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Study of Some Bacteriological and Immunological Aspects of Preterm Labor

A Thesis

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تيسير عبدالاله كاظم الحسيني

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بسم الله الرحمن الرحيم

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صدق الله العظيم

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1.1 Introduction

Preterm birth is defined as delivery of a baby before completed 37 weeks of pregnancy (Bennett, 2007).

The major risk factors for preterm birth are previous preterm birth, uterine over-distention and uterine abnormalities (Jones, 2004). The incidence of preterm birth in the developed world is between 7 and 12 %. The incidence of spontaneous preterm labor is at its lowest in women in their 20_s. The risk is increased in teenagers and in women over 30. There is a higher incidence of preterm labor in first pregnancies. Marital status, cigarette smoking, environmental stress, poor nutrition and use of alcohol, coffee and street drugs (especially cocaine) have all been linked to an increased risk of preterm birth (Bennett, 2007).

The main cause of preterm birth is infection which is possible cause in up to 40% of cases (Goldenberg *et al*., 2000).

Microbial colonization and inflammation in the maternal genital tract has emerged as one of the major risk factors associated with spontaneous preterm birth (Wadhwa *et al*., 2001). Infection may promote preterm labor by producing prostoglandins that in turn stimulate labor. Prostoglandins production by human amnion can be stimulated by bacterial endotoxins and that many organisms produce phospholipase and thus may potentially initiate preterm labor (Farraj, 2000).

Infection associated with or causing preterm labor are lower genital tract infection which include bacterial vaginosis (BV),

Group B *Streptococcus* (GBS), *Chlamydia* and *Mycoplasmas* or intrauterine which includes ascending (from genital tract), transplacental (blood-borne), transfallopian (intraperitoneal) and iatrogenic (invasive procedures); or extra-utrine which includes pyelonephritis, malaria, typhoid fever, pneumonia, *Listeria* and asymptomatic bacteriuria (Danielian and Hall, 2005). Preterm premature rupture of membranes (PPROM) accounts for 25% to 33% of all preterm deliveries (Petrova and Mehta, 2005).

Women with intra-amniotic infection are more likely to develop PPROM (Goswami and Thornton, 2006). Antibiotics may be of benefit in the prevention of preterm birth, so is the prophylactic use in women with abnormal genital tract colonization, for the prevention of PPROM (Lamont *et al*., 2001).

Aims of the study

1. Isolating and identifying the aerobic bacteria from the amniotic membrane of women with preterm labor.
2. Examining some virulence factors such as protease and lecithinase production.
3. Studying the effect of some antibiotics on bacterial isolates.
4. Studying the effect of some antibiotics on lecithinase.
5. Studying some aspects of humoral and cellular immunological parameters in women with preterm labor.

1.2 Literature Review

1.2.1 Definition of preterm labor

Preterm labor (PTL) is defined as regular contractions of the uterus, plus effacement and dilation of the cervix before the 37th week of gestation (Kimsey, 2008).

Most preterm births occur following preterm labor, preterm premature rupture of membrane (PPROM), or both. Such births are classified as spontaneous. Spontaneous preterm birth may be considered the clinical endpoint of an injury or stimulus to the fetoplacental unit sufficient to initiate labor or membrane rupture or both. The remaining preterm deliveries are classified as indicated, which means they follow induction or cesarean section undertaken because of a medical or obstetric disorder that places the fetus at risk (Gabbe *et al*.,1996; Goldenberg *et al*.,2001; Lackwood and Kuczynski, 2001).

1.2.2 Causes of preterm labor

One cause of preterm labor is premature (before labor begins), preterm (before full term) rupture of membranes, when the fluid around the baby leaks out, the baby and mother are exposed to infection. Labor usually occurs within a few days. Infection of the vagina, cervix, or uterus has been studied as possible causes of preterm rupture of the membranes. When infection is present in the vagina or cervix, toxins produced by the organism may weaken the membranes, making them more likely to leak or rupture. Inflammation from infections causes a local release

of a substance known as prostaglandin. Prostaglandins are found throughout the body, and the substance is believed to have some role in the beginning of labor (Kimsey, 2008).

Preterm labor has been linked to cervical incompetence, infection within the uterus, placental abruption or decidual haemorrhage, fetal or maternal stress and multiple pregnancy (Bennett, 2007).

1.2.3 Risk factors for preterm labor

The most common medical risk factors for a spontaneous preterm birth are infection in the urinary or reproductive tract (including the vagina), multiple pregnancy (pregnancy with twin, triplets or more), a past preterm delivery, vaginal bleeding in the second trimester, age younger than 18 years, mother's low body mass index, cigarette smoking during pregnancy and frequent contractions. The most common medical risk factors for an indicated preterm birth are preeclampsia, fetal distress, poor fetal growth that endangers the fetus's healthy survival and placental abruption (Healthwise, 2007).Other risk factors for preterm labor include : amnionitis, preterm premature rupture of membrane, maternal characteristics such as anemia, bacteriuria, stress, low socioeconomic status and poor nutrition (Gabbe *et al*.,1996) .

In addition to the risk factors discussed above, a variety of other factors have been associated with an increased risk for preterm labor. Asymptomatic bacteriuria is associated with an increased rate of prematurity. Systemic infection such as bacterial

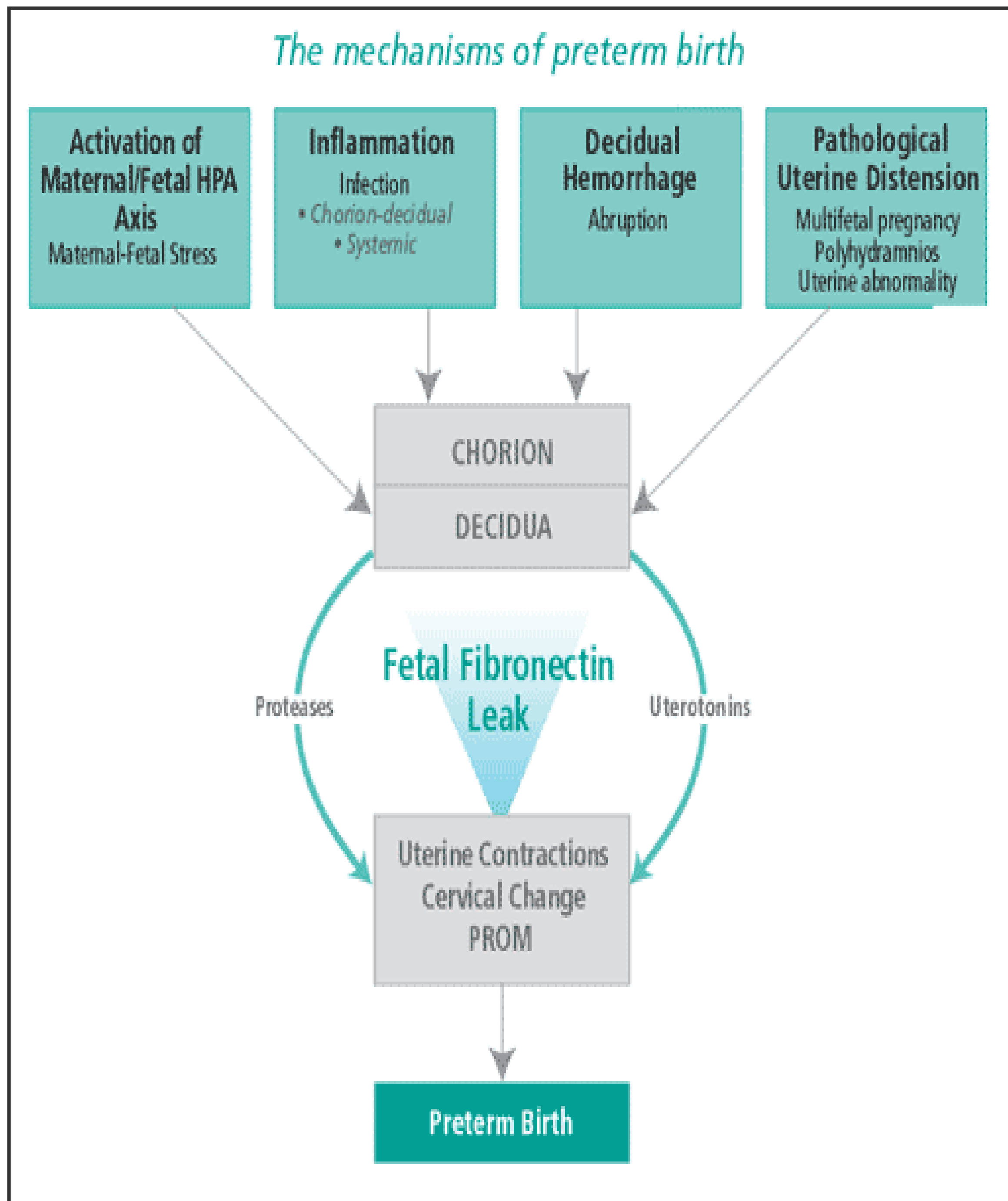
pneumonia, pyelonephritis, and acute appendicitis, often lead to increased uterine activity, potentially leading to premature delivery. Extremes in the volume of amniotic fluid, such as hydramnios or oligohydramnios, maternal abdominal surgery in the late second and third trimester can cause an increase in utrine activity that may culminate in preterm delivery (Robert and Goldenberg, 2002).

1.2.4 Symptoms of preterm labor

Symptoms of preterm labor include: menstrual-like cramps due to uterine contraction; low, dull backache; pelvic pressure; increase or change in vaginal discharge; and a general feeling that something is not right (Kimsey, 2008).

1.2.5 Mechanisms of preterm labor

One mechanism by which microorganisms might cause preterm labor is through ascension from the cervical/vaginal area and replication in the placenta, decidua and membranes (Lorie *et al*.,2002). Clinical studies have identified 4 major pathways for preterm birth (figure 1-1), all of which can cause the release of the biochemical marker fetal fibronectin (Gabbe *et al*.,1996; Goldenberg *et al*.,2001). Fetal fibronectin often can be detected before the biophysical markers (Healthcare professionals, 2007).



HPA=hypothalamo-pituitary adrenal

Figure (1.1) Mechanisms of preterm labor (Gabbe *et al*.,1996)

1.2.6 Bacterial Infections and preterm labor

In recent years, bacterial infection has come to be recognized as an important risk factor for preterm labor and for preterm premature rupture of membranes. Such infection can occur at any of several sites including: choriodecidual space, fetal membranes (amnion, chorion), placenta, amniotic fluid and umbilical cord (Goldenberg *et al.,*2001). Approximately, 40 percent of spontaneous premature births are thought to be caused by infection (Lettieri *et al.,*1993).

Infection can result from bacteria introduced into the abdominal cavity through the fallopian tubes, through blood entering the placenta, from the vagina through the cervix, via contaminated needles used for amniocentesis or chorionic-villus sampling (Goldenberg *et al.,*2001).

There is a strong correlation between infection within the uterus and the onset of spontaneous preterm labor. Infection within the uterus has the potential to activate all of the biochemical pathways, ultimately leading to cervical ripening and uterine contractions (Bennett, 2007).

Enterobacter microorganisms, i.e. *Escherichia coli*, *Klebsiella* spp. and *Proteus* spp., were found more frequent among the preterm group in both cord blood and placental tissue cultures. *Staphylococcus epidermidis* and *S. aureus* were also more prevalent in the preterm group as were streptococcal bacteria

(*Streptococcus milleri*, *Strep. viridans*, *Strep. sanguis* and *Strep. agalactiae*)(Farraj, 2000).

Bacterial infection of the amniotic membrane causes it to weaken and then break. However, most cases of PROM and infection occur in the opposite order, with PROM occurring first followed by an infection. The types of infections that can complicate PROM include amnionitis and endometritis. Amnionitis is an infection of the amniotic membrane. Endometritis is an infection of the innermost lining of the uterus. Amnionitis occurs in 0.5-1% of all pregnancies. In the case of PROM at term, amnionitis complicates about 3-15% of pregnancies. About 15-23% of all cases of preterm PROM will be complicated by amnionitis. The presence of amnionitis puts the fetus at a great risk of developing an overwhelming infection (sepsis) circulating throughout its bloodstream. Preterm babies are the most susceptible to this life-threatening infection. One type of bacteria responsible for overwhelming infections in newborn babies is group B streptococci (Health Me, 2004).

Asymptomatic bacteriurea, gonococcal cervicitis and bacterial vaginosis are strongly associated with preterm labor, and the role of species of *Chlamydia*, *Candida*, *Trichomonas* and *Ureaplasma* is less clear. *E. coli* and *Staphylococcus aureus* were the most commonly found microorganisms (32% each) in vaginal cultures followed by *Candida*, *Klebsiella*, and *Trichomonas* spp. (Gram *et al*., 2002).

Bacterial infection that spreads to the uterus and amniotic fluid is a major cause of preterm labor (Goldenberg and Rouse, 1998). Uterine infection triggers the release of substances that make the uterus contract, and can cause the amniotic sac to rupture (PROM). Either or both of these events will start preterm labor (Gallagher, 2007).

Infection can begin in the vagina, such as bacterial vaginosis (BV) often without symptoms. Studies showed that in most cases of preterm labor caused by infection, the bacteria found in the uterus came from the vagina (Goldenberg , 2000).

In the urinary tract, a urinary tract infection can lead to serious kidney infection that endangers both the mother and her fetus.

After amniocentesis or cervical cerclage is performed (this is very rare) (American Academy of pediatrics and American collage of Obstetrician and Gynecologists, 2002). Through the mother's bloodstream (this is rare) (Goldenberg, 2000).

Infection associated with or causing preterm labor may be in the lower genital tract, intrauterine or extra-uterine (generalised maternal infection) (Danielian and Hall, 2005).

A. Lower genital tract infection

Bacterial vaginosis(BV): Bacterial vaginosis arises as a result of a disturbance in the balance of normal maternal vaginal flora. The lactobacilli normally predominant in the vagina are replaced by other organisms, such as: Bacteroides species, *Gardnerella*

vaginalis, *Mycoplasma hominis*, Peptostreptococci, *Ureaplasma urealyticum* (Hillier and Holmes, 1999).

Bacterial vaginosis is usually diagnosed when it causes symptoms, usually a bad-smelling vaginal discharge. Antibiotic medication can be used to correct an overgrowth of problem bacteria in the vagina. BV. infection during pregnancy has been linked to a higher risk of preterm birth (McDonald, 2006).

Bacterial vaginosis is an abnormally of the normal vaginal flora characterized by a reduced number of lactobacilli, a higher pH, and 100 fold increased numbers of potential pathogens including *Gardenerella vaginalis, Bacteroides, Escherichia coli,* group B- Streptococcus, the anaerobes Peptostreptococcus, and Mycoplasma hominis. Since the presence of large number of lactobacilli and a low vaginal pH are important mechanisms to protect against the growth of potential pathogenic organisms, bacterial vaginosis represents a risk factor for preterm delivery (Bennet, 2007).

Around 50% of women with bacterial vaginosis are asymptomatic. If symptoms do occur, the most common is a thin, watery, malodorous, non-itchy discharge. The criteria used to diagnose bacterial vaginosis are vaginal pH >4.5, the presence of thin watery discharge, fishy odour (with 10% KOH), clue cells on saline wet mount, and/or Gram stain (Goswami and Thornton, 2006).The presence of BV. in pregnancy is associated with an approximately two-fold increase in the risk of preterm labor and

premature rupture of membranes (Hillier *et al*.,1995; Andrews *et al*.,1995 and Korn *et al*., 1995).

It has been found that bacterial vaginosis associated with premature rupture of membrane, preterm labor and infections of amniotic fluid (Gravett *et al*.,1986; McDonald *et al*.,1992). It has been also found that there were a significantly more cases of bacterial vaginosis among pregnant women who experienced preterm labor than among pregnant women who did not experience preterm labor (Al-Muk, 1992).

Preterm labor and preterm premature rupture of membrane are frequently accompanied by evidence of infection, in the amniotic fluid of organisms or inflammatory cytokines. Most of these microorganisms are thought to come from the vagina, especially among women with bacterial vaginosis. Mechanisms that may initiate preterm birth in these circumstances are not fully understood. Bacteria may induce prostaglandin synthesis in amniotic cells via several ways. Many genital tract organisms are associated with BV (but not Lactobacillus) produce phospholipase A2; an enzyme that liberates arachidonic acid. Bacteria may induce prostaglandin synthesis via direct invasion of the extraplacental membranes that will lead to disruption of the amniotic cells and release of lysosomal phospholipase. A third possible mechanism that may initiate labor is the migration of maternal inflammatory cells, which metabolize arachidonic acid (Shalev, 2008).

B.Intrauterine infection

Intrauterine infection may occur because of ascending infection from the vagina, blood-borne transmission via the placenta, transfallopian infection from the peritoneal cavity, or by iatrogenic introduction following invasive procedures such as amniocentesis, chorion villus biopsy, or fetal blood-sampling. The commonest cause of intrauterine infection is ascending infection from the lower genital tract. Ascending infection may follow rupture of the membranes, but can also occur with intact membranes. Pathogens ascend from the vagina through the cervix and cause infection of the decidua, the chorion, fetal blood vessels; and can infect and cross the amnion to the amniotic fluid and the fetus. The fetus may inhale the infected amniotic fluid leading to pneumonia, or may become septicaemic from haematological infection secondary to decidual and villous infection (Danielian and Hall, 2005).

Intrauterine infection has emerged as a major cause of premature labor and delivery. It has been estimated that 25% of all preterm deliveries occur to mothers who have microbial invasion of the amniotic cavity, although these infections are mostly subclinical in nature. Intrauterine infection and inflammation are frequently associated with preterm labor and delivery; and at least 40% (positive amniotic fluid & chorioamniotic space culture) of all preterm births have been estimated to occur with mothers who have an intrauterine infection, which is largely subclinical. The lower the

gestational age at delivery, the greater the frequency of intrauterine infection (Romero *et al.,*2003).

There is also strong evidence that intrauterine infection causes preterm labour and delivery. There is a plausible mechanism in that bacterial products are known to include proteases and collagenases, which could weaken the membranes; and phospholipase A2 and endotoxins known to be able to stimulate prostaglandin production *in vitro* and *in vivo*. Prostaglandins are known to be involved in the initiation of human labour, and are of course widely used for the pharmacological induction of labour. In addition, the host inflammatory response to infection causes the release of inflammatory cytokines which are involved in cervical ripening, and possibly membrane rupture (Danielian and Hall, 2005).

