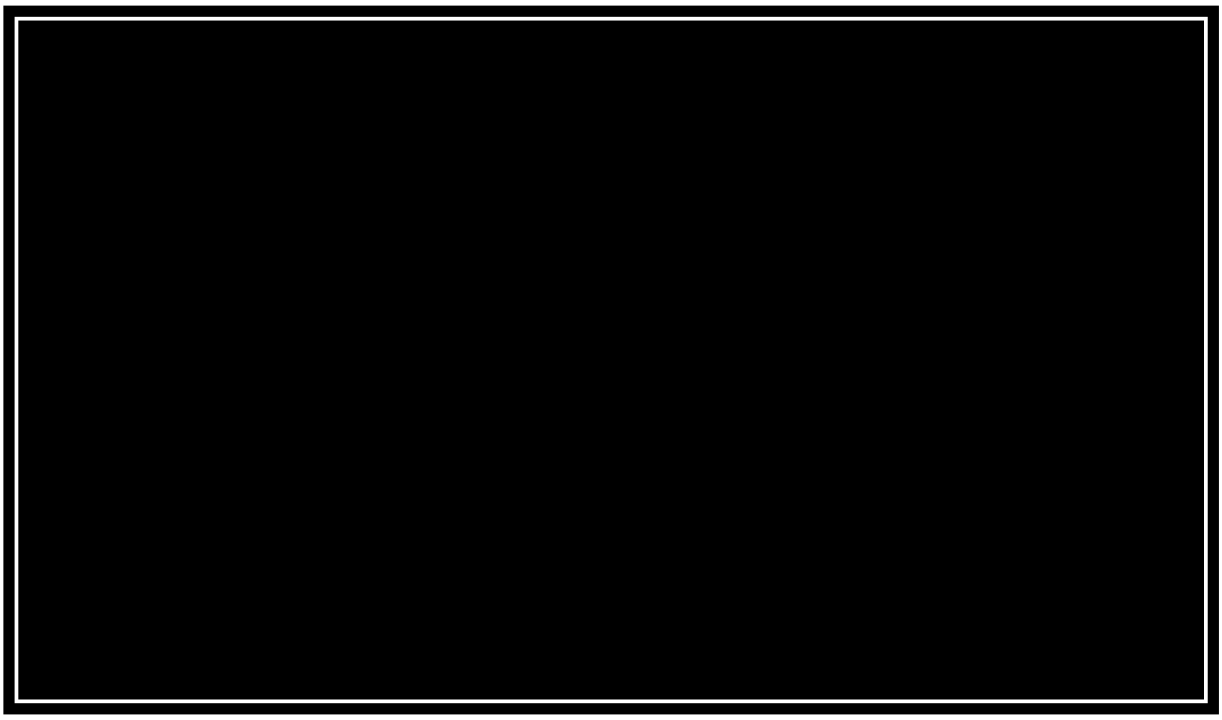


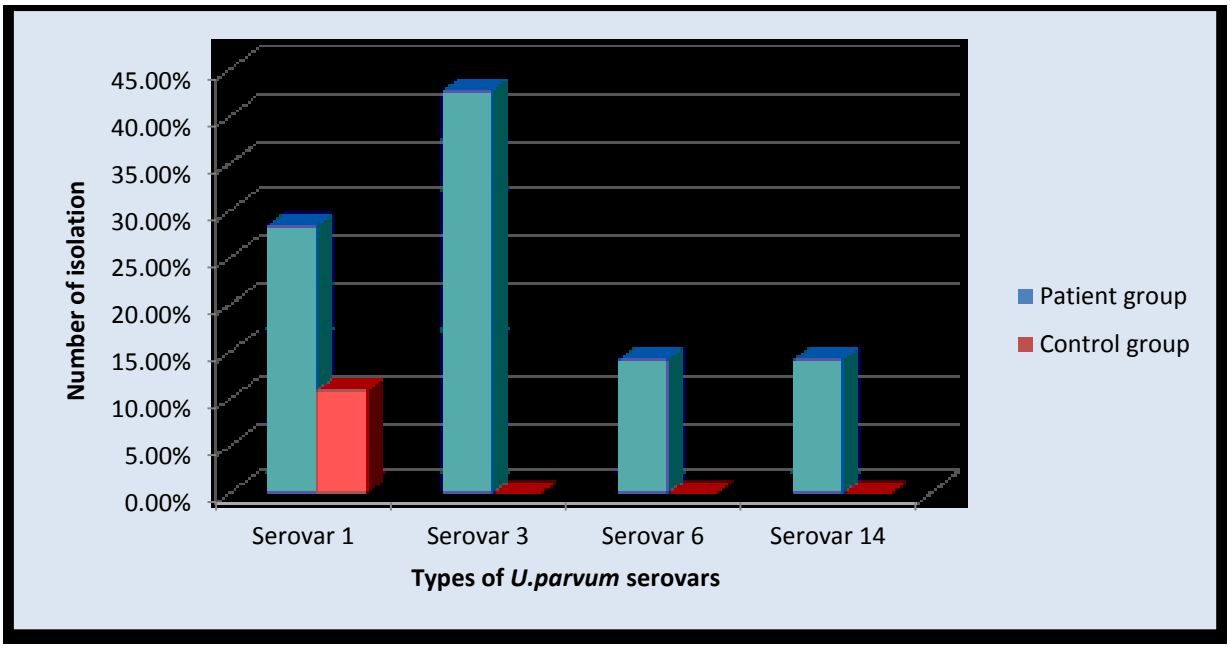
Figure (3-11) Ethidium bromide stained agarose gel showing PCR amplification product with (326 bp) primers for *U.parvum* . M 100 bp standard size reference marker . Lanes (1,3,5,6,7,9 &11): *U.parvum* positive results. Lanes (2,4,8,10 & 12) :*U.parvum* negative.(Agarose Con. 2% & Voltages 100).

