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Microbial and Molecular Study of Infections in High Vagina in Women from Kerbala

A Thesis Submitted to
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Dedication

To those …

Who removed the thorns from my path to pave my way to science

dear father;

Who makes me happy when I see her and my heart rejoices in her presence

dear mother;

Who coast to landed his love of my heart

My husband;

Who address me with love, cooperation and support my brother and sister;

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Enaas

Summary

A total of 100 female patients were enrolled in this study. The study conducted in the period from December 2013 to January 2015. All patients were attendants of the Gynecology and Obstetric Teaching Hospital in Kerbala , Province Kerbala. Iraq.

The majority of the patients with vaginal discharge (n=73,73%) were below 35 years old and 93% were in the fertility age (14-45 years). Discolorations of the discharges were noticed in 43 patients (43.4%), in 69 patients (69.7%),and dysuria in 62 patients (62.6%) . Around half of the patients (n=49,49%) were chronic (symptoms persisted for more than 1 month),and recurrent infections were reported in 37% of the patients. A 42 patients (42%) were pregnant, 15 patients (15%) mentioned that their partners were using condoms. Most of the patients (n= 59) reported a history of taking birth control pills.

A total of 174 microbial isolates were recovered from the 100 patients. The most predominant bacterial type isolate was *Escherichia coli* (n= 65, 37%) followed by *Proteus maribilis* (n=37, 21 %), Coagulase-negative staphylococci (C.N.S) (n= 33, 19 %) , *Klebsiella pneumoniae* (n=13, 7%), as well as other microorganisms in low frequencies(n=26).

Furthermore, polymicrobial colonization were reported in more than half of the patients (n=54). In addition, *Candida* spp. was detected in 3 patients only. No correlation was found between age of the patients and type of isolates. However, a negative correlation was found between discoloration of the discharge and presence of polymicrobial colonization (R= - 0.233, p= 0.020), however, no correlation was found with any single-isolate. The majority of infections were associated with bad odor, however, no specific type of isolates was statistically linked to the bad odor. Though, no significant correlation could be detected between using condoms by the patient's partners and type of isolates, nevertheless, among 15 patients associated with using condoms, 10 were found to be infected with *E. coli*. Furthermore, a statistically significant positive correlation (R= 0.292, p= 0.003) was found between history of taking contraceptives and colonization by *Klebsiella pneumoniae*. In this study, highly significant negative correlation was found between the presence of *E. coli* and *Klebsiella pneumoniae* (R= -0.291, *p*= 0.003)

where most of *E. coli* isolates (no= 56) were not associated with *Klebsiella pneumoniae* infection. Furthermore, all *Candida* spp isolates were recovered from patients not infected by *Escherichia coli*. On the other hand, a highly significant positive correlation (R=0.327, p=0.001) between *Escherichia coli* and polymicrobial infections. In addition, *P. mirabilis* was negatively correlated with C.N.S staphylococci (R=-0.216, p=0.031), however, it was positively correlated with polymicrobial colonization (R=0.394, p= 0.000).

Because *Escherichia coli* was the most prevalent microorganism in this study, further molecular characterizations of the isolates were performed. A phylogenetic analysis using PCR was performed. The most frequent phylogenetic of *Escherichia coli* was " B2" which comprised 60% (n= 36) followed by " A" that comprised 35.00% (n= 21). phylogenetic B2 was more prevalent among pregnant (16 versus 7) and non-pregnant non aborted (18 versus 6).Interestingly, 9 out of ten isolates of women who their husband using condoms were shown to be of B2 genotype. Virulence genes of adhesion factors in *Escherichia coli* isolates such as *Pap*C, *Afa* and *Sfa* were analyzed by the multiplex PCR assay; frequencies of *Pap*, *Sfa* and *Afa*, among *E.coli* isolates were 14, 42 and 5, respectively. A total of 65 isolates , 48 isolates had at least one virulence gene (34 isolates carrying just one virulence factor, and 14 isolates carrying 2 genes) and 17 isolates had none of the studied genes. however, none of the isolate found to carry all the three genes. *Pap* C and *Sfa* were found in higher rates in phylogenetic group B2 isolates, and this add evidence to the hypothesis that B2 is the pathogenic group.

In conclusion, *Escherichia coli* was the most prevalent microorganism that colonizing the vaginal epithelium, in addition to other potential pathogens. Most of the *Escherichia coli* isolates were belong to the virulent genotype “B2” and that those isolates harbor several virulence factors. In addition, *PapC* and *Sfa* adhesins genes were prevalent among the *Escherichia coli* isolates.