The amniotic cavity is normally sterile; and therefore, the isolation of any micro-organism from the amniotic fluid constitutes evidence of microbial invasion. This condition often exists in the absence of clinical signs and symptoms of infection (Romero *et al*.,2001).

C. Extra-uterine infection

Generalised maternal infections such as pyelonephritis and malaria remain relatively common antecedents of preterm delivery, though timely antimicrobial treatment usually reduces the risk. Infections such as typhoid fever and maternal pneumonia, although historically associated with preterm delivery, are usually sensitive to antibiotics, and are now less important in most regions (Danielian and Hall, 2005).

Although systemic maternal infection (i.e., pneumonia, pyelonephritis, malaria, typhoid fever) has been associated with preterm labor and delivery, the frequency of these conditions is low in developed countries. Thus, the attributable risk of systemic infection for prematurity is small (Offenbacher *et al.,* 1996.,Offenbacher *et al.,* 1998).

Untreated systemic maternal infection is associated with preterm labor and delivery and that treatment may decrease the rate of preterm delivery in some cases (e.g. pyelonephritis, typhoid fever)but not in others (e.g. pneumonia) (Romero *et al.*,2001).

1.2.6.1 Listeria monocytogenes:

Listeria monocytogenes is a Gram-positive rod-shaped bacterium. It is the agent of listeriosis, a serious infection caused by eating food contaminated with the bacteria .The disease affects primarily pregnant women, newborns, and adults with weakened immune systems (Todar, 2008).

Listeriosis is an uncommon infection that can cause intrauterine, fetal infection, and subsequent preterm delivery. Contaminated food is the usual source of infection, and transmission appears to be blood-borne following gastrointestinal infection (Danielian and Hall, 2005). Listeriosis during pregnancy usually occurs during the third trimester, when cell mediated immunity lowest (Mylonakis *et al*., 2002; Sheffield, 2004).

1.2.6.2 Asymptomatic bacteriuria

Asymptomatic bacteriuria, defined as more than 100.000 colonies of a single bacterial species per ml of urine, is cultured from mid-stream sample (U.S., 1996). The most commonly isolated bacteria is *E. coli* (Beckmann *et al*.,1995). Results of multiple studies have shown that women with asymptomatic bacteriuria have a higher preterm delivery rate than women without bacteriuria (Romero *et al*.,1989). Therefore, identifying and treating asymptomatic bacteriuria could decrease the risk of preterm delivery in affected patients (Lorie *et al*.,2002).

Asymptomatic urinary tract infection is common in pregnancy, and is associated with preterm delivery. Two meta-analyses have shown that antimicrobial treatment reduces the risk of preterm delivery, so causation appears likely. The exact mechanism is unknown, but there is evidence that there can be colonisation of the vagina with the same pathogen as found in the urine, and the bacteriuria may therefore be a surrogate marker for

abnormal vaginal flora that could be the cause of preterm delivery (Danielian and Hall, 2005).

1.2.6.3 Group B Streptococci

Group B Streptococcus is a gram-positive streptococcus, characterized by the presence of group B Lancefield antigen. These bacteria cause group B-Streptococcal infection. *Streptococcus agalactiae* is a species of normal flora of the gut and female urogenital tract (Gillespie *et al*.,2000; Brook *et al*.,2007).

Group B-Streptococcus (GBS) is an important cause of neonatal morbidity and death, especially in premature infants, but its role in the initiation of preterm labor is uncertain. The risk of preterm birth appears to be greatest in women with group B Streptococcus in urine; perhaps indicating a greater degree of colonization. Thus, treatment of the urinary tract infection may result in a reduction in preterm birth (Robert and Goldenberg, 2002).

1.2.6.4 *Chlamydia trachomatis*

Chlamydia trachomatis, an obligate intracellular bacteria, is considered to be the most commonly isolated sexually transmitted organism. The prevalence of *Chlamydia trachomatis* in pregnant patients ranges from 5 to 26 per cent (Bulletin,1994). Conflicting evidence exists regarding the relationship of chlamydial cervicitis and preterm labor (Gibbs *et al*.,1992). Patients infected with *Chlamydia trachomatis* are often asymptomatic, but they may present with a mucopurulent vaginal

discharge or cervicitis. Diagnosis is made by culture or by DNA probe, which is 90 per cent sensitive and 97 per cent specific (Bulletin, 1994).

1.2.6.5 *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is a gram-negative, intracellular, diplococcal organism that is sexually transmitted. This bacterium causes infections in the genital tract that may disseminate to organs. *N. gonorrhoeae* cervicitis is strongly associated with premature delivery (Romero *et al.,*1991).

Epidemiologic studies have demonstrated that the treatment of gonococcal cervicitis is associated with a decreased rate of preterm delivery (Gibbs *et al*., 1992).

1.2.7 Other infections (viral infections)

Routine screening for other infections such as hepatitis B, human immunodeficiency virus (HIV), and syphilis is important to prevent maternal and fetal-neonatal complications; however, the role of these infections in preterm labor is unclear (Lorie *et al*.,2002).

Although there is little evidence that viruses are a common cause associated to preterm delivery, they may be implicated in some cases. One possible mechanism is that viral infection of the trophoblast could play a role in placental dysfunction, leading to complications including spontaneous miscarriage, pre-eclampsia, fetal growth restriction, preterm birth; or preterm labour may occur

secondary to host inflammatory responses to the viral infection (Danielian and Hall, 2005).

1.2.8 Evidence supporting a role for infection in the onset of labor

Three lines of evidence support a role for infection in the onset of preterm labor (Romero *et al.*,2001)

a-Administration of bacteria or bacterial products to animals results in either abortion or labor.

b-Systemic maternal infections such as pyelonephritis, pneumonia, malaria and typhoid fever are associated with the onset of labor.

c-Intrautrine infection is associated with preterm labor and delivery.

1.2.9 The role of proinflammatory cytokines and other inflammatory mediators in preterm labor

A considerable body of evidence supports a role for inflammatory mediators in the mechanisms of preterm labor. Major attention has been focused on the role of proinflammatory cytokines such as IL-1*β*, TNF-α and IL-8. However, other proinflammatory and anti-inflammatory cytokines may also play a role, as can chemokines, platelet activating factors (PAF), prostaglandins and other inflammatory mediators. During the course of ascending intrauterine infection, microorganisms may reach the decidua, where they can stimulate a local inflammatory reaction and the production of proinflammatory cytokines and inflammatory mediators (platelet activating factor, prostaglandins,

leukotrienes, reactive oxygen intermediates and nitric oxide). If this inflammatory process is not sufficient to signal the onset of labor, microorganisms can cross intact membranes into the amniotic cavity, where they can also stimulate the production of inflammatory mediators by resident macrophages and other host cells. Microorganisms that gain access to the fetus may elicit a systemic inflammatory response syndrome, which is characterized by increased concentrations of IL-6 (Gomez *et al.,*1998) and other cytokines (Berry *et al.,*1998.,Romero *et al.,*2000), as well as cellular evidence of neutrophil and monocyte activation (Berry *et al.,*1995).

Evidence for the participation of IL-1 and TNF-α in preterm labor includes the following: IL-1*ß* and TNF-α stimulate prostaglandin production by amnion, decidua and myometrium (Romero *et al.,*1991); human decidua can produce IL-1*ß* and TNF-α in response to bacterial products (Gauldie *et al.,*1987; Casey *et al.,*1989); amniotic fluid IL-1*ß* and TNF-α bioactivity and concentrations are elevated in women with preterm labor and intraamniotic infection (Romero *et al*.,1989; Romero *et al*.,1992). In women with preterm PROM and intraamniotic infection, IL-1*ß* concentrations are higher in the presence of labor (Romero *et al*.,1989; Romero *et al*.,1992), IL-1*ß* and TNF-α can induce preterm parturition when administered systemically to pregnant animals (Romero *et al.,*1991). Fetal plasma IL-1*ß* is dramatically elevated in the context of preterm labor with intrauterine infection

(Gomez *et al.,*1997); placental tissue obtained from patients with labor, particularly those with chorioamnionitis, produces more IL-1β than tissue from women not in labor (Taniguchi *et al.,*1991).

There is considerable redundancy in the cytokine network and thus it is not clear that a particular cytokine is required to signal the onset of labor. Results of knockout animal experiments suggest that infection-induced preterm labor and delivery occur in subjects that lack a particular cytokine (Hirsch *et al.,*2002).

1.2.10 Immunology of preterm labor

During pregnancy, there is an alteration in maternal immunity within the uterus where innate immune responses are tightly regulated to prevent immunological rejection of the fetal allograft. Disruption of the delicate balance of cytokines by bacteria or other factors increases the production of proinflammatory cytokines at the maternal-fetal interface, and activates the parturition mechanism prematurely. Despite years of searching, there is still no broadly effective strategy for preventing preterm labor, and most therapies are directed at inhibiting myometrial contractions and improving neonatal outcome. Recent studies with progestins and interleukin-10 (IL-10), however, are showing promise in randomized clinical trials and animal studies. Furthermore, the identification of the toll-like receptors as upstream mediators of inflammation may offer alternative

therapeutic targets for preventing this common pregnancy complication (Peltier, 2003).

During pregnancy, the maternal immune response in the liver and spleen is not impaired, even in the presence of overwhelming placental infection. In the placenta itself, large inflammatory infiltrates are identified in the maternal deciduas; however, there is no inflammatory response in the fetal spongiotrophoblast and labyrinth layers of the murine placenta despite the presence of large numbers of bacteria. This leads to the conclusion that local events at the maternal-fetal interface prevented an effective immune response, and that the infected placenta might exert further detrimental effects by providing the bacteria a protected environment from which they could seed other maternal and fetal organs (Beckerman, 1994).

Specific immunity decreases markedly in pregnancy. The number of leukocytes and neutrophils, especially the number of young band neutrophils, increases gradually with the advancement of pregnancy. The phagocytic activity of neutrophils increases in the 2nd and 3rd trimesters. Neutrophils may compensate in part for the weakened specific immunity of pregnant women (Pramanik *et al*., 2007)

Preterm births that are triggered by a bacterial infection, have several toll-like receptors (TLR) that sense the surface components of living or dead bacteria. TLR2 and 4 are key receptors in recognizing bacterial surfaces. Investigators have

concentrated their study on these two receptors as a possible link in producing the inflammatory response that is believed to have brought about the fetal death(Han and Lui, 2007) .

Decidua is the functional layer of the endometrium of pregnancy, and is largely shed during parturition. The decidua contains a variety of immune cells, including T and B lymphocytes, granulocytes, natural killer cells and macrophages (Bulmer *et al,.* 1988., Vince *et al.*, 1990).

Decidual macrophages (DMs) are considered to be involved in protecting the fetus against intrauterine infections, which probably contributes to more than a third of total preterm deliveries occurring between 23 and 36 weeks of gestation (Lettieri *et al.*, 1993). Pathogens such as Group B *Streptococcus, Escherichia coli (E. coli), Neisseria gonorrhoeae* and *Chlamydia trachomatis* are known to cause chorioamnionitis during pregnancy. Bacterial lipopolysaccharide (LPS) introduced into amniotic fluid can stimulate DMs to generate phospholipase A_2 and, hence, increases the production of prostaglandins E_2 and F_2 (Casey *et al.*, 1989) which may lead to preterm labour. DMs may also produce cytokines such as IL-1, TNF-α and IL-6 in response to an infection, and therefore cause an intrauterine inflammatory reaction that could provoke pre-term parturition (McGregor *et al.*, 1988). Moreover, during implantation, DMs have an important role in the regulation of apoptosis which is critical for the invasion of the developing embryo (Abrahams *et al.,* 2004) and have several

biochemical functions that favour local immunological tolerance against fetal tissues (Heikkinen *et al.*, 2003).

In order to further understand the role of DMs in intrauterine immunity, their ability to interact with pathogenic bacteria and yeast zymosan has been examined. Using fluroscein-isothiocynate (FITC)-labelled bacterial particles and flow cytometry, it has been observed that DMs bind bacteria in a dose-dependent manner, which subsequently leads to phagocytosis. These DMs also produce superoxide radicals and the pro-inflammatory cytokine TNF-α when challenged with bacterial LPS, suggesting an important role for decidual macrophages in bacterial recognition and clearance during pregnancy (Singh *et al.,*2005).

1.2.11 Low risk of preterm labor

Because preterm labor and birth are so very hard to detect and prevent, early and consistent prenatal care is very important. Reducing exposure to sexually transmitted diseases, good nutrition and weight gain, quitting cigarette smoking and alcohol or drug use can also reduce the risk of preterm labor. In general, early treatment is most beneficial (Kimsey, 2008).

1.2.12 The role of vaginal flora in normal pregnancy and in preterm labor

1.2.12.1 Composition of vaginal flora

The vaginal flora varies considerably throughout a womon's life and is influenced by changes in hormonal levels. In women of reproductive age, the glycogen content of vaginal epithelial cells is

higher than in prepubertal girls, and this allows lactobacilli to flourish. Other organisms commonly isolated are *Diphtheroid, Corynebacterium*, coagulase negative *Staphylococcus, Streptococcus, Enterococcus* sp , *Gardenerella vaginalis*, genital *Mycoplasma,* and anaerobes (Hill *et al*.,1985). A number of potential pathogens may also be found as part of the endogenous vaginal flora. These include *Staphylococcus aureus* and organisms indigenous to the gastrointestinal tract, such as *E. coli* and occasionally *Klebsiella*, *Proteus*, and *Enterobacter* species. Group B *streptococcus* is present in the vagina of about 15 percent of women and may reside in the gut, which acts as a reservoir for the colonization of the vagina. Certain pathogens such as *Neisseria gonorrhoeae*, *Haemophilus influenzae* and group A *streptococcus* are not part of the endogenous vaginal / cervical flora, and their presence indicates an exogenous infection (McDonald, 1997).

1.2.12.2 Vaginal flora in normal pregnancy

The vaginal flora may be expected to change as a result of the substantial hormonal increases that occur during the first trimester of pregnancy. Early studies comparing pregnant women with non-pregnants of reproductive age have reported that the concentration of aerobic lactobacilli is ten-fold higher in pregnant women. An aerobic flora is thought to be less common in pregnant than in the non-pregnant women (McDonald, 1997).

Hillier *et al*.,(1993) studied the vaginal flora of 171 women who were admitted to the labor and delivery suite. The vaginal

flora was categorized as normal in 50 percent of cases, intermediate in 27 percent, and abnormal in 23 percent. Organisms commonly found in women with normal smears were lactobacilli, coagulase negative *Staphylococci, Ureaplasma, Diphtheroids, Viridans streptococci, Gardnerella vaginalis, Enterococcus spp., Candida albicans, E. coli*, group B *Streptococcus, and Staphylococcus aureus.*

1.2.12.3 Change in flora during normal pregnancy

Both aerobic and anaerobic flora remain fairly constant during pregnancy, and importantly, there is no general increase in the isolation of pathogenic bacteria as pregnancy progresses. The prevalence of *E. coli* appears to decrease with advancing gestation and is lower in each successive trimester (McDonald, 1997).

Vaginal flora in mid trimester and again in early labor with intact membranes reveals considerable differences in the persistence of vaginal organisms during late pregnancy. *Gardnerella vaginalis, Ureaplasma urealyticum, Bacteroides*, and to a lesser extent, *Mycoplasma hominis* tend to occur in mid-trimester and to persist throughout pregnancy. In contrast, a second group of organisms: *E. coli, Klebsiella* spp.*, Proteus* spp.*, Haemophilus influenzae, Staphylococcus aureus*, and *Peptostreptococcus* sp., when found in labor, are not present in the mid-trimester. They are almost always acquired late in pregnancy, especially in women who deliver preterm (McDonald *et al*.,1994).

1.2.13 Treatment of preterm labor

The therapeutic interventions considered in the setting of preterm labor generally have the following goals (Robert and Goldenberg, 2002):

a-To inhibit or reduce the strength and frequency of contractions, thus delaying the time to delivery.

b- Antibiotics are given in an attempt to prevent infection and prolong the pregnancy in order to optimize fetal status before preterm delivery (Gallagher, 2007).

Bedrest and intravenous fluids are believed to help stop contractions for some women, but clear benefit has not been shown. Treating preterm labor with medication has historically been unpleasant for the mother; at times dangerous, and has shown limited benefit in reducing the number of preterm births (Kimsey, 2008).

1.2.14 Prevention of preterm labor

Prevention of preterm labor may be divided into two major areas. The first involves a reduction in the presence of one or more of the specific risk factors, or, in a more general approach, an improvement in quality of life including income and nutritional enhancement, and a reduction in physical and emotional stress. Other programs attempting to decrease the rate of preterm delivery

have focused on screening for detection of preterm contractions or cervical change before the onset of true labor. These approaches include:

a-Patient education to recognize preterm contractions;

b-Provider surveillance for cervical changes;

c-Home uterine activity monitoring (Robert and Goldenberg, 2002).

At the present time, there is no prophylactic therapy which has been demonstrated to be unequivocally beneficial in preventing the onset of preterm labor in a high risk population. There is no evidence that oral beta-sympathomimetic drugs reduce the risk of preterm delivery, and their use has generally been abandoned in UK obstetric practice. Commonly used therapies include cervical cerclage, non-steroidal anti-inflammatory drugs, and more recently progesterone (Bennett, 2007).

Family physicians who provide obstetric care should include risk identification and education regarding the signs and symptoms of preterm labor in routine prenatal care. Although bed rest and decreased activity have not been shown to decrease the rate of preterm birth in high risk pregnancies, decreased activity is reasonable if the women experiences increased uterine contractions with activity. Because the data do not clearly show benefits of monitoring uterine activity at home, it is not

recommended at this time for the prevention of preterm delivery (Beverly and Von Der Pool, 1998).

Preventing the onset of preterm labor is generally unrewarding, but behavioural and life-style modification coupled with optimal management can reduce the incidence of delivery (Goswami and Thornton, 2006).

2. Materials and Methods

2.1 Materials

2.1.1 Patients

A total of sixty pregnant women with preterm labor whose ages range between (17-40) years have been included in this study. Those patients have been clinically diagnosed by gynecologists as having preterm labor, and were admitted to the labor room at Babylon Hospital of Maternity and Pediatrics, during the period from November/2007 to May/2008.

2.1.2 Controls

Twenty control women, whose ages range between (20-40) years, were divided into three groups:

a-Ten pregnants at term (37 weeks of gestation or more) with infection (vaginal infection or urinary tract infection).

b-Five pregnants at term (37 weeks of gestation or more) without infection.

c- Five normal, non-infected females-non pregnants.