The negative isolates may be due to that *Ureaplasma* are divided into two species. These are *U.parvum* and *U.urealyticum* , these two species can not identify by phenotypic and only identified by genotypic (kong *et al*., 2000; O'Sullivan *et al* 2011; Dando, 2012; Payne *et al*, 2014). So the negative results may be *U.urealyticum* rather than *U.parvum* and the results appeared to be attributable to a higher proportion of women with recurrent miscarriage in whom *U.parvum* were found the reason for this results is uncertain but it could be due to hormonal effects (estrogen hormone and progesterone hormone) which

could increase *U.parvum* counts and thus the likelihood of detection during pregnancy. From the results of this study, *Ureaplasma parvum* infection may be an important etiologic agent of recurrent miscarriage,. In study from Japan , PCR was used for determination the *Ureaplasma* spp. With non-gonococcal urethritis (NGU) and isolated *U.parvum* in rate 54% (21of 39) (Yamazaki *et al* 2012). Another study detected *U.parvum* in pairs of samples as many as 5 (18.5 %) of the 27 infertile women and 1 (7.7 %) of the 13 fertile women showed infection of the upper genital tract with *U. parvum*. (Kasprzykowska,2014). Other studies was isolated *Ureaplasma parvum* in rate (20%) from women with recurrent miscarriage and women with sexually transmissible diseases (STD) in china by using PCR technique. (Kong, *et al*, 1999). While *Ureaplasma parvum* was isolated in rate (25%) (Dhawan *et al* , 2012) from women with symptoms of urethral, cervical discharge, genital pruritis, dysuria in India. However, some other studies detected these organisms in high rate approximately (79%) from pregnant women and women with sexually transmitted disease in Australia (Kong ,*et al*, 2000; Payne, *et al*, 2014).

3.9. Subtype of *Ureaplasma parvum* Based on MBA Genes

Polymerase chain reaction (PCR) amplification occurred when DNA extracted from *Ureaplasma* species tested with primer pairs (UMS-57-UMA222) which specific *U.parvum* and amplified for all 4 serovars of *Ureaplasma parvum*. *U.parvum* isolates revealed positive results for primer pairs (UMS-57-UMA222) were further subtyped into different serovars. Serovars of *Ureaplasma parvum* could be identified with primer pairs as follows: UMS-83-UMA1A amplified serovar1, UMS3S-UMA269 amplified serovar3, UMS-54-UMA269 amplified serovar6 and UMS14S-UMA314A amplified serovar14. Statistical analysis showing highly significant between patient and controls group according to isolation of *U.parvum*. The results of this study revealed *Ureaplasma parvum* isolates were further subtyped into different serovars as shown in figure (3-12) .



*Statistical analysis include (P –value = 0.001 , Chi-square = 38.43) P-value < 0.05

Figure (3-12): Distribution of *Ureaplasma parvum* serovars among patient group and control group.

The results showed in figure (3-13) positive isolates for *U.parvum* serovar1 by using UMS-83-UMA1A primer pairs and positive isolates for *U.parvum* serovar3 by using UMS3S-UMA269 primer pairs. And the results revealed in figure (3-14) positive isolates for *U.parvum* serovar6 by using UMS-54-UMA269 primer pairs and positive isolates for *U. parvum* serovar14 by using UMS14S-UMA314A primer pairs and positive isolates for *Ureaplasma parvum* serovar3 by using UMS3S-UMA269 primer pairs.