In this this study, it is recommended that further studies be conducted to investigate the actual causes for the high prevalence of *E.coli* and to document the sources, if its external or endogenous the actual role of urinary system in *E. coli* transmission the vaginal epithelium.

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List of Abbreviations

CoNS	Coagulase-Negative Staphylococci
EDTA	Ethylene Dopamine Tetra Acetic Acid
EMB	Eosin Methylene Blue
PCR	Polymerase Chain Reaction
TSI	Triple sugar iron
PDA	Potato Dexrose Agar
SDA	Sabouraud Dexrose Agar
MR-VP	Methyl Red -Voges Proskauer
TE buffer	Tris-EDTA buffer
PBS	Phosphate Buffer Solution
APi	Analytical Profile index
papC	Pyelonephritis associated P fimbria C
Sfa	Type S fimbria adhesion
Afa	A fimbria adhesion
BHIA	Brain Heart Infusion Agar
TSA	Tryptose Soy Agar
PROM	Premature Rupture Of Membranes
FGT	Female Genital Tract
BV	Bacterial Vaginosis
AV	Aerobic Vaginitis
GBS	Group β Streptococci
PMNs	Polymorpho-nuclear leukocytes
MRSA	Meticillin-Resistant *S. aureus*
RBCs	Red Blood Cells
HA	Haemagglutination
MPCR	Multiplex PCR
SAPs	Secreted Aspartyl Proteinases
UTI	Urinary Tract Infection

Chapter One

Introduction

Introduction

The female genital tract could be split up into internal and external genitalia. The vulva comprises the external genitalia, while the internal genitalia are subdivided into three comportments; the lower genital tract (comprised of; vagina, and ecto cervix), the endo cervix, and the upper genital tract (comprised of endometrium and Fallopain tubes) (Cole, 2006). The lower part of the endocervix in addition to the ectocervix and vagina are considered non- sterile areas.The cervico-vaginal epithelium is extensively colonized by microorganisms, however, the upper genital tracts are usually careful to be sterilized (Teisala, 1987). Presence of microorganisms in these places is linked with development of several diseases (endometritis or pelvic inflammatory disease) (Goplerud *et al*.,1976; Gardner ,1935).

During pregnancy, the developing fetus is protected from invading pathogens by the placenta, fetal membranes, and cervical mucus. Introduction of microorganisms into the intrauterine cavity results in several obstetric conditions such as miscarriage (McDonald *et al* .,2000) chorioamnionitis, (Dong *et al*.,1987; Hillier *et al*.,1993) , premature rupture of membranes (PROM) (Naeye and Peter ,1980) and preterm birth (Divers,1992). These conditions are hypothesized to results from the maternal and, occasionally, fetal inflammatory response to bacterial pathogens. In addition, colonization of the birth canal may lead to neonatal sepsis. Early onset neonatal sepsis is linked to gaining of microorganisms from the mother, through transplacental route, or a climbing infection from the cervix, or may be caused by organisms that colonize the genito urinary tract of the mother (Balaska *et al*., 2003). Microorganisms currently associated with neonatal

sepsis include group *β Streptococcus*, *Escherici coli*, coagulase negative staphylococci, *Heamophilus influenzae, Listeria* spp (Balaska *et al*., 2003).

Furthermore, several studies suggested that ascending infections from the mother to the fetus might occur before or through labour when colonized bacteria from the maternal perineum feast through the vaginal channel, amniotic sac, and into the once-sterile amniotic fluid (Al Adnani and Seber,2007; Ayengar *et al* .,1991) .

In different parts of the world, cervicitis attracted a lot of attention due to their important public health and socio economic problems. In Iraq, many studies recorded vaginitis with other factors such as urinary tract infection, sexually transmitted disease and pregnancy (Miteb, 2000; Al-Zubiady,2001; Al-Saadi ,2003; Naji , 2005). Unfortunately, little attention was paid for the important causation of cervicitis in women suffering from it.

Aims of the study

1. Study the dissemination of entric and other bacteria in vagina and cervix .
2. To performing a molecular characterization of the most common organims isolated from the cervico-vaginal epithelium .

Chapter Two

Literature Review

2.1. Importance of Genital Tract Infections

The genital tract infections are among the most frequent disorders for which female patients seek care of gynaecologists. There are several infections and complications, sometimes with bad consequences, that could arise from infections such as bacterial vaginosis, aerobic vaginitis, cervicitis, endometritis, pelvic inflammatory disease, etc. Vertical transmission of vaginal infections during pregnancy may entail high risk on the fetus as well as the pregnant female. Furthermore, neonatal infections acquired during delivery through passage in the birth canal are of high clinical importance. Neonatal infections usually result from infection or colonization of the birth canal with potential pathogens. The cervico-vaginal epithelium is close to the fetus and, therefore, cervico-vaginal epithelium comprises the most important site from which microorganism can be transmitted to the fetus or newborn (Cole, 2006).