2.1.3 Specimens Collection

2.1.3.1 Amniotic Membrane Piece

When the patient become fully dilated she is placed in lithotomy position. Povidone iodine used to wash the vagina and perinem. After delivery of the baby the placenta and membrane are deliverd by schultz method and placed in asterilized dish. Apiece of membranes is taken from the inner side (maternal side) using asterilized pense and cissor to avoid contamination. Each piece was

placed in a sterile tube containing brain-heart infusion broth and incubated aerobically for 24-48 hours at 37C,° and then swab or loopfull was taken from this medium and inoculated on culture media (Blood agar, MacConkey agar and Nutrient agar) and incubated aerobically for 24-48 hours at 37C°(Collee *et al*., 1996).

2.1.3.2 Blood Samples

Blood samples were collected from 60 patients and 20 controls (six milliliters of venous blood) withdrawn by disposable syringe under aseptic technique. Each blood sample was divided into two parts:

a- Four milliliters were put in a tube containing anti-coagulant (EDTA) for E-rosette technique and phagocytic index.

b- Two milliliters were placed in a sterile plane tube and allowed to clot, then serum was separated by centrifugation at 300g for 15 minutes. The serum has been stored by freezing until used for serological tests to estimate the concentration of Immunoglobulins and TNF-α.

2.1.4 Laboratory Equipments and Apparatuses

Table (2-1) Equipments and Aapparatuses

Equipment	Company (Origin)
Autoclave	Stermite- Japan.
Bunsen burner	Germany
Centrifuge	Hermle- Japan
Hot air oven	Memmert-Germany
Hot plate	Classico-India
Incubator	Memmert- Germany
Light microscope	Olympus-Japan
Micropipette	Oxford, USA
Millipore filter paper	Satorius Membrane Filters GmbH- W. Germany
Ocular lens	Olympus-Japan
pH meter	Hoeleze&Cheluis,KG-Germany
Refrigerator	Concord- Italy
Sensitive electric balance	A & D-Japan
Sterile syringe	Discardit-Spain
Water bath	Memmert- Germany
Water distillator	GFL- Germany
ELISA	Beckman-Germany
ELISA Mixer	Denley-England
ELISA Washer	Beckman-Germany
ELISA Shaker	Jean Robin-France

2.1.5 Chemical and Biological Materials

Table (2-2) The Chemical and Biological Materials

Material	Company (Origin)
A-Chemical Materials Na_2HPO_4, KH_2PO_4, NaCL, $MgSO_4$, $CaCL_2$, K_2HPO_4	Merk, Switzerland.
- α-naphthol, KOH, ferric ammonium citrate, HCl, isopropyl alcohol, methyl red, tetramethyl-P-paraphenylene diamine dihydrochloride, Phosphate buffer saline(PBS-PH= 7.2) tablets.	B.D.H.-England
-99% ethanol, glucose, H_2O_2, *p*-dimethylaminobenzaldyde, 99% methanol, urea solution.	Fluka chemika-Switzerland
B-Biological Materials: - Culture media: Agar-agar , Blood agar base, Brain heart infusion agar, MacConcky agar, Mannitol salt agar, Nutrient agar, Müller-Hinton agar, Nutrient broth, peptone broth, Egg yolk agar, M9 agar.	Mast Lab.-England
Triple sugar iron agar(TSI agar), MR-VP broth, Simmon's citrate agar, Urea agar base .	Diffco-USA
-IgG, IgM endoplates	Biomaghreb-Tunisia
-TNF-α	Biosource-Europe S.A
C-Stains: -Gram's stain set -Geimsa stain	Crescent-Saudi Crescent-Saudi

2.2 Methods

2.2.1 Solutions

2.2.1.1 Phosphate buffer saline PBS (pH= 7.2)

This buffer was used in E-rosette test. It has been prepared by dissolving one buffered tablet in 100 ml distilled water and sterilized by autoclaving (121 °C, 15 pound/ inch^2, for 15 minutes) in accordance with the instructions of manufacturer company (BDH), and was kept at 4 °C.

2.2.1.2 Alsever's solution (pH= 6.1)

This solution was used as an anticoagulant, preservative and transport medium for sheep red blood corpuscles (SRBCs).

It was prepared by dissolving the following materials in 1200 ml of D.W. (Lewis *et al*., 2001).

Glucose	24.6 grams
Tri Sodium citrate	9.6 grams
Sodium chloride	5.1 grams

The solution was sterilized by autoclaving and then the pH was adjusted to 6.1 with drops of citric acid at concentration of 10% (Garvery *et al*., 1977; Lewis *et al*., 2001).

2.2.1.3 Normal saline

Normal saline was used in washing of SRBCs, and for making the bacterial suspensions. It has been prepared by dissolving 8.5 grams of sodium chloride (NaCl) in one liter of

D.W., and sterilized by autoclaving, and kept at 4°C. (Cruickshank *et al.*, 1975).

2.2.1.4 Ficoll- Hypaque (Lymphoprep, 1.077 D)

This solution was used for the isolation of lymphocytes for E-rosette test. It was stored at 4 °C in dark bottles.

2.2.1.5 Suspension of sheep red blood corpuscles (SRBCs)

The SRBCs suspension was used in E-rosette test and prepared as follows:

Fresh blood withdraw from sheep jugular vein into a sterile bottle containing Alsever's solution in a proportion 1:1 volume. The blood-Alsever's solution was centrifuged at 300g for 15 minutes. The sedimented (RBCs) were washed three times with normal saline, and re-suspended in Alsever's solution to a final concentration of (10%) (Garvey *et al.*, 1977).

2.2.2 Reagents

***Methyl red(MR) reagent**

0.1 gram of methyl red was dissolved in 300 ml of 99% ethanol and then completed the volume to 500 ml by distilled water. This reagent was used for differentiation of organism's ability to produce acid as an end product when fermenting dextrose (MacFaddin, 2000) .

*__Voges –Proskauer(VP) reagents__

Reagent A

Five grams of alpha-naphthol were dissolved in 100 ml of 99% ethanol .

Reagent B

Forty grams of KOH were dissolved in 100 ml of distilled water; and used for differentiation of organisms produced acetylmethylcarbinol end products when fermenting dextrose (Collee *et al.,*1996) .

***Oxidase reagent**

It was prepared by dissolving 0.1 gram of tetramethyl-paraphenylene diamine dihydrochloride in 10 ml of distilled water and stored in a dark container. It was used for the detection of the ability of bacteria to produce oxidase enzyme (Forbes *et al*.,2007).

***Catalase reagent**

Three percent solution of H_2O_2 was used to detect the ability of bacteria to produce catalase enzyme (Forbes *et al.,*2007). It was stored in a dark container.

***Kovac's reagent**

This reagent was prepared by dissolving 5 grams of (*P*-dimethyl-aminobenzaldehyde) in 75 ml amyl alcohol, and then 25 ml of concentrated hydrochloric acid was added. This reagent was used for detection of indole production (MacFaddin, 2000).

2.2.3 Culture Media

All culture media were prepared according to the instructions of the manufacturer's manual.

a- M9 media

Six gms of Na2HPO4, 3gms of KH_2PO_4, 0.5 gm of NaCL and 1gm of NH4CL were dissolved in 950 ml of D.W. with 2% agar, and then sterilized into autoclave. After cooling the mixture cooled to 50° C. 2ml of 1M of MgSO4 ,10 ml of 20% glucose and 0.1 ml of 1 M of CaCL2, sterilized separately by filtration were added; then the volume completed to 1000 ml (Miniatis *et al.*,1982).

b- Egg yolk agar medium

This medium was used to detect the ability of bacteria to produce lecithinase; prepared by adding 15 ml of egg yolk suspention to 85ml sterile nutrient agar after cooling it to 55^oC (Collee *et al*., 1996).

2.2.4 Stains

Gram's stain: This stain was used to differentiate Gram-negative from Gram-positive bacteria according to Collee *et al*.,1996.

2.2.5 Identification of Bacteria

A single colony was taken from each primary positive culture on blood agar, and on MacConckey agar and it has been identified depending on its morphology (colony shape, size, colour, borders, and texture), and then examined by the microscope after

being stained with Gram's stain. After staining, the biochemical tests have been done on each isolate to complete the final identification (Collee *et al.,* 1996; Benson, 1998; MacFaddin, 2000; Murray *et al.,* 2003, and Forbes *et al.,*2007).

2.2.6 Biochemical Tests

2.2.6.1 Catalase Test

A colony of the organism is transferred by sterile wooden stick to the surface of a clean, dry glass slide; and one drop of 3% H_2O_2 is added to it. The formation of gas bubbles indicates the positive result (Forbes *et al.,*2007).

2.2.6.2 Oxidase Test

A piece of filter paper was saturated with oxidase reagent; then a colony of organism was spread onto the filter paper. If the color turns rose to purple, the oxidase test would be positive (Forbes *et al.,*2007).

2.2.6.3 Coagulase Test

This test is used to detect the ability of an organism to clot plasma by the action of the enzyme coagulase. Coagulase slide method is used to detect the bound coagulase that is found on the surface of cell wall as follows :

After emulsifying staphylococcal colony with a drop of sterilized normal saline on a clean slide, one drop of human plasma has been added, then mixed gently. Coagulase positive organisms become clumped after a few seconds. To compare the result, control test was done by mixing saline and bacteria without plasma

to ensure that the organisms do not clump spontaneously (Bennerman, 2003) .

2.2.6.4 Indole Test

This test is used for the determination of the organisim's ability to produce indole from deamination of tryptophan by tryptophanase. The formation of red color ring at top of broth indicates a positive reaction, while a yellow color ring indicates a negative reaction (MacFaddin, 2000).

2.2.6.5 Methyl Red Test

It is employed to detect the production of sufficient acid during the fermentation of glucose. The change of color to orange indicates a positive reaction (Murray *et al.,* 2003).

2.2.6.6 Voges-Proskauer(acetoin production) Test

The VP test is used to detect acetoin (acetyl-methyl-carbinol), which is produced by certain bacteria during growth in peptone glucose broth (MR-VP broth). The positive result is indicated by changing the color of the medium to red (MacFaddin, 2000).

2.2.6.7 Simmon's Citrate Test

The citrate test is used to determine the ability of a bacterium to utilize citrate as its only source of carbon. The positive result changes the color of media from green to blue (Forbes *et al.,*2007).

2.2.6.8 Triple Sugar Iron(TSI) Test

The aim of this test is to differentiate the enterobacteriaceae according to carbohydrate fermentation and hydrogen sulfide production (Murray *et al.,* 2003).

2.2.6.9 Urease Test

Urease is an enzyme that breaks the carbon-nitrogen bond of amides to form carbon dioxide, ammonia and water. The urea base agar has been sterilized by autoclave, after that it was cooled to 50 C^o, and urea substrate was added to it. It was poured in sterile tubes; then it is inoculated by bacterial cultures, which were incubated for (24 - 48) hours at 37C^o. When urea breaks down, ammonia is released and the pH of the medium increases. This pH change has been detected by a pH indicator that turns pink in a basic environment. A pink medium indicates a positive test for urease. Failure of deep pink color to develop marks a negative reaction (Collee *et al.,* 1996).

2.2.6.10 Mannitol Salt Agar

The medium was inoculated with bacterial colonies, then incubated at 37C° for 24 hours. The color changes from pink to bright yellow when the bacteria is a lactose fermenter and this mean a positive result, while unchanging color of the medium indicates a negative result (Collee *et al.,* 1996).

2.2.6.11 Eosin Methylene Blue (EMB) Agar

Lactose fermenting colonies are either dark or possessed dark centres with transparent colorless peripheries, while

organisms that do not ferment lactose remain uncoloured (Murray *et al.,* 2003).

2.2.6.12 Motility Test (Semisolid Media)

Non motile bacteria give growth that is confined to the stab-line and have sharply defined margins, leaving the surrounding medium clearly transparent. Motile bacteria typically give diffuse hazy growth that spread throughout the medium, rendering it slightly opaque (Murray *et al.,* 2003).

2.2.7 Chlamydia rapid test device as recommended by manufacturing company (ACON Laboratories, Inc.)

principle

The Chlamydia rapid test device (swab) is a qualitative, lateral flow immunoassay for the detection of Chlamydia antigen from female cervical swab. In this test, antibody specific to the Chlamydia antigen is coated on the test line region of the test. During testing, the extracted antigen solution reacts with an antibody to Chlamydia that is coated onto particles. The mixture migrates up to react with the antibody to Chlamydia on the membrane and generate a red colored line in the test line region. The presence of this colored line in the test line region indicates a positive result, while its absence indicates a negative result. To serve as a procedural control, a colored line will always appear in the control line region, indicating that proper volume of specimen has been added, and membrane wicking has occurred .

Procedure

Allow the test; specimen, reagents, and/or controls to reach room temperature (15-30ºC) prior to testing.

a- The reagent (A) bottle was hold vertically and added 5 full drops of reagent (A) to the extraction tube. Reagent (A) is colorless. Immediately the swab was inserted, compress the bottom of the tube and rotate the swab 15 time. Let stand for 2 minutes.

b-Filled the quantitative pipette for reagent B up to the marked line, then added the reagent B to the extraction tube. The solution will turn cloudy. Compressed the bottom of tube and rotated the swab 15 time until the solution turns to a clear color with a slight green or blue tint. If the swab is bloody, the color will turn yellow or brown. Let stand for 1 minute.

c-Pressed the swab against the side of the tube and withdraw the swab while squeezing the tube. Keep as much liquid in the tube as possible. Fitted the dropper tip on top of the extraction tube.

2.2.8 Effect of Antibiotics on Lecithinase Production

Egg yolk agar medium was prepared, by adding 15 ml of egg yolk suspention to 85ml sterile nutrient agar after cooling it to 55ºC. Afterwards, the antibiotics solution at concentration 0.1mg/ml sterilized by filtration was added to the medium. The control medium was prepared but without antibiotics. The media were poured in sterilized petridishes and left to soulidify. By making holes were made in these media, and filled with bacterial broth. Then plates were incubated at 37ºC for 24-48hr. The effect

was read by disappearing the discoloration around these holes. (By supervisor communication).

2.2.9 Antibiotics Sensitivity Test

Antibiotic diffusion test (Kirby-Bauer susceptibility test) has been carried out according to MacFaddin, (2000). Antibiotic disc potency has been supplied from Bioanalyse (Turkey) (Table2-3).

Table (2-3): Antibiotic Disc Potency

Type	Abbreviation	Disc potency(µg)
Norfloxacin	NOR	10
Amikacin	AK	30
Azithromycin	AZM	15
Doxycycline	DO	30
Clarithromycin	CLR	15
Ceftazidime	CAZ	30
Ampicillin + Cloxacillin	APX	25 + 5
Cefotaxim	CTX	30
Gentamycin	GN	10
Erythromycin	E	15
Cephalexin	CL	30
Amoxicillin	AX	25
Amoxicillin + Clavulanic acid	AMC	20 + 10

2.2.10Detection of virulence factors

2.2.10.1 Lecithinase (phospholipase) test (pH:5-6)

Lecithinase test has been carried out in egg-yolk agar medium to determine the ability of microorganisms to produce the lecithinase after inoculation of the medium agar; plates were incubated overnight at 37°C. The appearance of opaque zone area around the colonies indicates a positive result for this test (Collee *et al*., 1996).

2.2.10.2 Protease test (pH:7.2)

This method was carried out by using M9 agar, supplemented with 2% agar. After sterilization in autoclave and cooling at 50ºC,0.25 gm/L glucose sterilized by filtration was added, and the media is supported by 1% Gelatin. After the inoculation of this media with bacterial strain and incubation for 24-48 hr, at 37ºC, 3ml of Trichloroacetic acid (5%) was added to precipitate the protein. The positive result is read by observing a transparent area around the colony (Piret *et al.,*1983).

2.2.10.3 CAMP Test

The CAMP reaction is eponymously named for its original descriptors: Christie, Atkins and Munch-Peterson (Christie *et al* .,1994). Certain organisms (including group B streptococci) produce a diffusible extra-cellular protein (CAMP factor) that acts synergistically with the beta-lysin of *staphylococcus aureas* to cause enhanced lysis of red blood cells (Forbes *et al* .,2007).

2.2.10.4 Haemolysin production

Haemolysin production has been carried out by inoculating a blood agar medium with bacterial isolate at 37ºC for 24-48 hrs. An appearance of clear zone around the colonies refers to complete haemolysis (β-haemolysis). Greenish zone around the colonies refers to partial haemolysis (α-haemolysis); while no change refers to non-haemolysis (γ- haemolysis) (Doboy *et al*.,1980).

2.2.10.5 Coagulase factor test

This test was carried out as described in (2.2.6.3) .

2.2.11 Immunological tests

2.2.11.1 Erythrocyte–rosette formation (E-rosette test)

E-rosette means the clustering of sheep erythrocytes around a leukocyte or other cell. This test is used as a marker for T-lymphocytes of humans and most mammals. E-rosette test is a method used to identify, isolate and estimate the T-lymphocyte (Parslow *et al*.,2001).

This test has been carried out according to Burrell, 1979; Madsen *et al*., 1980 ; Frank, 1997; and Gengozian *et al.*, 2002) as follows:

a- Three ml of lymphoprep (Ficoll-Hypaque) have been pippetted into centrifuge tube .

b- Two ml of freshly drown blood have been carefully layered onto the surface of centrifuge tube without mixing.

c- Centrifugation has been carried out at 400g for 30 minutes at room temperature.

d- The leukocytes appears as a fluffy white coating at the plasma medium interface, while the erythrocytes appears on the bottom of the tube, the upper plasma layer has been carefully collected and saved for further experiments.

e- The white cells have been collected with a Pasteur's pipette and placed into another centrifuge tube.

f- Centrifugation is performed at 400 g for 30 minutes and the supernatant is discarded.

g- The preparation is washed three times in PBS at 400 g for 15 minutes.

h- The pellet is re-suspended in 0.5 ml of saline to which 0.05ml autologous plasma was added .

i- To another centrifuge tube containing 0.1 ml of plasma lymphocyte mixture, 0.1 ml of SRBCs (10%) has been added and mixed gently.

j- The centrifuge tube containing the mixture is incubated at 37°C for 15 minutes, then centrifuged at 200 g for 15 minutes.

k- Incubation is carried-out for one hour at 4°C with the supernatant stilled on the pellet .

l- The cells are resuspended very gently by tilting the tube back and forth 2-3 times.

m- Blood film is prepared, fixed with ethanol for 10 minutes, and stained with Geimsa's stain for 15 minutes, the film is washed with D.W., dried in air, and examined microscopically under oil immersion lens (100x).

n- One hundred lymphocytes have been counted, and the percent of rosette forming lymphocytes with three or more sheep erythrocytes adhered to it, have been recorded.