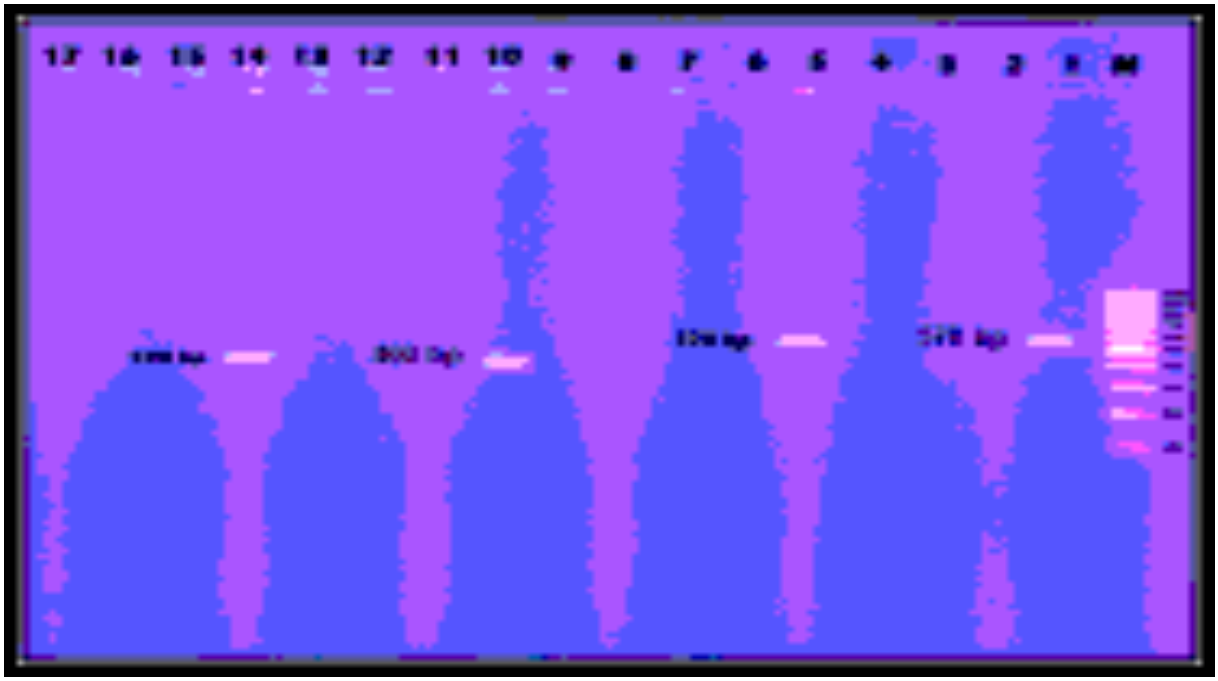


Figure (3-13) Results of PCR amplification for identification of serovar 1 (578 bp) and serovar 3 (400 bp). M 100 bp standard size reference marker. Lane (1,5): serovar 1 positive results. Lane (10, 14): serovar 3 positive results. Lane (2, 3, 4, 6, 7, 8, 9,11, 12, 13, 15, 16, 17): Negative samples. (Agarose Con. 2% & Voltages 100).

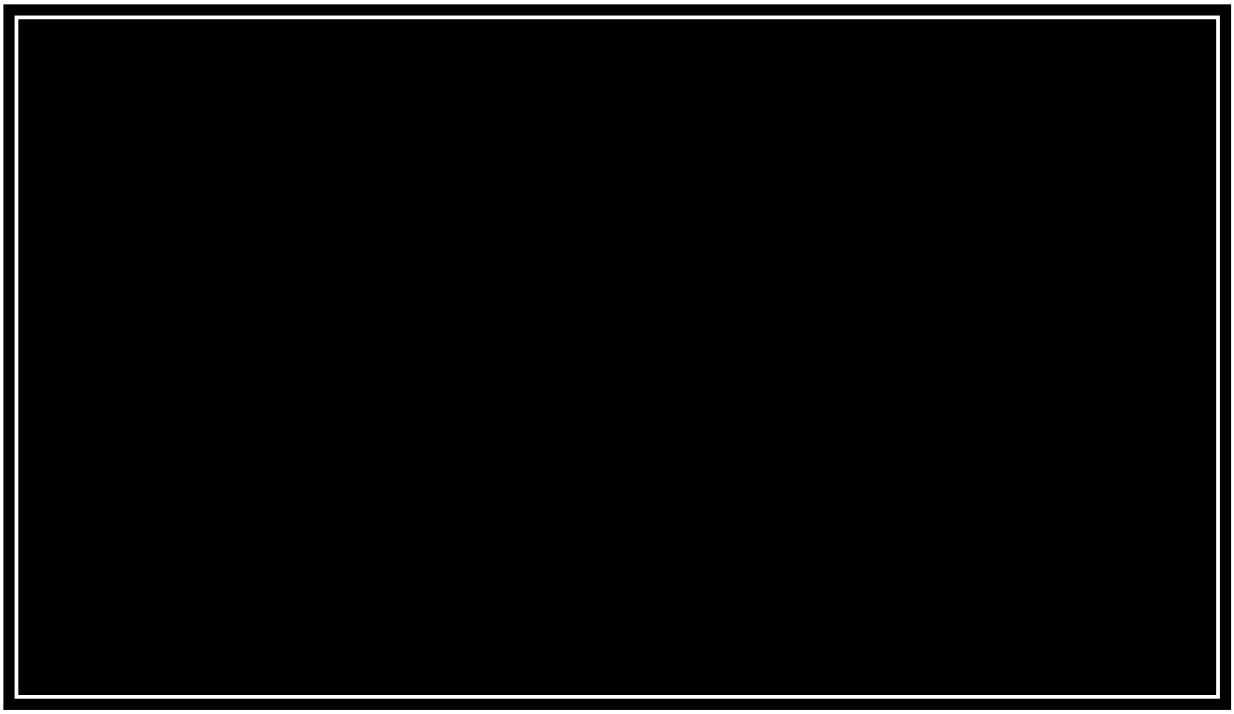


Figure (3-14) Results of PCR amplification for identification of serovar 3 (400 bp) , serovar 6 (370 bp) and serovar 14 (572 bp). M 100 bp standard size reference marker. Lane (1, 3, 4, 5, 6, 8, 9, 10, 11, 13, 14): Negative samples. Lane 2 : serovar 3 positive results. Lane7: serovar 6 positive results. Lane12: serovar 14 positive results. (Agarose Con. 2% & Voltages 100).