2.2. Anatomic Sites of Genital Tract Associated with Infections

Anatomically, the female genital tract (FGT) could be split up into internal and external genitalia (Figure 2.1).

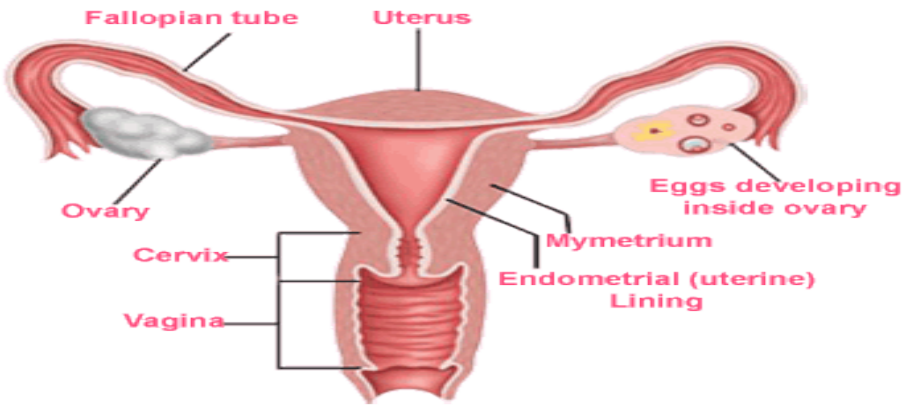


Figure (2-1):Anatomy of the human genital tract (Cole, 2006).

2.2.1. Vagina and Cervix

The vagina is a thin-walled fibro muscular tube, about 8-10 cm long, lies between the vestibule and the cervix. It is located between the bladder and the rectum. The upper part of the vagina surrounds the end of the cervix, and forms a vaginal recess called the vaginal fornix (posterior, lateral and anterior fornices) (Paavonen,1983) .

The cavity of the cervix, called the cervical canal, forms a connection between the vagina and the uterine cavity. The cervical canal opens into the vagina via the external *orifice* and to the uterine body, via the internal *orifice*. The part of the cervical canal which protrudes into the vagina is called the ecto cervix and the lining of the lumen is called the endo cervix (Llewellyn *et al.*,2004).

The vagina and cervix form a complex and dynamic ecosystem of epithelia, excretions, microbiota and innate immunity factors that depend on the stages of steroidal hormones. These levels influence the changes in the female genital tract not only from the childhood to menopause but also at different stages in the monthly cycle (Stevens and Lowe, 2005).

2.2.2.Uterus

The uterus (also named the womb) is the principal generative organ of female. The basic role uterus is to provides the developing embryo (weeks 1 to 8) and fetus (from week 9 until the delivery) with mechanical protection, nutritional support, and removal of waste. In addition, uterus plays a role during delivery where contractions in its muscular wall help in pushing out the fetus(Cole, 2006).

2.2.3. Fallopian Tubes

The female of humans has two tubes leading from the ovaries into the uterus. Those tubes are called Fallopian tubes or oviducts. When the ovum become mature, the follicle and the wall of the ovary will rupture and the ovum is released and enters the Fallopian tube. With the aid by movements of cilia on the inner lining of the tubes, the ovum travels toward the uterus(Cole, 2006).

2.3. Microbiota of the Female Genital Tract

Disturbance of normal vaginal microbiota, predisposes for obstetrical and gynaecologic complications, in addition, to increasing the risks of acquisition of sexually transmitted diseases (Todar, 2012).

2.3.1. Vaginal Microbiota

Vaginal microbiota composed of several microbial types,however, *Lactobacillus* bacteria is the dominant vaginal microorganism found in premenopausal women (Todar, 2012). Several exogenous and endogenous factors are known to influence the vaginal microbiota. Bacteria type in women is variable with stage of the menstrual cycle (Antonio *et al* ., 1999). Genetics and race factors may have impact on vaginal microbiota . For instance, the prevalence of hydrogen peroxide-producing lactobacilli in African American women is lower than other ethnic groups, and vaginal pH is higher. Furthermore, other factors may play roles, for instance, sexual intercourse and use of antibiotics have been linked to the loss of lactobacilli (Onderdonk *et al* ., 1986). Some others demonstrated evidences that use of condoms during sexual intercourse may lead to changes in *lactobacilli* levels, and may cause an increase the level of *E. coli* within the vaginal microbiota. Disruption of vaginal microbiota can lead to infections (Onderdonk *et al* ., 1986) .