2.2.11.2 Determination of Immunoglobulins Concentration:

Principles of single radial immunodifution (SRID) test

Equal volumes of control and test serum samples have been added to wells in an agarous gel-containing a mono-specific antiserum. The sample diffuses radically through this gel and the substance being assayed (antigen) forms a precipitation ring with the mono-specific device (ocular). Unknown concentrations have been determined from the tables supplemented with each type of endoplate which contains 12 wells (Lewis *et al*., 2001).

Procedure

Endoplates and the serum (of patients and control) have been removed from refrigerator. Reagents were equilibrated to room temperature.

a-Plate has been removed from ziplock bag. After lid removed, the wells were inspected for moisture. If moisture was present, plates were left uncovered to remain at room temperature (approximately 15 minutes) until moisture evaporated.

b-Sera of patients were thoroughly shaken (in their own containers) by inversion. Each patient's sample was dispensed onto the appropriate wells. Each well required 5 µl of serum.

c-After lid was replaced, it was incubated at room temperature on a level surface. Incubation times were 72 hours for IgG test and 96 hours for IgM test.

d-Immunopreciptin ring diameters were microscopically measured by ocular lens to the nearest 0.1mm. The calculated diameters were compared to the standard diameter to calculate the concentrations of serum humoral factors (Lowell, 2001).

2.2.11.3 Phagocytic index

Principle

Phagocytes involves the ingestion of foreign materials. Ingestion can be determined by incubation of neutrophils with *staphylococcus aureus* then intracellular *Staphylococcus aureus* can be seen microscopically (Furth *et al* .,1985).

Polymorph nuclear lymphocyte separation (PMNLS)

PMNLS were isolated from the pellet as described by Bij *et al.,*(1988) with slight modification as follows:

a-Five milliliters of heparinzed blood were mixed with 5% solution of dextran and phosphate buffer solution (PBS) in a 4:1 ratio and subsequent sedimentation at 37°C.

b-The supernatant was then harvested and holding with isotonic sterile calcium and magnesium free Hank`s balanced salt solution (HBSS).

c-Cell suspension was then centrifuged at 2000g for 10 minutes in a conical test tube and the supernatant discarded. After washing

twice in HBSS, the pellet was gently resuspended in 200µl of HBSS, and then adjusted to ($5x10^6$ cell/ml).

Bacterial culture:

Staphylococcus aureus isolate was grown on nutrient agar over 37°C. The bacterial growth was harvested and washed 3 time with PBS. The pellet resuspended in PBS and diluted to appropriate cell count ($1x10^8$ cell/ml).

Assay procedure

This was performed according to the procedure outlined (Macki and Cartney,1995) by adding 0.25ml of bacterial suspension ($1x10^8$ cell/ml) to suspension of PMNLS ($1x10^6$ cell/ml) in 0.25ml HBSS. The mixture was mixed and incubated in water bath at 37°C for 30 minute with continuous slow mixing then centrifuged at 1500g for 5 minute. The pellet suspended in 200µl of HBSS. A drop was delivered to prepare thin smear and fixed with methanol. Smears were stained with diluted Giemsa stain for 10 minutes. The slides were then allowed to dry after washing with tap water and examined microscopically.

Calculation of the results

Two hundred PMNS were counted and the percentage of phagocytic cells were determined.

2.2.11.4 TNF–α EASIA Test as recommended by manufacturing company(BIOSOURCE)

Principle

The BIOSOURCE TNF-α EASIA is a solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA) performed on microtiter plate. The assay was based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs) directed against distinct epitopes of TNF-α are used. Antibody-producing cells are immortilized using the myeloma cell fusion method of Kohler and Milstein. A hybridoma cell was produced which secretes specific homogeneous antibodies. The use of a number of distinct MAbs avoids hyper specificity and allows high sensitive assay with extended standard range and short incubation time. Standards or samples containing TNF-α react with capture monoclonal antibodies (MAbs 1) coated on the microtiter well. After incubation, the occasional excess of antigen was removed by washing. Mab 2, the horseradish peroxidase (HRP)- labelled-antibody, was then added. After an incubation period allowing the formation of a sandwich: coated MAbs 1- TNF-α –Mabs 2 –HRP , the microtiter plate is washed to remove unbound enzyme labelled antibodies. Bound enzyme-labelled antibodies are measured through a chromogenic reaction. Chromogenic solution ($TMB+H_2O_2$) was added and incubated. The reaction is stopped with the addition of stop solution (H_2SO_4) and the microtiter plate is then read at the appropriate wavelength. The amount of

substrate turnover number was determined colourimetrically by measuring the absorbance which is proportional to the TNF-α concentration. A standard curve was plotted and TNF-α concentrations in a sample is determined by interpolation from the standard curve. The use of the EASIA Reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) result in high sensitivity in the low rang and in an extended standard rang.

Assay Procedure:

a-Selected the required number of strips for the run. The unused strips should be resealed in the bag with desiccant and stored at 2-8° C.

b-Secured the strips into the holding frame.

c-Pipette 50µl of incubation buffer into all wells.

d- Pipette 200µl of each standard, control, or sample into the appropriate wells.

e-Incubated for 2 hours at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.

f-Aspirated the liquid from each well.

g-Washed the plate three times by :

1-dispensing of 0.4 ml of BioSource wash solution into each well.

2-aspirating the content of each well.

h- Pipette 100µl of standard 0 into all well.

i- Pipette 50µl of anti- TNF-α conjugate into all the wells.

g-Incubated for 2 hours at room temperature on horizontal shaker set at 700 rpm ± 100 rpm.

k- Aspirated the liquid from each well

l- Washed the plate three times by :

1-dispensing of 0.4 ml of BioSource wash solution into each well.

2-aspirating the content of each well.

m- Pipette 200µl of freshly prepared chromogenic solution into each well within 15 min. following the washing step.

n- Incubated the plate for 30 min. at room temperature on horizontal shaker set at 700 rpm ± 100 rpm, avoiding direct sunlight.

o- Pipette 50µl of stop solution into each well.

p-Read absorbance at 450 nm and 490 nm (reference filter:630 or 650 nm) within 3 hours and calculate the results.

2.2.12 Statistical analysis:

Mean, standard deviation, and T-test ($p < 0.05$) were carried out according to Bowers (1997).

3.1 Clinical study

3.1.1 Age distribution of pregnants with preterm labor

As shown in table (3-1), a total of 60 pregnants with preterm labor were included in this study. 57 (95%) of the total had positive amniotic membrane bacterial culture, whereas 3(5%) of the total had negative culture. This high percentage of bacterial growth agrees with Robert and Goldenberg (2002) who found choriamnion colonization to be associated with 83% of the very early spontaneous preterm labor. The high percentage of positive bacterial culture may be due to genitourinary tract infection of pregnant women, as well as to the low level of immunresponse of pregnant women.

Table (3-1) Distribution of pregnants with preterm labor according to age and bacterial culture

Age range (years)	Number	Number of positive culture
≤20	22	21
21-25	11	10
26-30	12	11
31-35	9	9
≥40	6	6
Total	60	57

The results in the table (3-1) also reveals that the most affected age group is that of ≤20 years 22:60 cases (36.7%), and that the least affected group is ≥40 years 6:60 (10%).

This study has found that the preterm labor occurs in an age range of (17-40) years old. This results is in agreement with the results obtained by Goldenberg, (2002); Ross and Robert, (2007); and Kimsey, (2008) who have noticed that women younger than 17 and older than 35 carry a higher risk of preterm delivery.

3.1.2 Preterm premature rupture of membrane (PPROM) and preterm labor

The results indicated that 35 (58.4%) out of 60 pregnants with preterm labor have positive PPROM, while 25 (41.6%) have no rupture. PPROM is often due to an infection in the uterus (Health Topics, 2008).

At term, programmed cell death and the activation of catabolic enzymes such as collagenase and mechanical forces result in ruptured membranes.

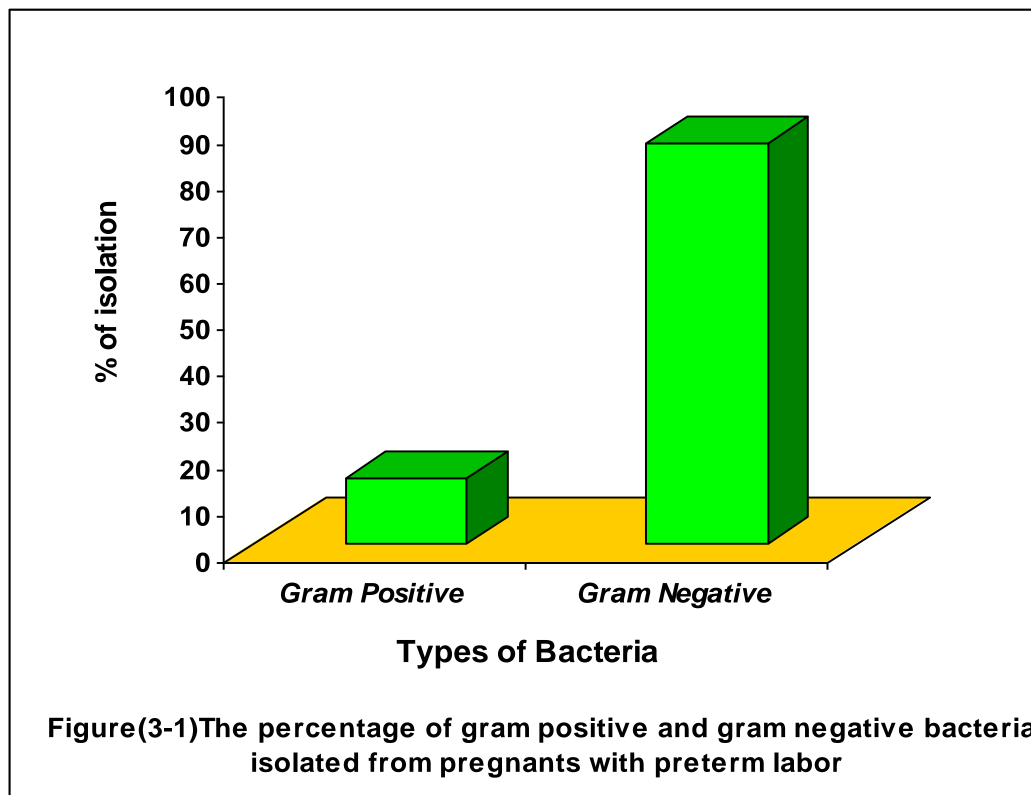
Preterm PROM occurs probably due to the same mechanisms and premature activation of these pathways. However, early PROM also appears to be linked to underlying pathologic processes, most likely due to inflammation and/or infection of the membranes. Clinical factors associated with preterm PROM include low socioeconomic status, low body mass index, tobacco use, preterm labor history, urinary tract infection, vaginal bleeding at any time in pregnancy, cerclage, and amniocentesis (Jazayeri *et al.*,2006).

3.2 Bacteriological study

3.2.1 Bacterial isolation

A total of 60 amniotic membrane pieces have been subjected to aerobic culturing on different types of culture media. The results reveal that 57 samples have positive bacterial culture, whereas 3 samples have showed no bacterial growth. This negative growth may be due to the consumption of antibiotics by the patients or the presence of another causative agents for preterm labor such as multiple pregnancy, poly hydramnions, uterine abnormalities, intrauterine death, iatrogenic and cervical incompetence (Steer and Flint, 1999).

The results shown that Gram negative bacteria are the predominant bacterial isolates and constitutes about 85.96% (49:57) from the total isolates and compared with Gram positive bacteria which constitutes only 14.04 % (8:57) as shown in figure (3-1) .



Figure(3-1)The percentage of gram positive and gram negative bacteria isolated from pregnants with preterm labor

The high percentage of Gram negative bacteria may be due to the presence of endotoxin (lipopolysaccaride) which associates with the onset of preterm labor (Romero *et al.*, 1988). Deb *et al.,* (2005) have reported that genito-urinary tract or systemic infections of the Gram negative bacteria in pregnant women causes preterm labor. Lipopolysaccaride is the most potent antigenic component of the Gram negative bacterial cell wall, and is known to modulate the expression of various proinflammatory cytokines. Therefore, women with preterm labor had ahigh concentration of endotoxin in amniotic fluid than patients who were not in labor (Romero *et al.*,2008).

3.2.1.1 Gram negative bacteria

The Gram negative bacteria isolated in this study are listed in table (3-3) .

Table (3-3) Number and Percentage of Gram Negative Bacteria Isolates from Pregnants with Preterm Labor

Bacterial isolate	NO.	%
E coli	29	50.9
Acinetobacter baumanii	9	15.75
Enterobacter sp.	4	7.0
Klebsiella sp.	3	5.3
Pseudomonas aeruginosa	2	3.25
Burkholdrea pseudomonallie	1	1.75
Providancia sp.	1	1.75
Total	49	85.96

E. coli is the most common bacterial species isolated from pregnants with preterm labor (50.9 %). This result matches with Uma *et al* ., (2007) who reported 75% *E. coli* has been found to be the most commonly organisms associated with preterm labor in many studies such as those of by Peltier (2003) and Lorie *et al.,* (2002). This may be due to the antibiotic resistance of *E. coli* which are considered the main cause of asymptomatic bacteriuria and urinary tract infection in pregnant women.

Acintobacter baumanii was the second type of Gram negative bacteria isolated from pregnants with preterm labor (15.75%).

The other bacteria that has been isolated in this study were *Enterobacter sp.* and *Klebsiellia sp.*, which represents 7.0% and 5.3% respectively.

Other Gram negative bacteria isolated from preterm pregnant but in low frequency were *Pseudomonas aeruginosa*, *Burkholdrea pseudomonallie,* and *Providancia sp.*, at 3.25 %, 1.75% and 1.75% respectively.

3.2.1.2 Gram positive bacteria

Table (3-4) shows the Gram positive bacteria isolated in this study. Coagulase negative *Staphylococci* and *Staphylococcus aureus* constitute 7.0 % and 1.75 % respectively. These results correspond with Farraj (2002) who had found that *Staphylococcus aureus* represents 2%. Group B streptococci (*Streptococcus agalactiae*) represents 3.5% in this study. *Streptococcus agalactiae* commonly associate with preterm labor. This association may be because *Streptococcus agalactiae* forms a part of normal vaginal flora and can cause ascending infection. In addition it can produce many virulence factors associated with its pathogenicity (Hillier *et al*., 1991; Beverly and Vonderpool, 1998).

The other bacteria that were isolated in this study was *Listeria monocytogenes* which represents 1.75%. Marchiano and Gandhi (2006) had reported that once bacteria infect the blood stream, they can spread to the placenta. This can lead to chorioamnionitis and may cause multiple, small infections. It may ultimately lead to premature rupture of the membrane and preterm labor.

Table (3-4) Number and Percentage of Gram Positive Bacteria Isolates from Pregnants with Preterm Labor

Bacterial isolate	NO.	%
Coagulase negative Staphylococci	4	7.0
Streptococcus agalactiae	2	3.5
Staphylococcus aureus	1	1.75
Listeria monocytogenes	1	1.75
Total	8	14.04

3.2.2 Virulance factors of bacterial isolates

3.2.2.1 Lecithinase (phospholipase) production

The vast majority of bacteria isolated from women with preterm labor were lecithinase producer (94.64%) as shown in table (3-5). This enzyme acting on phospholipids catalyzes the hydrolysis of phospholipids in cell membrane (Encarta, 2007). Bacterial products such as phospholipase were known to be able to stimulate prostaglandins production *in vitro* and *in vivo*, and prostaglandins were known to be involved in the initiation of human labor (Danielian and Hall, 2005) .

Table (3-5) Lecithinase Production by Bacterial Isolates Recovered from Pregnants with Preterm Labor

Bacterial Isolates	No. of Isolates	Lecithinase Production
E. coli	28	27
Acintobacter baumanii	9	8
Coagulase negative Staphylococcus	4	3
Staphylococcus aureus	1	1
Enterobacter sp.	4	4
Klebsiellia sp.	3	3
Pseudomonas aeruginosa	2	2
Burkholdrea pseudomonallie	1	1
Streptococcus agalactiae	2	2
Providancia sp.	1	1
Listeria monocytogenes	1	1
Total	56	53(94.64%)

3.2.2.2 Extracellular protease production

As shown in table (3-6), most bacteria isolated from women with preterm labor were negative for protease production. Only 30.77% were positive. A protease is an enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. Protease is also a type of exotoxin, which is a virulent factor in bacteria pathogenesis (Southan, 2001; Hedstrom, 2002 ;Hooper, 2002; Barrett *et al.*,2003; Puente *et al.*,2003; Ross *et al.*,2003 and

Puente and Lopez-Otin, 2004). Protease production by many microorganisms reduce the strength and elasticity of amniochorion membranes (McCoy *et al*., 1995).

Table (3-6) Extracellular Protease Production by Bacterial Isolates Recovered from Pregnants with Preterm Labor

Bacterial Isolates(No. of Isolates)	Protease Production
E coli (7)	3
Acintobacter baumanii (1)	1
coagulase negative Staphylococcus (1)	0
Staphylococcus aureus (1)	0
Enterobacter sp. (2)	0
Listeria monocytogenes (1)	0
Total (13)	4(30.77%)

3.2.2.3 Coagulase production

As shown in table (3-7), one strain of *Staphylococcus* is able to produce coagulase which is considered as a virulent factor for pathogenicity of these bacteria by clumping the fibrin around the bacteria (Hall, 1991; Kenneth, 2002). Possibly coagulase could provide an antigenic disguis if it clotted fibrin on the cell surface or could make the bacterial cells resistant to phagocytes or tissue bacterial target (Humphreys, 2004).