Ureaplasma parvum serovar 3 isolated in rate 15(42.8%) was the most frequent isolate in woman with recurrent miscarriage followed by serovar 1 10 (28.5%) while serovar 6 5(14.2%) and serovar 14 5(14.2%) in patient group, however in control group *U.parvum* was isolated serovar1only in rate (11%). Among the different serovars of *U.parvum* , serovar 3 was the most frequent serovar detected in patient group. Therefore *U.parvum* (biovar 2) serovar 3 was predominant among woman with recurrent abortion and suggest the *U.Parvum* serovar 3 there is evidence that it may play a role in recurrent miscarriage and prematurity also may be related with intra-amniotic inflammatory response to *Ureaplasma parvum* and that this is related not only to recurrent miscarriage but also to early onset sepsis in the baby. The results of the present study similarity with the results of other studies demonstrated that *U.parvum* is consistently isolated more frequently from lower genital tract (Al-Karawy, 2015; Al-Talqani & Al-Musawi, 2015). And serovar 3 was the most common serovar isolated from miscarried women, preterm birth, infertility. (Reyes L, *et al* , 2009 ; Robinson, *et al*, 2013; Paralanov, *et al*, 2012; Zeighami, *et al*, 2009; Zimmerman, *et al* ,2011). (Dando, 2012) demonstrated serovar 3 was the most common serovar isolated from male and female in Australia and united states.

Through the difference in detection rates of the different serovars of *U.parvum* was statistically significant , predominance of serovar3 was consistent with previous reports (Moss , *et al*, 2008 ; Dhawan *et al* , 2012) and suggest a possible pathogenic role of *U.parvum* serovar3. Another study detected *U. parvum* serovar 3 is the most prevalent serovar detected in reproductive humans (Knox ,*et al* , 2003; Novy, *et al*, 2009; Issley, 2011). Other study isolated the complete genome sequence of *U.parvum* serovar 3, clinical strain SV3F4, isolated from a Japanese patient who had an infectious miscarriage during the 13th gestational week in her previous pregnancy. (Ning , *et al*, 2014; Collins, 2010; Ekiel, *et al*, 2009; Paralanov, *et al.*, 2012). Also isolated *U. parvum* serovar 3/14 in 86% of women with symptomatic genital tract infections. Similarly have

identified *U.parvum* serovar 3/14 in vaginal swabs of 87 % pregnant women. It is possible that the combination of variable serovar specific genes of *Ureaplasma* with generally Known virulence factors determines the development of pathological processes on the mucosal surface of the human genital tract. (Kasprzykowska, *et al* ,2013; yoshida, *et al*, 2005; Polglase, *et al*, 2010) therefore Further studies are needed to confirm the serovar 3 distribution in different clinical setting and their possible pathogenic role.

3.10. Antimicrobial Susceptibility Testing

Many reports have suggested that *Ureaplasma parvum* may be associated with urogenital infections, infertility, and adverse pregnancy outcomes (Kamiya, *et al* 2013). According to previous studies used in the present study eight antibiotic agent included (Doxycycline, Azithromycin, Gentamicin, Ciprofloxacin, tetracycline , Levofloxacin , erythromycin ,Clarithromycin) were tested on 35 samples of *Ureaplasma parvum.* as these are the major antibiotics used in the treatment of genital tract infection caused by *U.parvum*. A further purpose of choosing these antimicrobial agents was these are conventionally being used for the routine treatment of sexually transmitted infection, (Krausse , *et al* , 2010; Kawai, *et al*, 2015; Miura, *et al*, 2014; Raynes, *et al*, 2012; Beeton, *et al*, 2016). In addition, a new macrolide Clarithromycin was also tested. The results revealed in table (3-1) that *Ureaplasma parvum* isolates were (80%) susceptible to Doxycycline (MIC 8ug/ ml), (71.4%) to Clarithromycin (MIC 8ug/ml), (60%) to Tetracycline (MIC 8ug/ml), (42.8%) to Erythromycin (MIC 16ug/ml) , (65.7%) to Levofloxacin (MIC 4ug/ml), (74.2%) to Ciprofloxacin (MIC 256ug/ml).

Table (3-1) : Antibiotic susceptibilities profile (MIC) of *Ureaplasma parvum* isolated from women with recurrent miscarriage.