2.3.2. Disruption of the Genital Tract Microbiota

Several factors might be complicated in the disruption of the vaginal microbiota. These factors include attack of an exogenous organism, or by overgrowth of one or more species of the normal vaginal microbiota, as in bacterial vaginosis or aerobic vaginitis(Antonio *et al*.,1999).

2.4. Infections of the lower FGT

The lower genital tract infections are categorized according to the site in which the symptoms and clinical findings appear, *e.g*., vaginitis, and cervicitis. Lower genital tract complaints, such as abnormal discharge, odour, vaginal itching and vaginal burning, among women may be the results of bacterial vaginosis (BV), vulvovaginal candidiasis trichomoniasis, gonorrhoea and *Chlamydia* infections (Holmes,1999 ; Edwards,2004;Petersen,2006).

2.4.1. Bacterial Vaginosis (BV)

In bacterial vaginosis (BV), the normally protective *Lactobacillus* spp. is a replacement with a massive overgrowth of anaerobic and facultative organisms such as *Gardnerella vaginalis*, *Atopobium vaginae, Bacteroides* spp., *Mobiluncus* spp., and genital mycoplasmas (Eschenbach *et al.*, 1989; Hillier,1993). This change in microbial community of the vagina may result in the development of symptomatic vaginitis, nevertheless, in most of the cases, symptoms does not appear on the affected women (Klebanoff *et al*., 2004). BV is implicated in the development of several adverse consequences such as miscarriage, (Llahi *et al* .,1996) preterm birth (Eschenbach *et al* ., 1984), chorioamnionitis (Silver *et al* .,1989) postpartum endometritis (Watts *et al*.,1989) and higher risks of acquiring HIV infection (Taha *et al*.,1998).

2.4.2. Aerobic Vaginitis (AV)

Aerobic vaginitis (AV) is considered as the second chief anomaly affecting the vaginal microbiota. AV is a condition in which the usually existed Lactobacillus spp. are substituted by aerobic microorganisms, most frequently commensals or pathogens acquired from the intestine. Indeed, one of the important features of AV is the presence of coliforms or cocci bacteria (Donders *et al* ., 2002).

Moreover, in >70% of affected women the AV is associated with clinical signs and symptoms of vaginitis. The microorganisms that is frequently associated with AV include; Group β streptococci (GBS), *Escherichia coli*, and *Staphylococcus aureus* (Donders *et al.*, 2002). Several studies were able to demonstrate a link between AV and several important pregnancy-related difficulties such as late miscarriage, chorioamnionitis, and preterm birth (Rezeberga *et al*., 2008; Donders *et al* .,2009; Donders *et al* .,2011).

2.4. 3.Cervical infection and Cervicitis

In women, genital infections are major causes of morbidity in the gynecologic patients (Miteb, 2000). The most important type of lower genital tract infection is cervicitis (inflammation of the lower part of the uterus) (Crosby *et al*., 2000).

A beneficial sign of cervical function is cervical mucus. In common, cervical mucus is clear through ovulation and cloudy white through post ovulation. It is imperative to know whether a woman is using oral contraceptives, because this could also affect the presence of cervical mucus. Yellow (purulent) mucus designates the existence of great numbers of polymorpho-nuclear leukocytes

(PMNs).Cervicitis is usually caused by an infective agent (Sobal,1990 ;Becker,2000). Moreover, other conditions such as using contraceptive, douching, abortion, injury, and hormones imbalance may lead to cervicitis (Martino and Vermund,2002). Indeed, cervicitis is regarded as a clinical diagnosis; characterized by friability of the cervix, mucopurulent discharge from the exocervix, and increased numbers of polymorphs in endocervical secretion (Chandran, 2006). Cervical erosion are caused prolonged cervicitis. If the genital tract infection successfully ascends to the upper genital tract, several complications may develop including infertility, ectopic pregnancy and chronic pelvic pain (Jennifer and Apicella, 2004). If left untreated cervicitis in pregnant women may entails risks of miscarriage, premature delivery, or infections of new born baby, which can lead to pneumonia, severe eye infection, or blindness (Chandran,2006).

When an antibiotic or any of other factor disturb the *Lactobacillus*, the acidic pH is no longer maintained and potential pathogens may grow (Escheabaeh *et al.,* 1983), causing vaginal inflammation that may transmitted to the cervix causing cervicitis (Varkey , 2004).