Table (3-7) Hemolysin and Coagulase Production by some Bacterial Isolates Recovered from Pregnants with Preterm Labor

Bacterial Isolates (No. of Isolates)	Coagulase	Hemolysin Production
E coli (5)	0	2(β)
Acintobacter baumanii (4)	0	0
Staphylococcus (5)	1	0
Streptococcus agalactiae (2)	0	2(β)
Klebsiellia sp. (1)	0	0
Pseudomonas aeruginosa (2)	0	0
Burkholdrea pseudomonallie (1)	0	1(β)
Providancia sp. (1)	0	0
Listeria monocytogenes (1)	0	1(β)

3.2.2.4 Hemolysin production

Table (3-7) also shows hemolysin production by some bacteria isolated from women with preterm labor.Two out of five isolates of *E. coli* expressed β-hemolytic. Both of the two isolates of *Streptococcus agalactiae* show narrow zone β-hemolysis. This result approximately fits with Gillespie *et al*., (2000) and Brook *et al*., (2007) who have reported that *Streptococcus agalactiae*

displays β-hemolysis when cultured on a blood agar plate, and produces zones of hemolysis that are only slightly larger than the colonies themselves.

One isolate of *Listeria monocytogenes* and *Burkholdrea pseudomonallie* also show β-hemolysis. *Acintobacter baumanii, Staphylococcus, Klebsiellia sp., Pseudomonas aeruginosa,* and *Providancia sp.* were γ –hemolysis (non hemolysis) pattern, with no color change around the bacterial colonies. Production of hemolysin by *E. coli* is considered the important virulence factor which are cytotoxic due to the formation of transmembranous pores in host cell membrane (Todar, 2008).

3.2.2.5 CAMP test

The isolates of *Streptococcus agalactiae, E. coli, Listeria monocytogenes* and *Burkholdrea pseudomonallie* show positive results for CAMP test when streaked adjacent to colonies of *Staphylococcus aureus* cultivated on sheep blood agar plates.

CAMP test used for the identification of *Listeria monocytogenes* were CAMP positive with *Staphylococcus aureus* (Hanson, 2006).

Table (3-8)CAMP Test of Beta Hemolytic Bacterial Isolates Recovered from Pregnants with Preterm Labor

Bacterial Isolates (No. of Bacterial Isolates)	CAMP Test
Listeria monocytogenes (1)	+
Streptococcus agalactiae (1)	+
E. coli (2)	+
Burkholdrea pseudomonallie (1)	+

3.2.3 Effect of antibiotics on bacterial isolates

The results of this study reveal that there is a remarkable increase in bacterial resistance (except for *Listeria monocytogenes*) to B-lactam antibiotics: amoxicillin, amoxiclave, ampiclox, and doxycycline as shown in figure (3-2) , (3-3) , (3-4) and (3-5) respectively. These results agree with Forbes (2007) who has reported that *Staphylococci* are the Gram positive bacteria that most commonly produce beta-lactamase. Gram negative bacteria including *Enterobacteriaceae*, *P. aeruginosa,* and *Acintobacter spp*., produce dozens of different beta-lactamase types that mediate resistance to one or more of the beta-lactam antibiotics .

Mechanisms of beta-lactam resistance may be due to: beta-lactamase production and altered target (penicillin binding protein PBP) among Gram positive bacteria. Among Gram negative bacteria resistance can be mediated by decreased uptake through

the outer membrane porins. In addition to the above factores, antibiotics effective against *Listeria monocytogenes* include ampicillin and azithromycin (Todar, 2008).

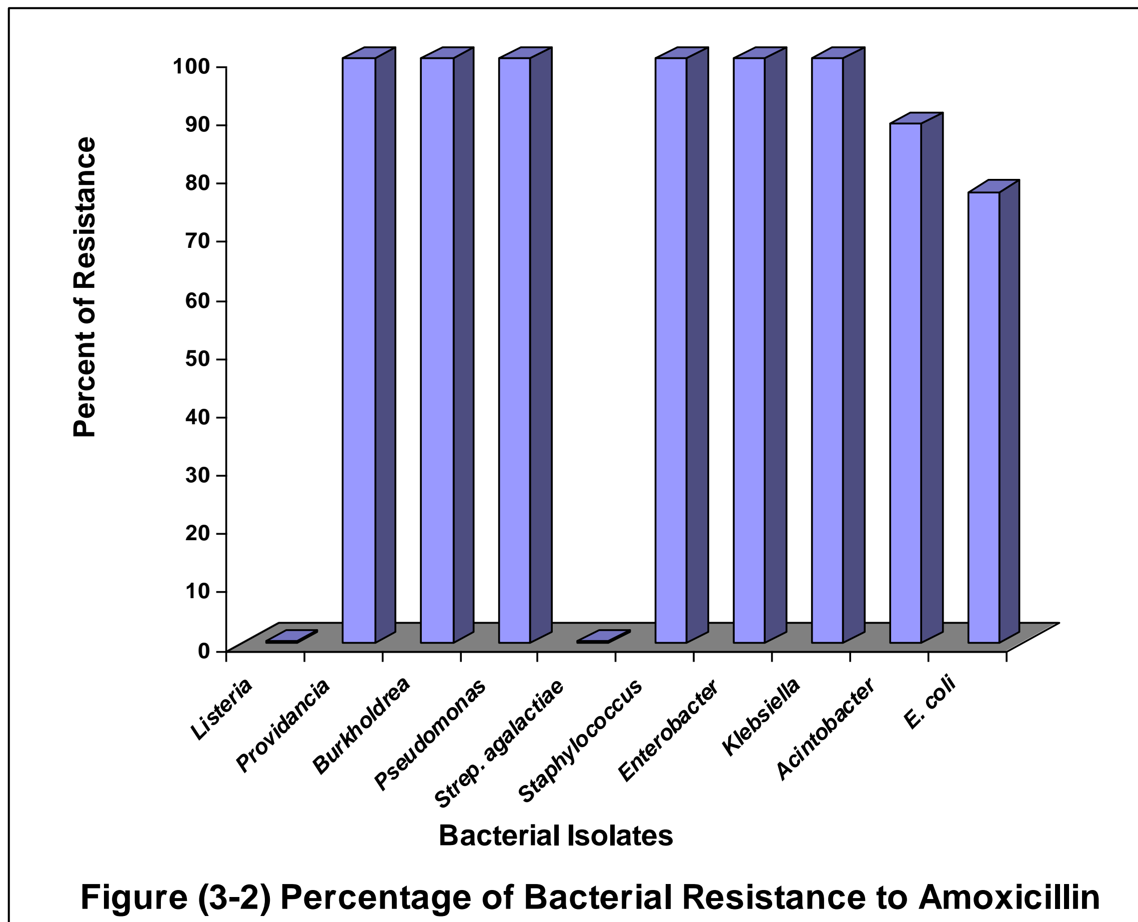


Figure (3-2) Percentage of Bacterial Resistance to Amoxicillin

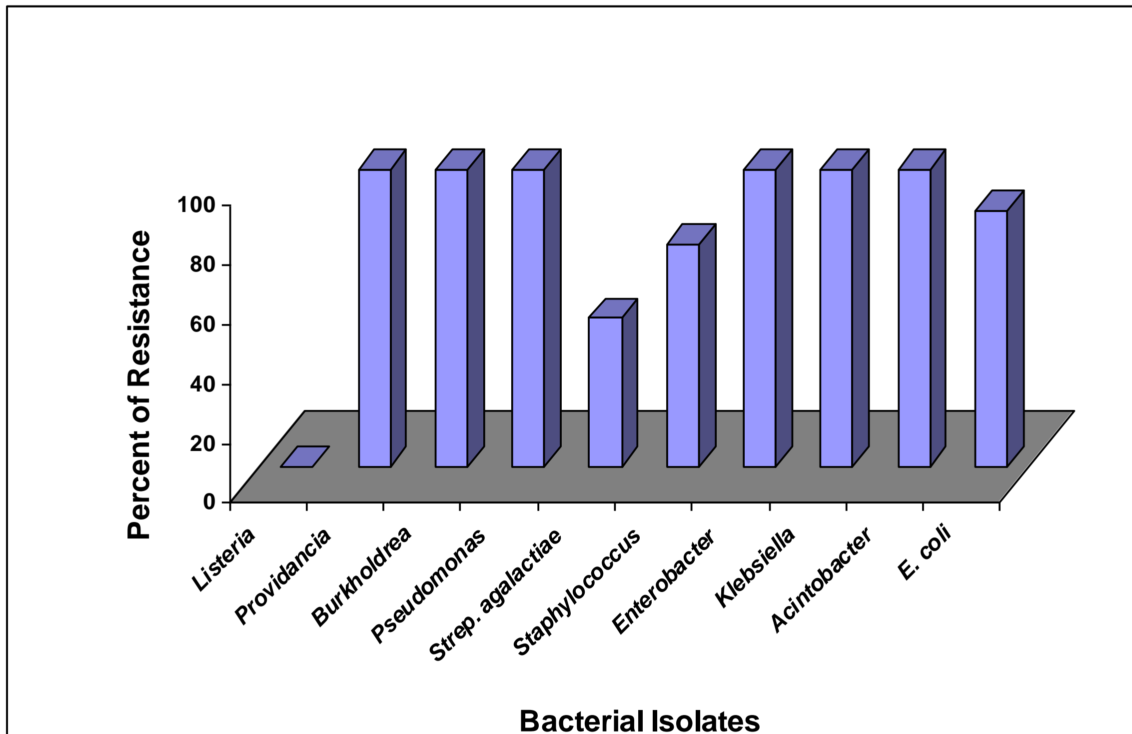


Figure (3-3) Percentage of Bacterial Resistance to Amoxiclave

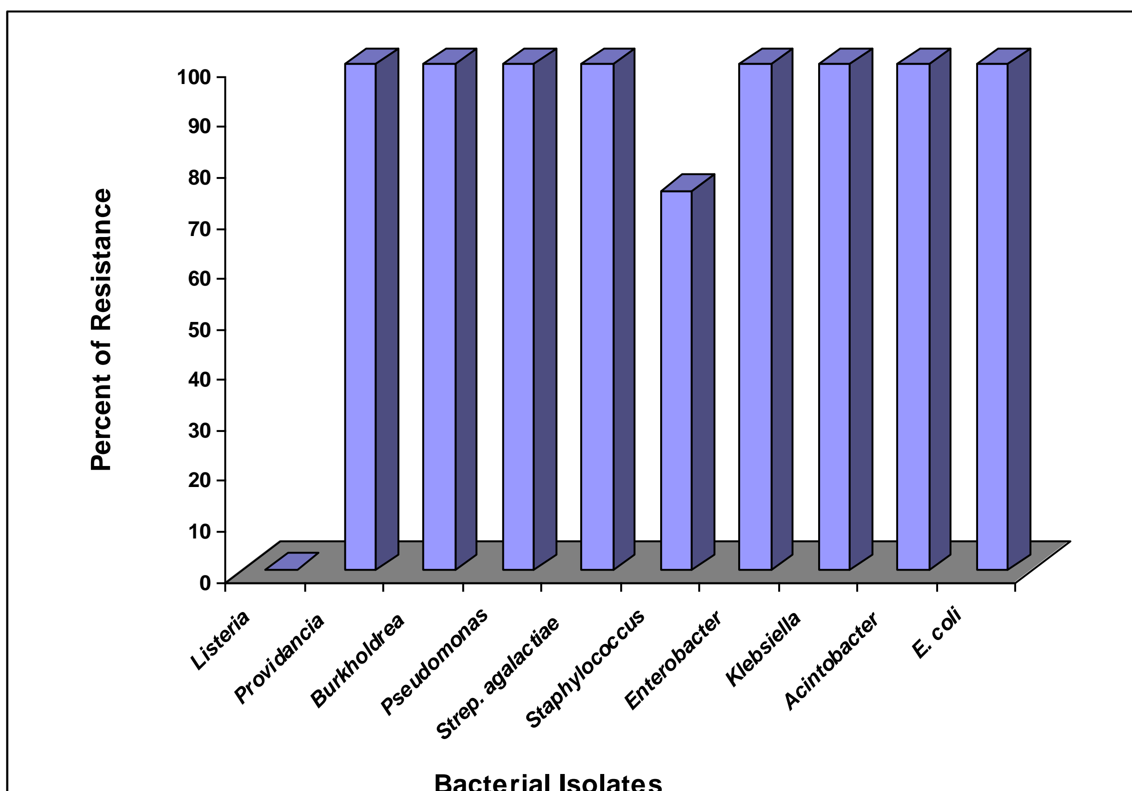


Figure (3-4) Percentage of Bacterial Resistance to Ampiclox

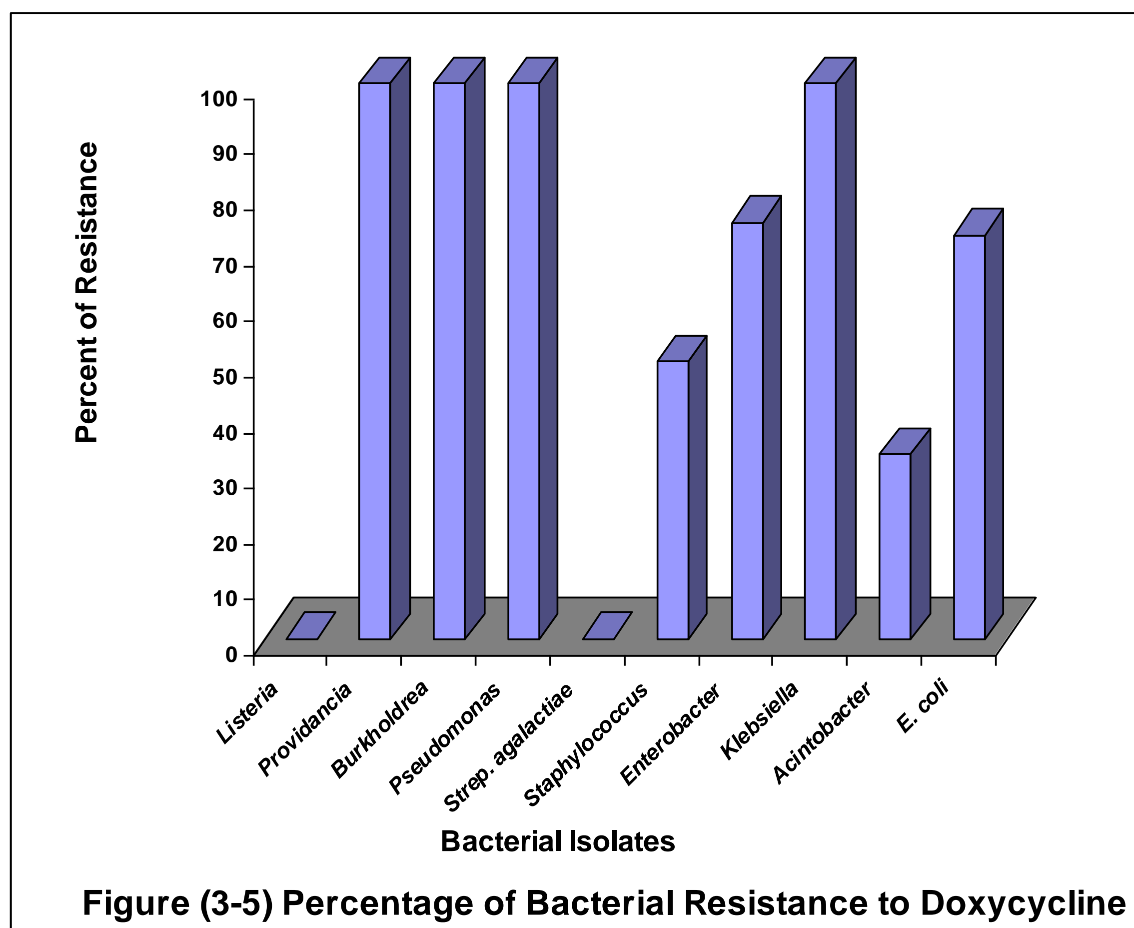


Figure (3-5) Percentage of Bacterial Resistance to Doxycycline

The effect of some cephalosporin which include ceftazidiume, cephalexin and cefotaxim on bacterial isolates has been also investigated as shown in figures (3-6), (3-7) and (3-8) respectively. Most bacteria resist the action of ceftazidime (except *Providancia sp.*) and cephalexine (except *Listeria monocytogenes*) .

Cefotaxime is a third generation cephalosporin. The results show that all isolates of *Klebsiellia sp., Staphylococcus, P. aeruginosa* and *B. pseudomonallie* are (100%) resistant to cefotaxime. *E. coli* and *Strep. agalactiae* are both (50%) resistant. The resistance decreased gradually to (44% and 25%) for *Acintobacter* and *Enterobacter*, respectively .

On the other hand, *Providancia sp.* and *Listeria monocytogenes* are completely (100%) sensitive to cefotaxime.

The resistance of *P. aeruginosa* to cephalosporin may be due to the synthesis of B-lactamase as well as loss of PBP by mutation.

P. aeruginosa exhibits intrinsic or acquired resistance to many antibiotics. These bacteria are highly inherently resistant, and this arises from combination of unusually restricted outer membrane permeability and chromosomally encoded B-lactamase. This agrees with results mentioned by Hankok and Speert (2000) and Bisiklis *et al* ., (2005).