Antibiotics/n=35	Susceptibility	MIC	Rate Susceptibility
Doxycycline	S	8ug/ml	28 (80 %)
Ciprofloxacin	S	256ug/ml	26 (74.2 %)
Gentamicin	R	-	0
Clarithromycin	S	8ug/ml	25 (71.4 %)
Azithromycin	R	-	0
Tetracycline	S	8ug/ml	21 (60 %)
Erythromycin	S	16ug/ml	15 (42.8%)
Levofloxacin	S	(4ug/ml)	23 (65.7 %)

Also The results showed that *Ureaplasma parvum* isolates were (91%) susceptible to Doxycycline (16ug/ml), (85.7%) to Clarithromycin (16ug/ml), (65.7%) to Tetracycline (16ug/ml), (54.2%) to Erythromycin (32ug/ml), (82.8%) to Levofloxacin (8ug/ml). The *Ureaplasma parvum* isolates were highly resistant (100%) in this investigation recorded to Ciprofloxacin (4-128ug/ml), Azithromycin (4-256ug/ml) and Gentamicin (4-256ug/ml). as shown in figure (3-15).The results of the present study similarity with the results of other studies in susceptibility rates of *Ureaplasma parvum* to antibiotics.(Dhawan *et al*,2012; Krausse ,*et al*, 2010; Kawai, *et al*, 2015).

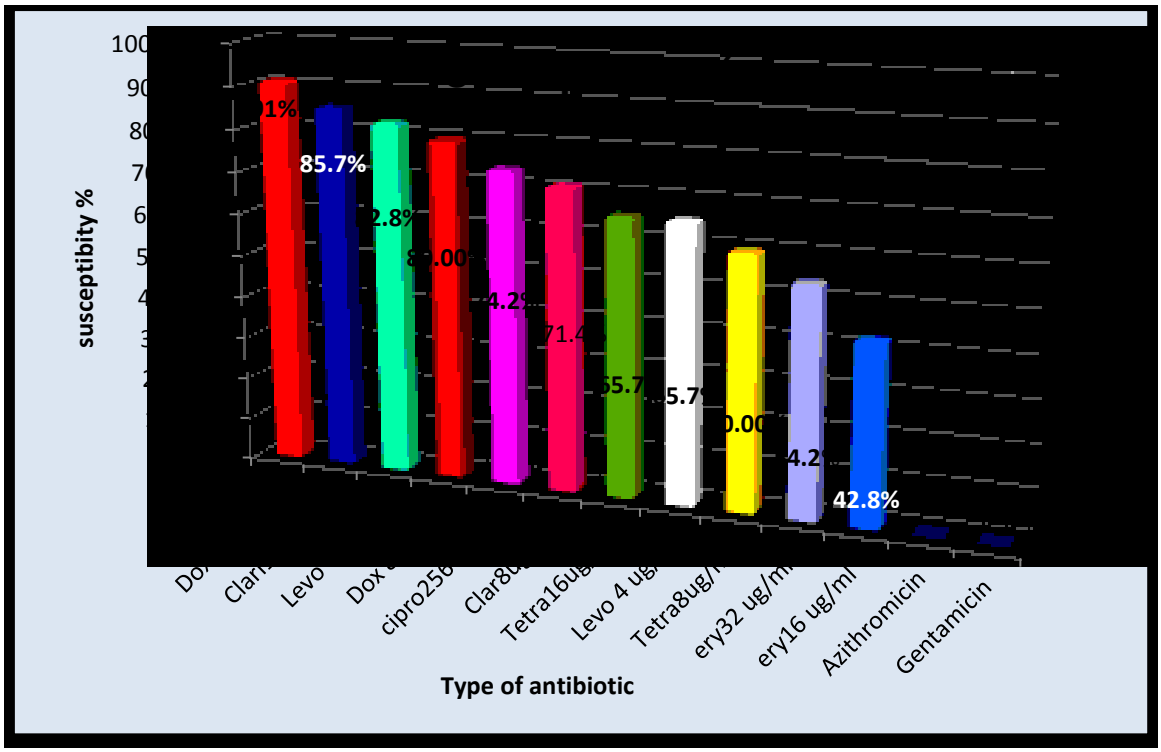


Figure (3-15): Susceptibility results of *Ureaplasma parvum* to different antibiotics

Ureaplasma parvum has been considered susceptible to macrolides , However in present study *Ureaplasma parvum* was resistance to azithromycin and erythromycin . Similar to finding (Xiao, *et al* ,2010), azithromycin resistant strains of *Ureaplasma parvum* are now being reported with increasing frequency. azithromycin resistance development in *U. parvum* A point mutation in par C and two novel mutations in par E were found in an ofloxacin-resistant strain (Bayraktar, 2010). While erythromycin resistant strains of *Ureaplasma* may be belong to mutations in the L22 ribosomal protein were seen in three strains that were resistant to erythromycin. (Govender, *et al*, 2012). No resistance was seen against new macrolide , Clarithromycin for *Ureaplasma parvum* and the results are similarity with those reported earlier (Stellrecht, 2004; Miura, *et al*, 2014).