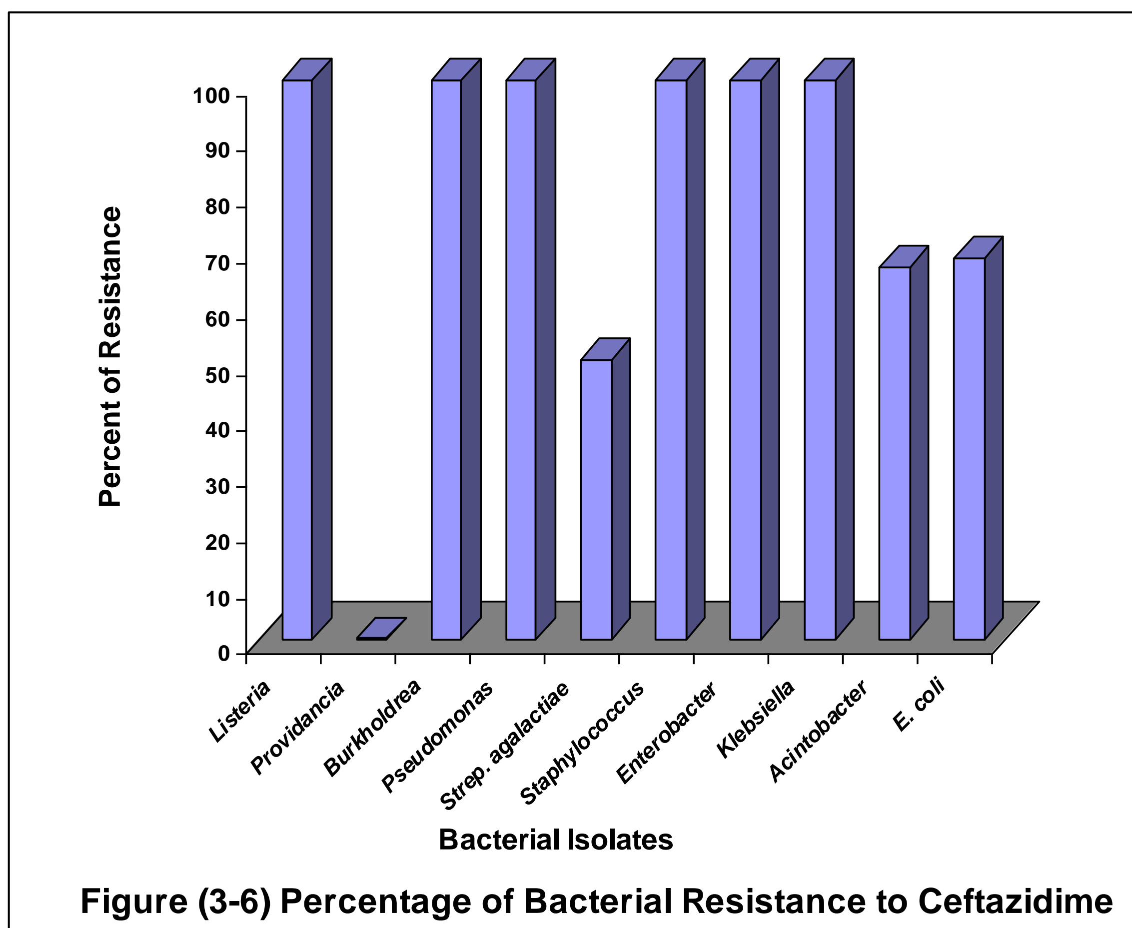


Figure (3-6) Percentage of Bacterial Resistance to Ceftazidime

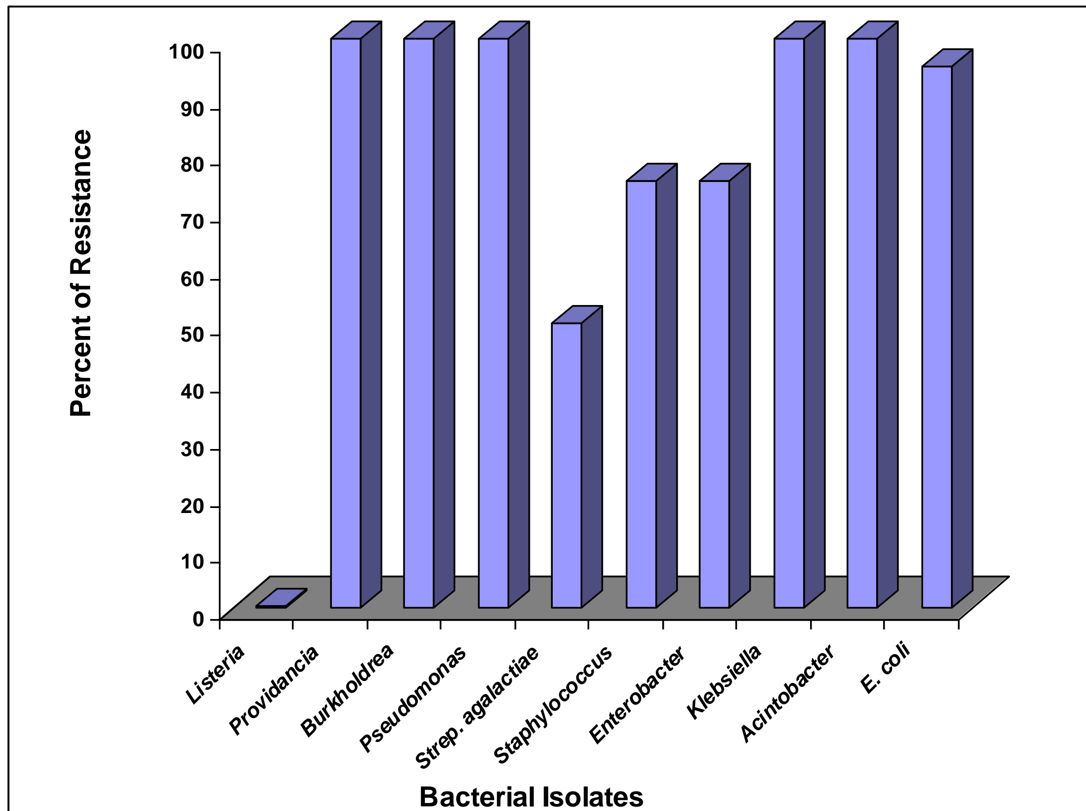


Figure (3-7) Percentage of Bacterial Resistance to Cephalexin

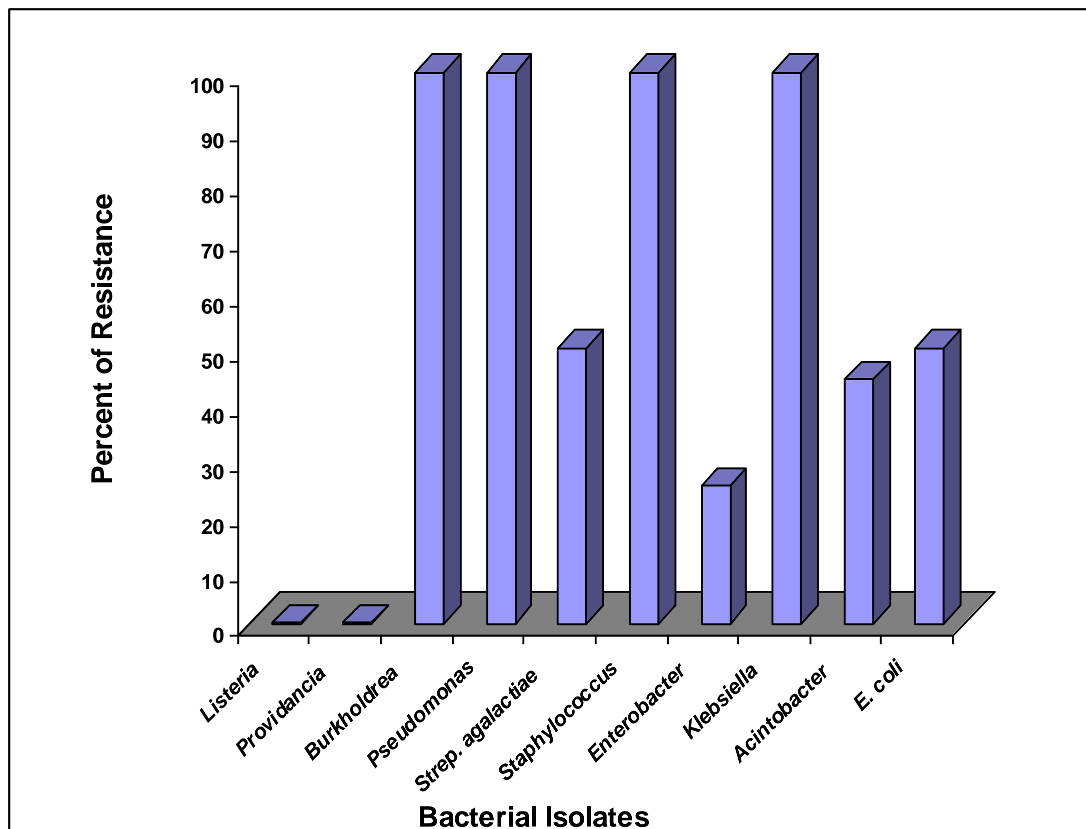


Figure (3-8) Percentage of Bacterial Resistance to Cefotaxim

Bacterial resistance to macrolide group, which include azithromycin, clarithromycin and erythromycin, has also been investigated as shown in figures (3-9), (3-10) and (3-11) respectively. Most bacteria are resistant to these antibiotics, except *Listeria monocytogenes* and *Strep. agalactiae* which were completely susceptible (100%) to macrolide group, followed by *Staphylococcus*.

The results show the effect of this antibiotic is more on Gram positive bacteria than on Gram negative. This agrees with Forbes (2007) who has reported that macrolides are not effective against most genera of Gram negative bacteria. However, they are effective against Gram positive bacteria in contrast with the uptake difficulties associated with Gram negative outer membrane.

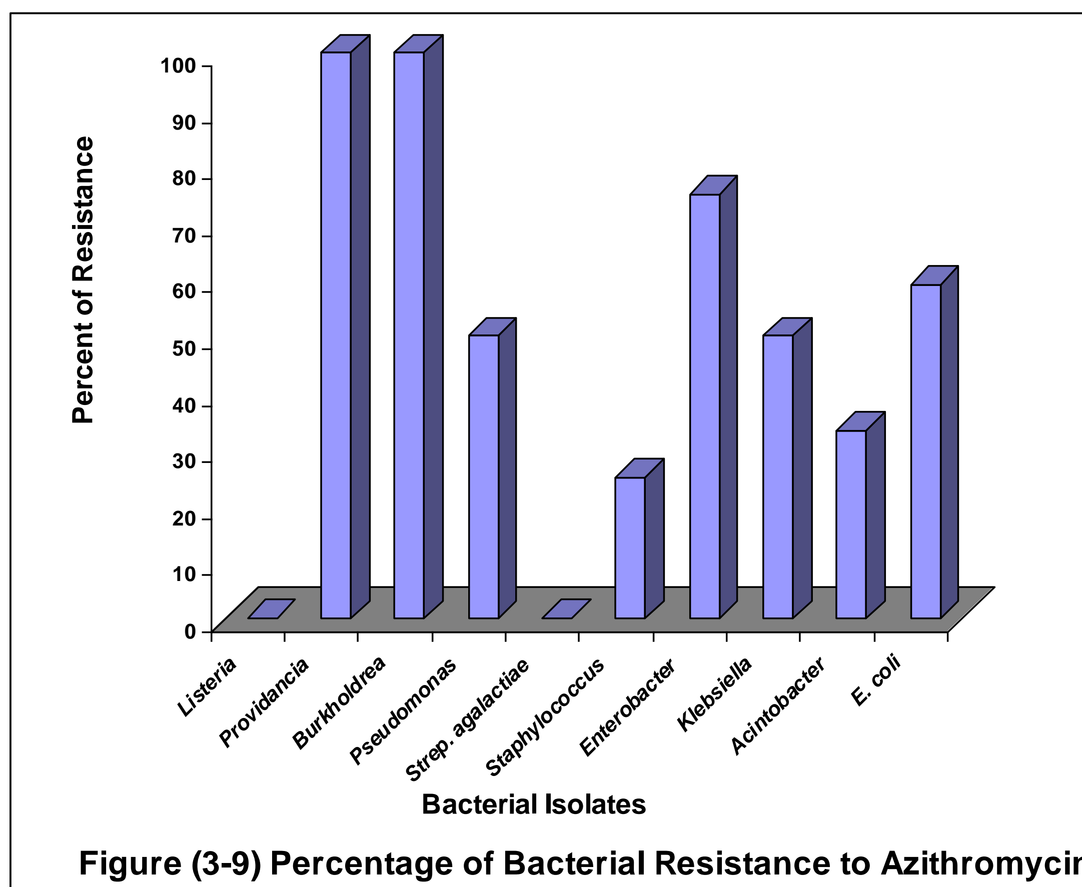


Figure (3-9) Percentage of Bacterial Resistance to Azithromycin

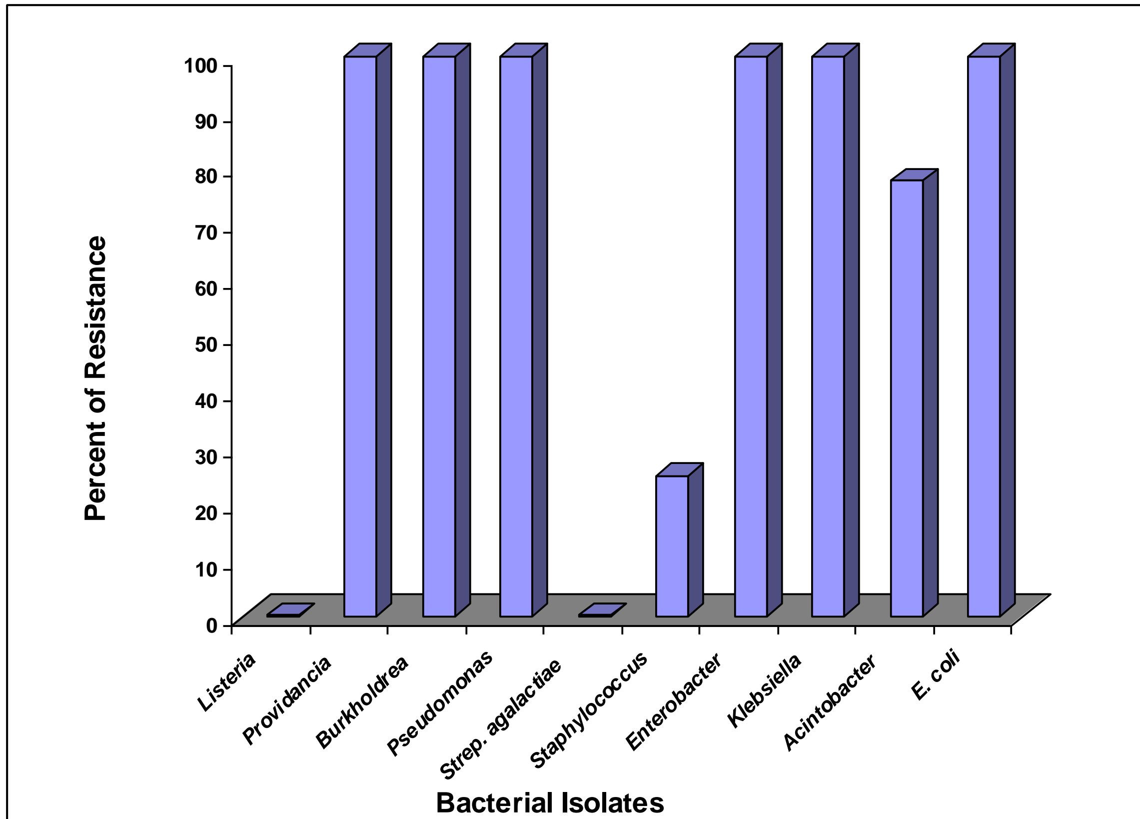


Figure (3-10) Percentage of Bacterial Resistance to Clarithromycin

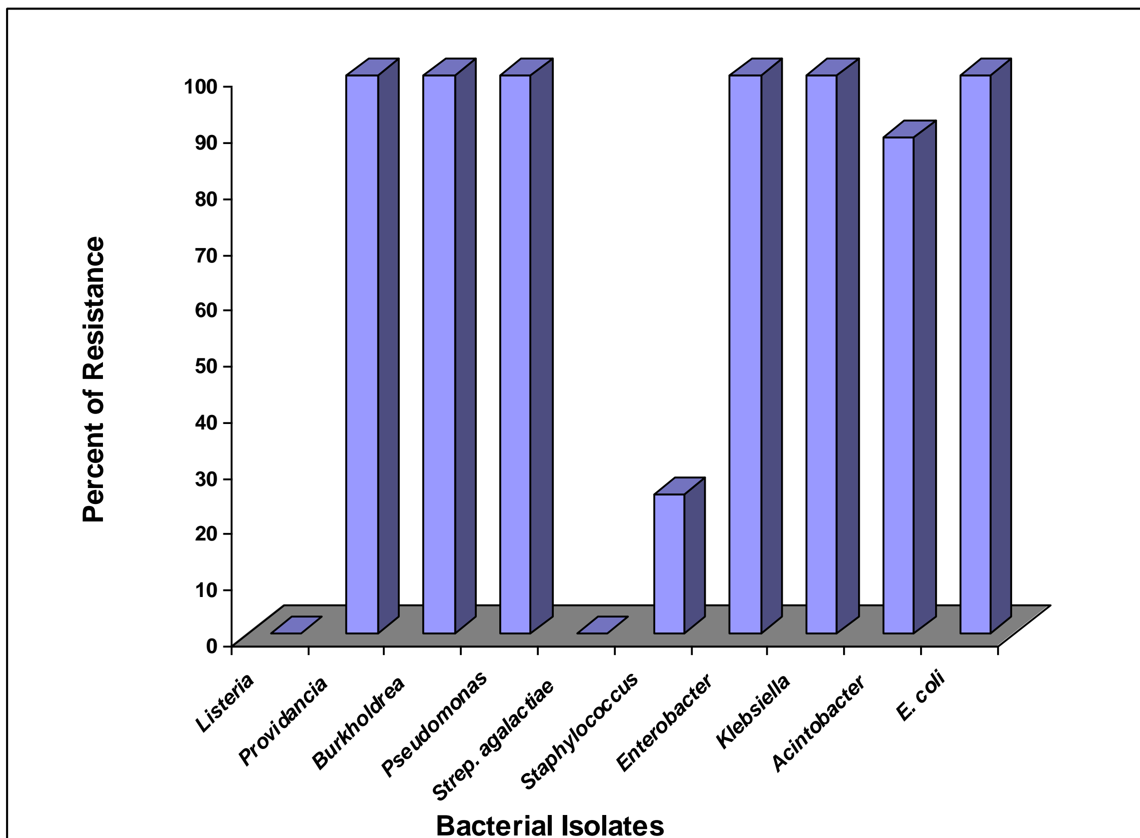


Figure (3-11) Percentage of Bacterial Resistance to Erythromycin

Bacterial resistance to aminoglycosides has also been studied. These include gentamicin and amikacin as shown in figures (3-12) and (3-13), respectively. Most bacteria are resistant to gentamicin except *Listeria monocytogenes* and *Strep. agalactiae* which are completely (100%) susceptible to gentamicin followed by *Acintobacter*. Amikacin can be considered the more effective antibiotics on Gram positive and Gram negative bacteria isolated from pregnants with preterm labor. All strains are susceptible to amikacin, which agrees with Forbes (2007) who has reported that the spectrum of activity of aminoglycosides includes a wide variety of Gram positive and Gram negative bacteria.

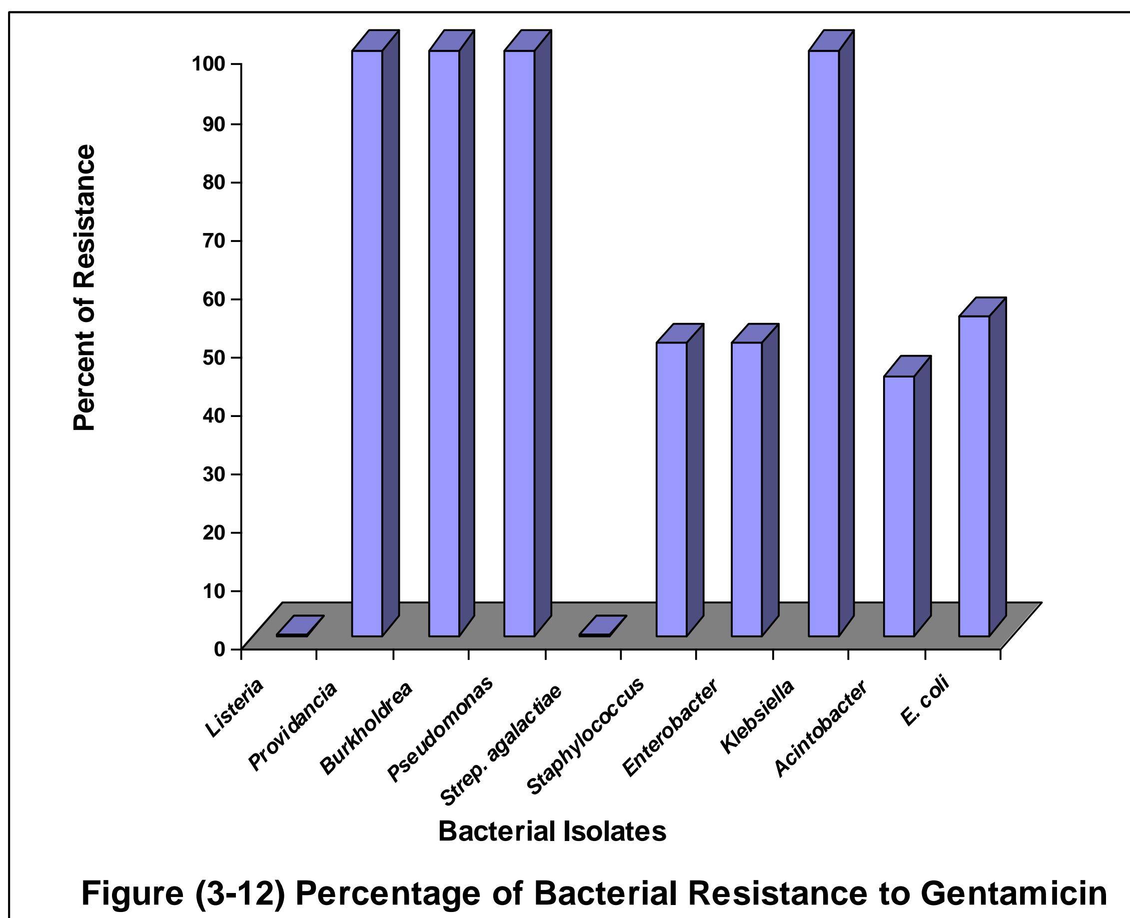


Figure (3-12) Percentage of Bacterial Resistance to Gentamicin

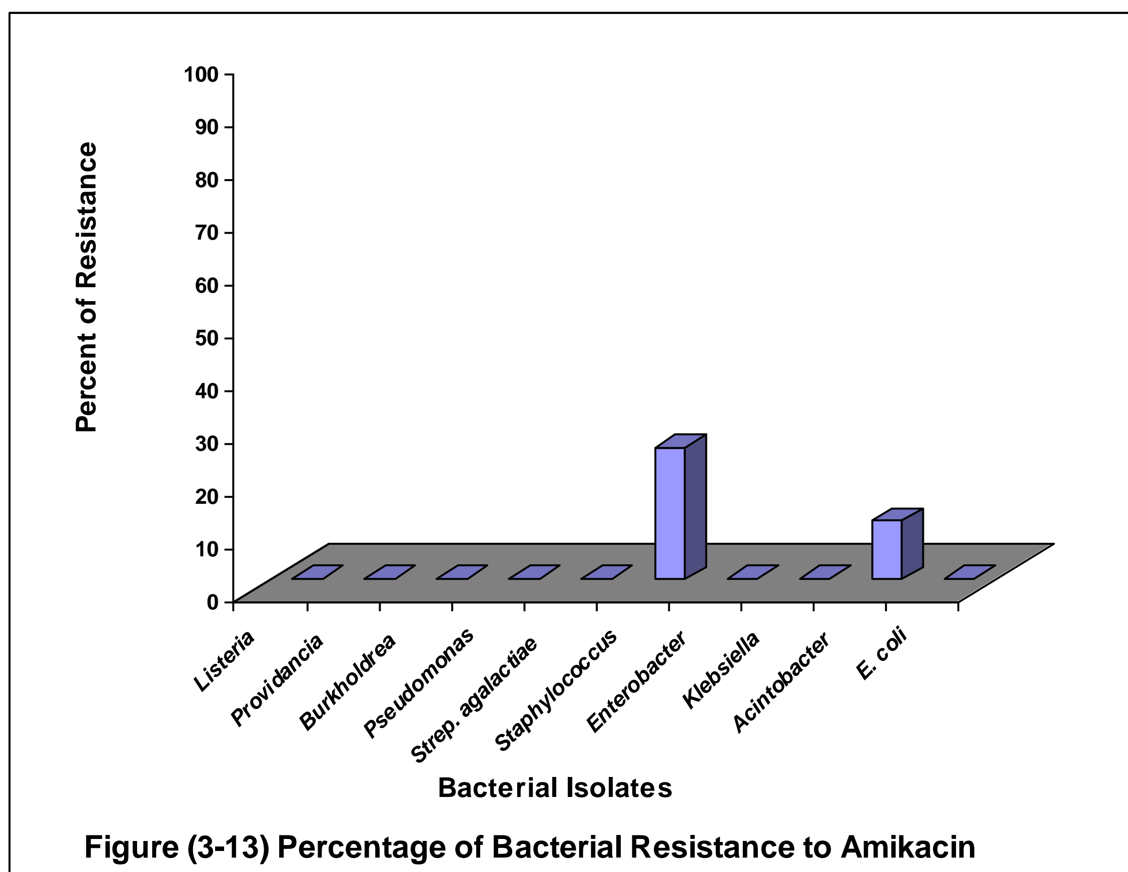


Figure (3-13) Percentage of Bacterial Resistance to Amikacin

On the other hand, the effect of norflaxacin comes next to the effect of amikacin as shown in figure (3-14). Most bacteria are susceptible to norflaxacin except *Providancia sp.* which is completely (100%) resistant, followed by *P. aeruginosa* (at 50%). This agrees with Forbes (2007) who has reported that the fluoroquinolones are potent bactericidal agents, and that they have a broad spectrum of activity that includes Gram positive and Gram negative bacteria.

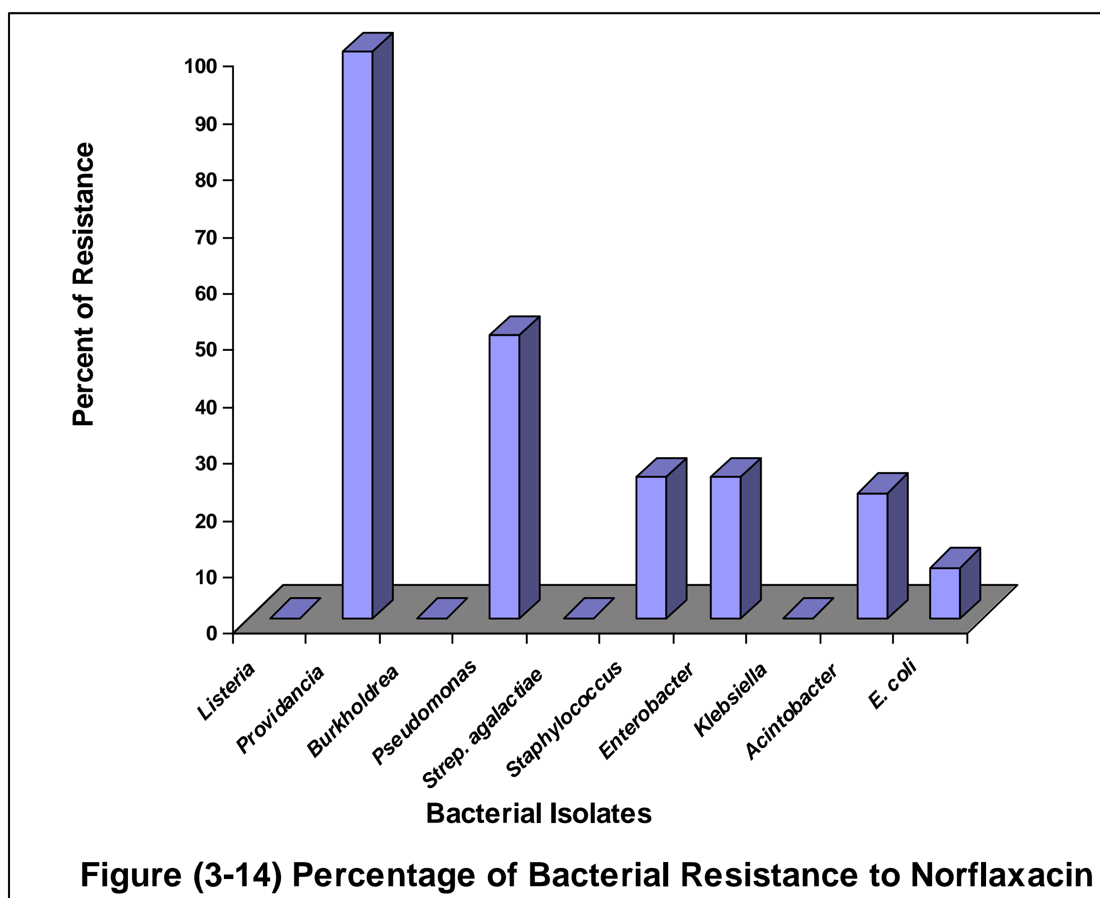


Figure (3-14) Percentage of Bacterial Resistance to Norflaxacin

3.2.4 The effect of antibiotics on lecithinase production by bacterial isolates recoverd from pregnants with preterm labor

As shown in table (3-9), the effect of cefotaxim, ampiclox, amoxiclave, amoxicillin, and nalidixic acid on lecithinase has been studied.

The results show no effect of cefotaxim (1%), ampiclox (1%) and amoxicillin (1%) on the lecithinase, which can grow in the presence of this antibiotics in the media, while nalidixic acid (1%) show effect on one isolate only. Most isolates are effected by amoxiclave (1%). This may be due to the presence of clavulanic acid.

Table(3-9)The effect of antibiotics on lecithinase production by some bacterial isolated recovered from pregnants with preterm labor

Bacterial Isolates (No. of Isolates)	Lecithinase + Cefotaxim(1%)	Lecithinase + Ampiclox(1%)	Lecithinase + Amoxiclave(1%)	Lecithinase + Amoxicillin(1%)	Lecithinase + Nalidixic acid(1%)
E coli (a)	+	+	-	+	-
E coli (b)	+	+	-	+	+
E coli (c)	+	+	-	+	+
E coli (d)	+	+	+	+	+
E coli (e)	+	+	+	+	+
Enterobacter (a)	+	+	-	+	+
Enterobacter (b)	+	+	-	+	+
Acintobacter baumanii	+	+	+	+	+
Staphylococcus	+	+	-	+	+

+ Presence of growth
- Absence of growth

3.3 Immunological parameters

3.3.1 Immunoglobulines concentration

Table (3-10) shows the levels of IgM in pregnants with preterm labor and control subjects which include infected pregnants at term, non infected pregnants at term, and normal female. The results are 477.06 mg/dl ,520.66 mg/dl,512.20 mg/dl and 524.96 mg/dl, respectively (figure 3-15a). Also the levels of IgG in the same groups are 2926.33 mg/dl, 2643.33 mg/dl, 2537.23 mg/dl and 490.80 mg/dl, respectively (figure 3-15b). These results indicate that there is no significant increase in IgM level between preterm labor and all control subjects(P <0.05), while IgG level increases significantly in preterm labor as compared with normal female, but is non significant as compared with other control subjects (P <0.05). This result inducates that the production of IgM in infected preterm labor patient is unaffected. Least level of IgM may indicate the low level of protective immunoglobuline in acute infection. Moreover, the high level of IgG may indicate the presence of chronic infection, particularly in pregnants with preterm labor whose IgG is higher than at term. This chronic infection may contribute to the preterm labor (Doan *et al*., 2008).

Table (3-10) Concentration of Immunoglobulins IgM and IgG (mg/dl) in Pregnants with Preterm Labor and Controls

Testing group		IgM	IgG
A-Preterm Pregnants	M*	477.0667	2926.3333
	SD**	87.4243	689.9726
B-Infected Pregnants at term	M	520.6667	2643.3333
	SD	10.0664	51.3160
	Significance between A,B	Not significant (P <0.05)	Not significant (P <0.05)
C-Non infected Pregnants at term	M	512.2000	2537.2333
	SD	35.5070	1529.5163
	Significance between A,C	Not significant (P <0.05)	Not significant (P<0.05)
D-Normal Female	M	524.9667	490.8000
	SD	6.7870	408.7829
	Significance between A,D	Not significant (P <0.05)	significant (P <0.05)

*** Mean**
**** Standard deviation**

-a-

-b-

Figure (3-15) Single Radial Immunodiffusion Test (SRID) For IgM and IgG In Pregnants with Preterm Labor

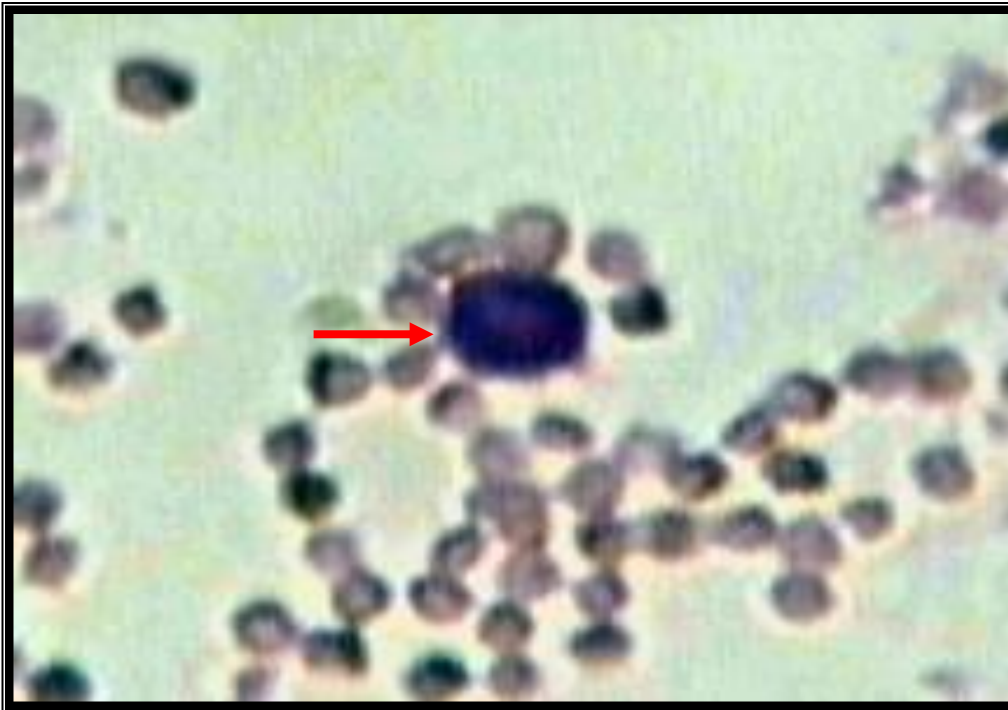
3.3.2 T-cell counts

E-rosette test was used in this study to estimate T-cell count in pregnants with preterm labor and control subjects. T-lymphocytes are considered E-rosette positive forming T-cells when three or more sheep erythrocyte adhere to it (figure 3-16a), otherwise they are considered E-rosette negative forming T-lymphocytes (figure 3-16b).

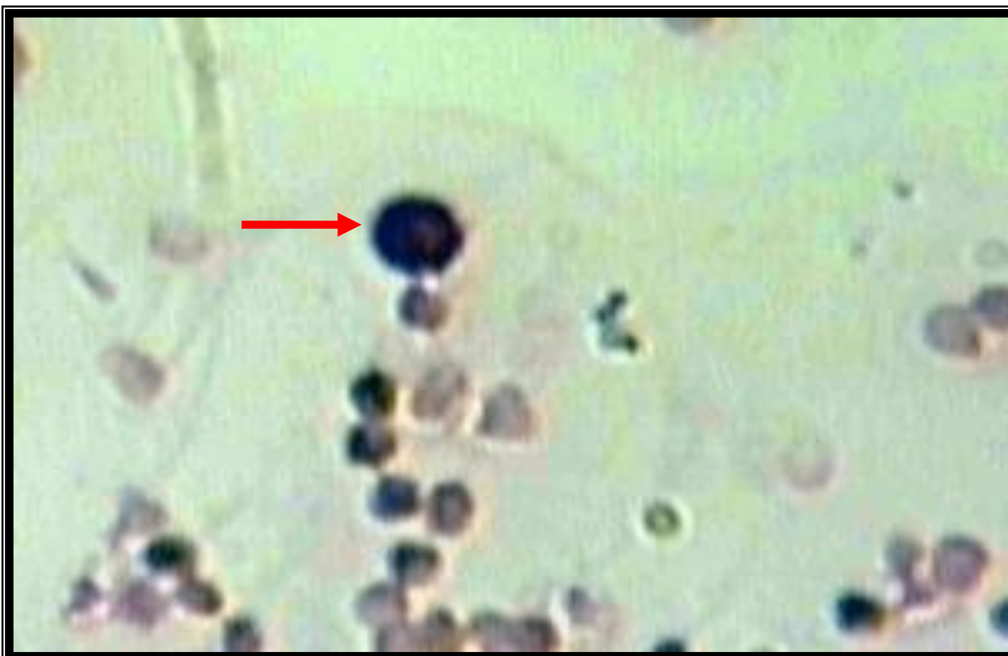
Table (3-11) shows the results of E-rosette test. The mean value of E-rosette positive of T-lymphocytes in preterm labor is at 9%. In control subjects which include infected pregnants at term it is 16%. Non infected pregnants at term is at 15%, and normal female is at 23.6%, which represents the normal comparing value for this test.