Tetracycline & Doxycycline was the most active agents against *Ureaplasma parvum*. This finding is consistent with those of other studies conducted in China (Lin, *et al*.,2007; Govender, 2012) and Turkey (Kilic, *et al*, 2004 ; Xiao, *et al*, 2010; Xiao, *et al*, 2011). The majority of *Ureaplasma parvum* serovars were susceptible to Doxycycline, Clarithromycin and Levofloxacin while *Ureaplasma parvum* serovars fully resistance (100%) to Gentamicin and Azithromycin. Resistance to Gentamicin and Azithromycin was of intermediate nature in all the *Ureaplasma parvum* isolates. The results revealed the SV3 isolates fully resistance (100%) to GEN and AZM, and ERY while susceptible to DOX in rate (80%), (60%) to LVX, (60%) to CLR but SV14 isolate revealed fully susceptible (100%) to CLR, CIP, DOX. And fully resistance (100%) to GEN and AZM. While SV1,SV6 showed fully resistance (100%) to AZM , GEN. And SV1 susceptible to DOX in rate (70%),TET (60%), CIP (90), LVX (70), ERY (70), CLR (70%), but SV6 susceptible to DOX in rate (80%),TET (100%), CIP (100), LVX (80), ERY (80), CLR (80%). as shown in figure (3-16).

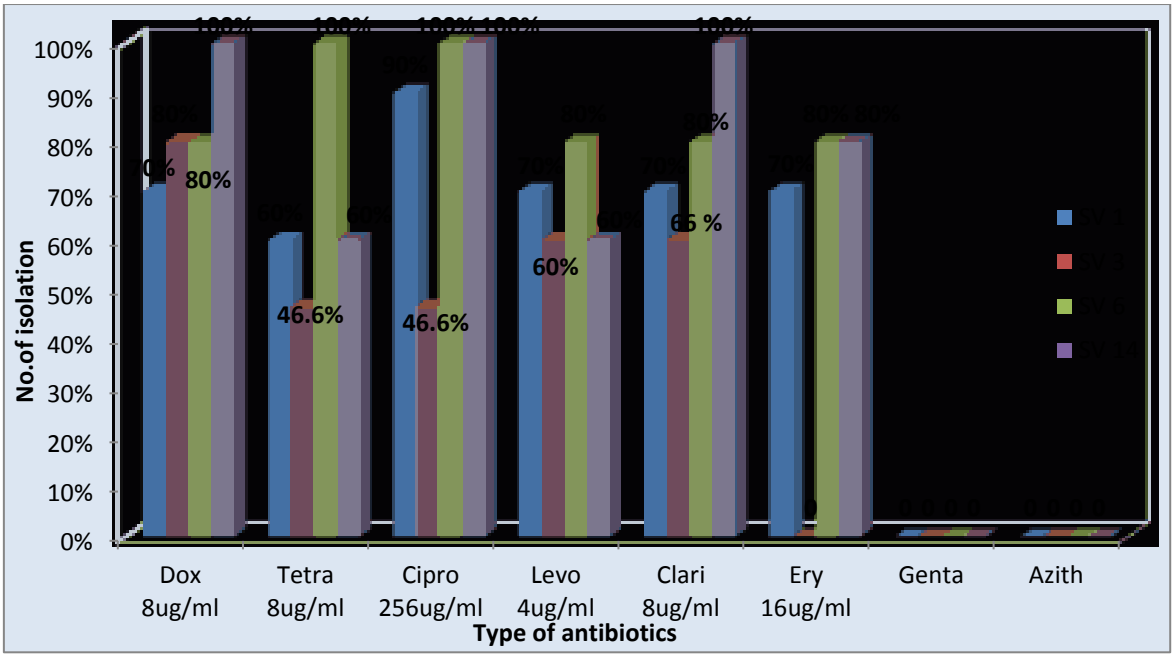


Figure (3-16) Susceptibility (MIC) of *Ureaplasma parvum* serovars to different antibiotics .

The study extends the observations of *U.parvum* serovar 3 the most frequent serovar detected in patient group was susceptible to Doxycycline, Levofloxacin and Clarithromycin. While resistance to, erythromycin, azithromycin, and gentamicin. This finding is consistent with those of other studies (Mardassi, *et al*, 2012; Beeton, *et al*, 2016; xiao,*et al*, 2011).The fluoroquinolones are considered useful in the treatment of *Ureaplasma* infection as these are potentially effective against pathogenic species and also including strains resistant to other drugs such as doxycycline. Fluoroquinolones are used for treating urogenital infections and interact in bacteria with the type II topoisomerases DNA gyrase and topoisomerase IV, both of which are composed of two A and two B subunits; these subunits are encoded by the gyrA and gyrB genes for DNA gyrase and parC and parE genes for topoisomerase IV (Kawai, *et al*, 2015).

The results in present study showed that *Ureaplasma parvum* isolate susceptible to Levofloxacin However *Ureaplasma* was resistance to Ciprofloxacin (fluoroquinolones) . Similar rates of resistance to quinolones has been observed in clinical isolates of *Ureaplasma* in most of the studies (Kilic, *et al*, 2004; Xiao, *et al*, 2012; Santos, *et al*, 2012). There is few report of fluoroquinolones -resistant *Ureaplasma* in the field of urology in Japan (Kamiya**,** *et al*, 2013). Fluoroquinolones resistance in *Ureaplasma parvum* may occur to some degree due to the widespread use of these drugs for the treatment of respiratory and urogenital infections. *Ureaplasma parvum* resistance to fluoroquinolones has been attributed to substitution mutations, principally in the *gyrA* and *parC* genes and, to a lesser extent, in the *gyrB* and *parE* genes of the DNA gyrase/topoisomerase IV complex (Glass, 2000; Biasini, et al , 2014; Mardassi, *et al*, 2012). Six mutations were identified in the *U. parvum parC* gene, five mutations in *parE*, and one mutation in *gyrA* that resulted in amino acid substitutions. There were only three silent mutations observed in *gyrB*. The *gyrA* mutation that resulted in the substitution of glutamine for lysine at amino acid 103 in *U. parvum* may be near enough to the tyrosine active site of the

protein at amino acid 122 to contribute to the resistance Ciprofloxacin observed (Beeton, *et al*, 2016; Duffy,2006; Uchida, *et al* , 2013; xiao,*et al*, 2011) . However a high rate of resistance to gentamicin was observed for *Ureaplasma* parvum , gentamicin belong to Aminoglycosides group are not active against anaerobes bacteria ,Three mechanisms of resistance have been recognized, namely ribosome alteration, decreased permeability, and inactivation of the drugs by aminoglycoside modifying enzymes. The latter mechanism is of most clinical importance since the genes encoding aminoglycoside modifying enzymes can be disseminated by plasmids or transposons.(Ramirez *et al*, 2010; Redelinghuys, *et al* , 2014; Beeton, *et al*, 2016). Ribosome alteration can result from single mutations in chromosomal genes encoding ribosomal proteins : *rpsL* (or *strA*) , *rpsD* (or *ramA* or *sud*) , *rpsE* (*eps* or *spc* or *spcA*). Decreased permeability alteration in the aminoglycoside transport system, inadequate membrane potential, modification in the LPS (lipopolysacchaccarides) phenotype can result in a cross resistance to all aminoglycosides. In activation of aminoglycosides these enzymes are classified into three major classes according to the type modification: AAC (acetyltransferases) , ANT (nucleotidyltransferases or adenyltransferases) , APH (phosphotransferases). (xiao,*et al*, 2010; xiao,*et al*, 2011).The results revealed in the present study may be indicate that *Ureaplasma* colonizing the genital tract epithelium may form biofilms that protect the organisms from host defenses and antibiotic treatment. To confirm the biologic relevance of the current in vitro studies, biofilm-formation of clinical *Ureaplasma* isolates should be tested in vivo experimental models. Treatment of *Ureaplasma* infection is imperative to prevent the occurrence of complications . Empirical therapy is important in the treatment of *Ureaplasma*, since culture and antimicrobial susceptibilities of *Ureaplasma* are not routinely done in Iraq laboratories. The results indicate that doxycycline , Clarithromycin and Levofloxacin should be the first choice drug when empirical treatment is required.

Chapter Four

Conclusions and Recommendations

Conclusions

1- *Ureaplasma parvum* may be an important etiologic agent of recurrent miscarriage.

2- *Ureaplasma parvum* are smaller than conventional bacteria in cellular and have specific nutritional requirements, their identification, isolation and characterization require molecular techniques to complement culture.

3- The IH medium enhancing the growth of *Ureaplasma spp.* Therefore , the study strongly recommended these media for cultivation and isolation of *Ureaplasma* spp.

4- The antibiotic susceptibility revealed that *Ureaplasma parvum* isolates were sensitive to Doxycycline, Clarithromycin, Tetracycline, Erythromycin, Levofloxacin and Ciprofloxacin and resistant to Azithromycin and Gentamicin .

5- *Ureaplasma parvum* serovar (3) was the most frequent serovar detected in present study and susceptible to Doxycycline, Clarithromycin and Levofloxacin. And *U.parvum* serovar3 is very important etiologic agent of recurrent miscarriage.

Recommendations

1. Further studies are needed to confirm the *U.parvum* serovar 3 distribution in different clinical setting and their possible pathogenic role.

2. Identification of the virulence factors of *U.parvum* serovar 3 which determine the development of pathological processes on the mucosal surface of the female genital tract.

3. The culturing of *Ureaplasma spp.* should be carried out for all patient with miscarriage and unexplained infertility.

4. Further study on *Ureaplasma parvum* to determine the exact effect on female genital tract .

5. More studies are needed to determine the relationship of *Ureaplasma parvum* virulence factors, host immune factors affecting pathogen susceptibility, and inflammatory variability, and interactions with environmental factors such as oxygen exposure.

6. Furthermore, a study analyzing antimicrobial therapy of *Ureaplasma parvum* and serovars colonized in women with recurrent miscarriage to determine whether these antibiotics are effective in reducing recurrent miscarriage rate.

Chapter Five

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Appendix

Appendix

Case Report

- Date : / / 2016
- Patient No. :
- Age :
- No. of baby :
- No. of abortion :
- Time of abortion :
- Type of specimen :
- Clinical signs :

bleeding ☐ Fever ☐ Anemia ☐

Acute pain in abdominal ☐

الخلاصة

تضمنت هذه الدراسة جمع 130 عينة تشمل (72 عينة نزيف مهبلي ,41عينة مسحات من المهبل و 17 عينة ادرار) من نساء متزوجات تعاني من الاجهاض المتكرر و40عينة سيطرة شملت هذه العينات 15عينة مسحات من المهبل و25 عينة ادرار من النساء اللواتي يراجعن مستشفى النسائية والاطفال التعليمي والعيادات الخاصة في محافظة المثنى خلال الفترة الممتدة من تشرين الاول / 2015 الى شباط / 2016 .