T-cell count decreases significantly between preterm labor and all control subjects ($P < 0.05$).

The decreasing of T-cell count in preterm may increase the susceptibility of pregnants to microbial infection, depending on the fact that T-cell play a vital role in the defense against these infections, directly as cytotoxic T-cells, or indirectly as helper T-cells for other components of immune system (Rich *et al.,*2003).



Figure(3-16a):photomicrograph of E-rosette positive T-lymphocyte (→)(1000X)



Figure(3-16b):photomicrograph of E-rosette negative T-lymphocyte (→)(1000X)

3.3.3 Phagocytic index

Phagocytic index has been used in this study to evalute the neutrophils function in pregnants with preterm labor and control subjects. Table (3-11) show the result of phagocytic index. The mean value of phagocytic index in preterm labor is at 3.4%, while in control subjects which include infected pregnants at term is at 9%, non infected pregnants at term is at 8.6%, and normal female is at 11.8%.

Neutrophils function decreases significantly between preterm labor and all control subjects ($P < 0.05$).

The innate immune system is one component of the uterine environment, and has a role in the prevention of uterine infection (King *et al.*,2007). Neutrophils play a major role in the body's defense against acute infection. The major role of the neutrophil is to ingest, kill, and digest invading microorganism, particularly bacteria. Failure to fulfil this role leads to infection. Defects in neutrophil function can be quantitative-neutropenia-or qualitative-neutrophil dysfunction (Chapel *et al.*,1999).

3.3.4 Concentration of tumor necrosis factor-alpha (TNF-α)

Table (3-10) show the levels of TNF-α which is considered as an important immunological parameter that enhances the mechanism of preterm labor.

The results show that the mean of TNF-α in preterm labor is 91.287 pg/ml, while control groups which include infected pregnants at term is 57.485 pg/ml. Non infected pregnants at term is 42.239 pg/ml, and normal female is 27.883 pg/ml. Thus, there is significant increase in the level of TNF-α in preterm labor when compared to other control groups ($P < 0.05$).

This agrees with Html (2007) who has reported that the amniotic fluid proinflammatory mediators (TNF-α) increase during intra-amniotic infection, preterm labor, or preterm premature rupture of membrane.

Spaziani *et al* (1998) suggest that TNF-α may play a role in infection-induced preterm labor by its pleiotropic ability to simultaneously stimulate cyclooxygenase-2 activity, prostaglandin E2 production, and expression of the prostaglandin E2 production receptor sub type Ep1 in human amnion. Thus, TNF-α is the major mediators that may be responsible for the induction of preterm labor in this study, and the production of this cytokine is stimulated by bacterial infection; mainly gram negative bacteria (Abbas *et al.*,2007).

Table(3-11) T-cell Count, Phagocytic Index and TNF-α for Pregnants with Preterm Labor and Controls

Testing group		T-cell %	Phagocytic index %	TNF-α pg/ml
A-Preterm Pregnants	M*	9.000	3.4000	91.2870
	SD**	3.8079	1.1402	33.7728
B-Infected pregnants at term	M	16.000	9.0000	57.4858
	SD	1.2247	1.2247	41.2440
	Significance between A,B	significant (P <0.05)	significant (P <0.05)	significant (P <0.05)
C-Non infected Pregnants at term	M	15.000	8.6000	42.2398
	SD	2.7019	1.6733	12.5475
	Significance between A,C	significant (P <0.05)	significant (P <0.05)	significant (P <0.05)
D-Normal Female	M	23.6000	11.8000	27.8838
	SD	1.3416	3.1937	35.8213
	Significance between A,D	significant (P <0.05)	significant (P <0.05)	significant (P<0.05)

*** Mean**
**** Standard deviation**

List of Abbreviations

AK	Amikacin
AMC	Amoxicillin+cloxacillin
APX	Ampicillin+cloxacillin
AX	Amoxicillin
AZM	Azithromycin
BV	Bacterial vaginosis
CAMP	Christie , Atkins and Munch-Peterson
CAZ	Ceftazidime
CL	Cephalexin
CLR	Clarithromycin
CN	Gentamycin
CoNS	Coagulase negative staphylococci
CTX	Cefotaxim
DM	Decidual macrophages
DNA	Deoxyribonucleic acid
DO	Doxycycline
DW	Distilled water
E	Erythromycin
EASIA	Enzyme amplified sensitivity immunoassay
EMB	Eosin methylene blue
E-rosette	Erythrocyte-rosette
FITC	Fluroscein-isothiocynate
GBS	Group B *Streptococcus*
HBSS	Hank's balanced salt solution
HIV	Human immunodeficiency virus
HPA	Hypothalamo-pituitary adrenal axis
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IgG and IgM	Immunoglobulin G,and M
IL-1,6,8,10	Interleukin-1,6,8,10
LPS	Lipopolysaccharide
M	Mean
MR	Methyl red reagent
MAbs	Monoclonal antibodies
NOR	Norfloxacin
PAF	Platelet activating factors
PBP	Penicillin binding protein

PBS	Phosphate buffer saline
PMNLS	Polymorph nuclear lymphocyte separation
PPROM	Preterm premature rupture of membranes
PROM	Preterm rupture of membranes
PTL	Preterm labor
SD	Standard deviation
SRBCs	Sheep red blood corpuscles
SRID test	Single radial immunodiffusion test
TLR2,4	Toll-like receptor 2,4
TNF-α	Tumor necrosis factor-alpha
TSI	Triple sugar iron
UTI	Urinary tract infection
VP	Voges-Proskauer reagent

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Last, but not least, I am really indebted to the staff members of the Labor Room at Babylon Hospital of Maternity and Pediatrics for their sincere cooperation and generous help.

Appendix (1)
Morphological and Biochemical Features for Identification of Gram Negative Isolates

Tests	*E.coli*	*Acinetobacter baumannii*	*Enterobacter sp.*	*Klebsiella sp.*	*P.** aeruginosa*	*Burkholdrea pseudomonallie*	*Providancia sp.*
Gram's stain	G-ve, short rods	G-ve Coccobacilli (Diplococci)	G-ve rods	G-ve, short rods	G-ve rods	G-ve rods	G-ve rods
Capsule	–	–	+	+	+	*Nd	+
Oxidase	–	–	–	–	+	+	–
Catalase	+	+	+	+	+	+	+
Indole	+	–	–	–	–	–	*Nd
MR	+	–	–	–	–	–	–
VP	–	–	+	+	–	–	*Nd
Citrate	–	+	+	+	+	*Nd	*Nd
Urease	–	–	–	+	–	–	–
TSI	A/A with gas	ALK/ALK	A/A with gas	A/A with gas	ALK/ALK	*Nd	*Nd
H2S	–	–	–	–	–	–	–
Motility	+	–	+	–	+	+	+
Hemolysis		–	–	–	+	+ (beta)	–
EMB	Metalic sheen	Pale	Centrally dark	Centrally dark	Pale	*Nd	*Nd
Lactose fermenter	+	–	+ (slow)	+	–	+	+ (slow)

*Nd: non determined

**The major diagnostic test for *P. aeruginosa* is the production of blue(pyocyanin) pigment with the yellow / green pyoverdin(fluorescein) giving the characteristic blue-green appearance of culture.

Appendix (2)
Morphological and Biochemical Features for Identification of Gram Positive Isolates

Test	*Coagulase negative Staphylococcus*	*Staphylococcus aureus*	*Streptogoccus agalactiae*	*Listeria monocytogenes*
Gram's stain	G+ve cocci (clusters)	G+ve cocci (clusters)	G+ve cocci (chains)	G+ve rod or coccobacilli (often pairs)
CAMP	–	–	+	+
Oxidase	–	–	–	–
Catalase	+	+	–	–
Coagulase	–	+	–	–
Haemolysis	–	–	+ (beta)	+ (beta)
Urease	–	–	–	–
Growth on MacConkey	–	–	–	–
Mannitol fermenter	–	+	–	*Nd
Motility	–	–	–	+

*Nd: non determined

Appendix(3)
Antibiotics resistant of Gram negative bacterial isolates

Antibiotic Type	Bacterial Isolates (No.)													
	E.coli (22)		*Acint-obacter* (9)		*Entero-bacter* (4)		*Pseud-omonas* (2)		*Burk-holdrea* (1)		*Klebi-sella* (2)		*Provid-ancia* (1)	
	S*	R**	S	R	S	R	S	R	S	R	S	R	S	R
Norfloxacin	20	2	7	2	3	1	1	1	1	0	2	0	0	1
Amikacin	22	0	8	1	4	0	2	0	1	0	2	0	1	0
Azithromycin	9	13	6	3	1	3	1	1	0	1	1	1	0	1
Doxycycline	6	16	6	3	1	3	0	2	0	1	0	2	0	1
Clarithromycin	0	22	2	7	0	4	0	2	0	1	0	2	0	1
Ceftazidime	7	15	3	6	0	4	0	2	0	1	0	2	1	0
Ampicillin + Cloxacillin	0	22	0	9	0	4	0	2	0	1	0	2	0	1
Cefotaxim	11	11	5	4	3	1	0	2	0	1	0	2	1	0
Gentamicin	10	12	5	4	2	2	0	2	0	1	0	2	0	1
Erythromycin	0	22	1	8	0	4	0	2	0	1	0	2	0	1
Cephalexin	1	21	0	9	1	3	0	2	0	1	0	2	0	1
Amoxicillin	4	18	1	8	0	4	0	2	0	1	0	2	0	1
Amoxicillin + Clavulanic acid	3	19	0	9	0	4	0	2	0	1	0	2	0	1

***Sensitive**

****Resistant**

Appendix (4)
Antibiotics resistant of Gram positive bacterial isolates

Antibiotic Type	Bacterial Isolates (No.)					
	Coagulase negative Staphylococcus (4)		*Strep. agalactia* (2)		*L. monocytogenes* *(1)*	
	S*	R**	S	R	S	R
Norfloxacin	3	1	2	0	1	0
Amikacin	3	1	2	0	1	0
Azithromycin	3	1	2	0	1	0
Doxycycline	2	2	2	0	1	0
Clarithromycin	3	1	2	0	1	0
Ceftazidime	0	4	1	1	0	1
Ampicillin + Cloxacillin	1	3	0	2	1	0
Cefotaxim	0	4	1	1	1	0
Gentamicin	2	2	2	0	1	0
Erythromycin	3	1	2	0	1	0
Cephalexin	1	3	1	1	1	0
Amoxicillin	0	4	2	0	1	0
Amoxicillin + Clavulanic acid	1	3	1	1	1	0

***Sensitive**

****Resistant**

Conclusions

1. Preterm labor found mostly in women who are 20 ≤ years of age.
2. Gram negative bacteria are the predominant micro-organisms in pregnant women with preterm labor, and *E. coli* is the most common one.
3. Amikacin and norflaxacin are the most effective antibiotics against bacterial isolates.
4. Amoxiclave (1%) is the most effective antibiotic on lecithinase produced by bacteria isolated from pregnants with preterm labor.
5. IgM does not increase significantly in preterm labor compared to all control groups.
6. IgG increases significantly in preterm labor as compared with normal female, and is non significantly as compared with other control subjects.
7. T-cell count decreases significantly in preterm labor compared to all control subjects.
8. Neutrophils function decreases significantly in preterm patients compared to normal delivery.
9. TNF-α increases significantly in preterm labor when compared to other control groups.

Recommendation

1. Pregnants with high risk should be screening for bacterial infection as well as other microbial infection as a prophylactic measure to prevent preterm labor.
2. Amoxiclave may reduce the severity of bacterial pathogenicity.
3. The use of TNF-α level as one of the laboratory test to detect women at risk of preterm labor.

Certification

We certify that this thesis was prepared under our supervision at the Department of Microbiology, College of Medicine, University of Babylon in partial fulfillment of the requirements for the Degree of Master of Science in Microbiology.

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SUMMARY

This study was conducted in Babylon Teaching Hospital of Gynecology and Pediatrics from November 2007 to May 2008.

A total of 60 pregnant women with preterm labor admitted to Labor Room and 20 control women (10 of them were pregnant at term with bacterial infection, 5 were pregnant at term without bacterial infection, and 5 were normal females not pregnants and not infected) were included in this study. The ages of patients and controls ranged from (17-40) years.

Samples (pieces of amniotic membrane) were taken aseptically after delivery of the fetus and placenta from patients with preterm labor. The collected samples were processed for bacterial isolation and determination of virulence factors (lecithinase, protease, coagulase, hemolysin and CAMP (Christie, Atkins and Munch-Peterson) test). Antibiotic sensitivity tests were assessed for each isolate as well as the effect of some antibiotics on lecithinase produced by bacterial isolates from pregnants with preterm labor.

Blood samples were collected from both patients and controls to estimate T-cells count by E-rosette test, neutrophils function by phagocytic index, IgM and IgG concentration by single radial immunodiffusion (SRID) test, and tumor necrosis factor alpha (TNF-α) by EASIA (Enzyme Amplified Sensitivity Immunoassay) method.

Women of 20 years of age or less are the most commonly affected by preterm labor.

The results of bacterial culture were positive in 57 (95%) of amniotic membrane samples from pregnants with preterm labor. Gram negative bacteria were more predominant 49:57 (85.96%) than Gram positive bacteria 8:57 (14.04%); *E. coli* was the main etiological agent (29:57) of Gram negative bacteria, while coagulase negative *Staphylococci* was the main etiological agent (4:57) of Gram positive bacteria in pregnants with preterm labor, followed by *Streptococcus agalactiae* (2:57).

Amikacin and Norflaxacin were the most effective drugs on both Gram negative and Gram positive bacteria. Amoxiclave (1%) was the most effective antibiotic on lecithinase production by bacterial isolate from pregnants with preterm labor.

There is a significant reduction ($p<0.05$) in T-cells count and neutrophils function in patients with preterm labor compared with all control groups. No significant increase ($p<0.05$) in IgM level in patients with preterm labor compared to all control groups while IgG level was significantly higher ($p<0.05$) in patients with preterm labor compared with normal female and non significant compared with other control groups. TNF-α was significantly higher ($p<0.05$) in patients with preterm labor compared to all control groups.

الخلاصة

أجريت الدراسة في مستشفى بابل للنسائية والأطفال للفترة من تشرين الثاني 2007 ولغاية أيار 2008.

تضمنت الدراسة 60 امرأة حامل يعانين من ولادة مبكرة وتم ادخالهن إلى صالة الولادة و20 امرأة أخرى كمجموعة سيطرة (10 منهن كن حوامل في الشهر التاسع ويعانين من التهابات بكتيرية و 5 كن حوامل في الشهر التاسع ولايعانين من التهابات بكتيرية و5 كن نساء طبيعيات غير حوامل وليس لديهن التهابات).

كانت أعمار المريضات و مجموعة السيطرة تتراوح بين 17 و 40 سنة.

تم اخذ عينات من الغشاء الامينوسي وبطريقة معقمة تجنبا للتلوث بعد ولادة الطفل و المشيمة للمريضات اللائي يعانين من ولادة مبكرة.

تم فحص العينات بالعزل البكتيري وتحديد عوامل الضراوة (انتاج اللسيثينيز، وتحلل البروتينات، و إنتاج انزيمات التجلط، وتحلل الدم واختبار CAMP). كما تم اختبار الحساسية للمضادات الحيوية وكذلك تأثير المضادات الحيوية على انزيم اللسثينيز الذي يفرز بواسطة بعض أنواع البكتيريا المعزولة من المريضات اللائي يعانين من ولادة مبكرة.

تم جمع عينات الدم من المريضات ومجموعة السيطرة لحساب اعداد الخلايا التائية بطريقة فحص E-rosette وقياس وظيفة كريات الدم البيض العدلة بواسطة مؤشر البلعمة وقياس مستوى الكلوبينات المناعية نوع IgM و IgG بطريقة الانتشار المناعي الشعاعي المفرد وقياس تركيز TNF-α بطريقة EASIA .

كانت أغلبية النساء اللائي يعانين من ولادة مبكرة يبلغن من العمر 20 سنة أو اقل .

نتائج الزرع البكتيري كانت ايجابية عند 95٪ من عينات الغشاء الامينوسي المأخوذة من الحوامل اللائي يعانين من ولادة مبكرة.

إن البكتريا السالبة لصبغة غرام كانت هي السائدة 57:49 (85.96٪) في حين لم تتجاوز البكتريا الموجبة لصبغة غرام 57:8 (14.04٪) ، وجد ان بكتريا *E. coli*

هي المسبب الرئيسي (57:29) لحالات الولادة المبكرة المتسببة عن البكتريا السالبة لصبغة غرام بينما كانت بكتريا *Staphylococci* Coagulase negative هي السائدة (57:4) بين حالات الولادة المبكرة المتسببة عن البكتريا الموجبة لصبغة غرام يليها بكتيريا *Streptococcus agalactiae* (57:2).
الاميكاسين و النورفلاكساسين هما المضادان الحياتيان الأكثر تأثيرا على كلا البكتريا الموجبة والسالبة لصبغة غرام .
الاموكسكلاف (1٪) هو المضاد الحياتي الأكثر تأثيرا على انزيم اللسثينيز المنتج من بعض العزلات البكتيرية المعزولة من الحوامل اللائي يعانين من ولادة مبكرة.
اظهرت النتائج انخفاض معنوي ($P<0.05$) في عدد الخلايا التائية وفي وظيفة كريات الدم البيض العَدلة في المريضات اللائي يعانين من ولادة مبكرة مقارنتا بكل مجاميع السيطرة. لا توجد زيادة معنوية ($P<0.05$) في مستوى الكلوبيولين المناعي IgM بين المريضات اللائي يعانين من ولادة مبكرة مقارنتا بكل مجاميع السيطرة بينما يرتفع مستوى الكلوبيولين المناعي IgG بشكل معنوي ($P<0.05$) في المريضات اللائي يعانين من ولادة مبكرة مقارنتا بالنساء الطبيعيات وبشكل غير معنوي ($P<0.05$) مقارنتا بباقي مجاميع السيطرة .
مستوى TNF-α يرتفع بشكل معنوي ($P<0.05$) في المريضات اللائي يعانين من ولادة مبكرة مقارنتا بكل مجاميع السيطرة

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Dedication

To . . .

My memory of my father;

The kind heart: my mother;

My support in life: my husband, Ammar;

The tender hearts that surrounded me with care and courage: my husband's parents and his sister; and all my brothers and sisters;

My flowers: Murtadha and Safa .

Taiseer

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