خلال هذه الدراسة تم استعمال نوعين من وسط IH (بروث IH واكارIH) لغرض عزل وتسريع ودعم نمو بكتيريا *Ureaplasma* حيث تم عزل *Ureaplasma spp.* على وسط IH بمعدل (% 52.3) من النزيف و (30.7%) من مسحات المهبل و(7.6%) من الادرار من النساء اللواتي تعاني من الاجهاض المتكرر واعطى النزيف المهبلي من النساء متعددة الاجهاضات اعلى نسبة عزل لبكتيريا *Ureaplasma spp.* مقارنة بعينات الادرار و مسحات المهبل حيث تم الحصول على 118 (90.7%)عينة موجبة في الزرع من نساء متعددة الاجهاضات مقابل 9 (22.5%)عينات موجبة بالزرع من السيطرة.

واظهرت النتائج ان نسبة الاصابة ببكتريا *Ureaplasma spp.* تتناسب عكسيا مع عمر المرأة حيث كان اعلى نسبة اصابة (46%) في الفئة العمرية التي تراوحت اعمارها من 17 الى 26سنة تلتها الفئة العمرية من 27 الى 36 بنسبة (40.7%) ومن ثم الفئة العمرية المتقدمة بالسن من 37 الى 46 سنة بنسبة (13.3%).

استعملت تقنية تفاعل سلسلة البلمرة (PCR) في تشخيص بكتيريا *Ureaplasma parvum* باستخدام جين خاص بتشخيص وعزل (UMS-57/UMA222 target gene 5' end of MBA gene) *Ureaplasma parvum* حيث تم عزلها بنسبة (29.6%) من النساء متعددة الاجهاضات و بنسبة (11.1%) من عينات السيطرة .

خلال هذه الدراسة شخصت اربعة serovar من بكتيريا*Ureaplasma parvum* باستخدام Gradient PCR technique بلاعتماد على جين MBA الخاص بتشخيص بكتيريا *Ureaplasma parvu*m و البرايمرات الخاصة بعزل serovar وفحصت بهذه التقنية جميع عزلات *Ureaplasma parvum* التي اظهرت نتيجة موجبة بالجين الخاص بعزل النوع

(UMS-57/UMA222 target gene 5' end of MBA gene) حيث سجلت النتائج اعلى نسبة عزل (42.8%) ل serovar 3 ثم serovar 1 بنسبة (28.5%) و serovar 6 بنسبة (14.2%) و serovar

14 بنسبة (14.2%) حيث اعطى serovar 6 & serovar 14 اقل نسبة عزل . بينما تم عزل بكتيريا *Ureaplasma parvum* بنسبة (11.1%) في السيطرة وشملت (1) serovar فقط.

شملت الدراسة الحساسية الدوائية عزلات serovar وسجلت نتائج اختبار الحساسية للعزلات *U.parvum* SV3 حيث اظهرت مقاومة عالية بنسبة (100%) ل Erythromycin Gentamicin, Azithromycin. واظهرت عزلات SV3 حساسة ل Doxycycline بنسبة (80%) و ل Levofloxacin بنسبة (60%) و لClarithromycin بنسبة (60%) واظهرت عزلات SV14 حساسة بنسبة (100%) لClarithromycin, Ciprofloxacin , Doxycycline . ومقاومة عالية بنسبة (100%) لGentamicin, Azithromycin و سجلت عزلات SV1,SV6 مقاومة عالية بنسبة (100%) لGentamicin, Azithromycin واظهرت عزلات SV1حساسة بنسبة (70%) ل Doxycycline و لTetracycline بنسبة (60%) ولCiprofloxacin بنسبة (90%) ول Levofloxacinبنسبة (70%) و لClarithromycin بنسبة (70%) و لErythromycin بنسبة (70%). بينما عزلات SV6حساسة لDoxycycline بنسبة(80%) ولTetracycline بنسبة (100%) ول Ciprofloxacin بنسبة (100%) و Levofloxacin بنسبة (80%) ولClarithromycin بنسبة (80%) ولErythromycin بنسبة (80%).

وزارة التعليم العالي
والبحث العلمي
جامعة القادسية
كلية الطب
فرع الاحياء المجهرية

دراسة جزيئية و مايكروبايولوجية لبكتيريا *اليوريابلازما* *يارفم* في النساء متعددة الاجهاض

رسالة مقدمة إلى
مجلس كلية الطب-جامعة القادسية
وهي جزء من متطلبات نيل درجة الماجستير علوم في
ا لأحياء المجهرية الطبية

تقدمت بها

غفران كاظم مسلم
بكلوريوس علوم الحياة-جامعة المثنى (2013)

إشراف

أ.م.د.ابتسام حبيب العزاوي
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2016 م