2.5. Infections of the Upper Genital Tract

However, several researcher were able to recover organisms from the endometrium of healthy women and thus give evidences that oppose the hypothesis of sterility (Stray *et al* .,1982;Hemsell *et al* .,1989). Nevertheless, the finding of bacteria in these assumed sterile places may be result from faulty specimen collection, where pollution with bacteria from the lower genital tract may occur during sample collection by transcervical techniques (Duff *et al* .,1983). However, cultures of endometrial samples collected via surgical hysterotomy gave similar

results, where around 25–30% of women were found to harbor one or more micro-organisms in their uteri (Cowling *et al* .,1992;Moller *et al*., 1995).

Furthermore, some bacteria were shown to be able to attach to human spermatozoa, and this enable them to transportation during the cervix and into the intrauterine cavity (Svenstrup *et al*., 2003).

During pregnancy, the developing fetus is protected from the invading organisms by several defense lines that include; placenta, fetal membranes, and cervical mucus block function. Introduction of microorganisms into the intrauterine cavity is associated with several obstetric conditions such as miscarriage, (McDonald *et al* .,2000) chorioamnionitis (Dong,1987;Hillier *et al* .,1993),premature rupture of membranes (Naeye,1980) and preterm birth (Divers,1992). These bad consequences are thought to develop as result of maternal and, to some extent, fetal inflammatory response to the presence bacterial pathogens (Naeye and Peters ,1980).

2.6. Ascending Infection and Chorioamnionitis

Chorioamnionitis is defined as inflammation of the fetal membranes and placental chorion. Frequently, chorioamnionitis results from due ascending bacterial infection in which microorganisms come from vagina and attack the choriodecidual space (the space between the fetal membranes and the maternal tissues) and then amniotic fluid is infection occur by microorganisms crossing intact chorioamniotic membranes (Goldenberr *et al*., 2000). Other routes for microbial transmission are also suggested but are less frequent, these include; migration of the microorganisms from the abdominal cavity during the Fallopian tubes, contamination through taking samples by amniocentesis or from chorionic

villus, or hematogenous feast from detached places (Goldenberr *et al*., 2000; Gibbs,1991). Introduction of microbes into the chorioamnion may cause maternal as well as, in cerain cases, a fetal inflammatory reply manifested by the production of pro inflammatory mediators such as cytokines and chemokines (Hillier *et al* .,1993) that may result in several complications including cervical ripening, membrane damage, or premature birth at earlier gestational ages.

2.7. Cervico – Vaginal Pathogens Causing Neonatal Infections

It has been shown that Gram-negative bacteria that are residents in the gastrointestinal tract can colonize vaginal mucosal surfaces. These kinds of microorganisms may get the opportunity to be transmitted to the fetus or newborn in the perinatal period and, thus, may cause substantial neonatal morbidity.

Escherichia coli; most of the cases of early onset sepsis in preterm infants are caused by *E coli*, in addition, this microorganism is the most prevalent cause of early onset meningitis (Stoll *et al*., 2011). A recent study has reported that vaginal delivery is related with a 4-fold greater risk of colonization by *E. coli*, suggesting that these microorganisms are probably originated from the mothers (Parm *et al*., 2011). It is also has been shown that *E. coli* strains having the K1 capsular antigen are accountable for mainstream of neonatal cases of *E. coli* meningitis because these strains shows specific tropism for the central nervous system (Glode *et al*., 1977). Actually, it is known for long time that vertical transmission of the K1 serotype from mother to infant is very common, with 50% of infants colonized if their mothers were positive for this organism at the time of delivery (Saff *et al*., 1975).

In addition, several other Gram-negative pathogens have been detected in the motherly genital tract and shown to cause neonatal early onset sepsis including *Haemophilus*, *Klebsiella*, and *Enterobacter spp*. (Stoll *et al*.,2011;Kinney *et al*.,1993;Tariq *et al* .,2006). However, in comparison to *E coli* these are less frequent.

Staphylococcus aureus; most of the newborns become colonized with *Staphylococcus aureus* within the first few days of life (Martyn, 1949) and most of the infants acquire *S. aureus* during and after delivery through direct skin to skin contact with nurses and other healthcare personnel. Nevertheless, vagina of the mother has become an important reservoir of neonatal colonization and, indeed, it has been shown that rates of colonization by *S. aureus* in newborns are very much higher (up to tenfold) when the vagina tract of the mother carry this microorganism (Bourgeois *et al*., 2010). On the other hand, it was found that in carrier mothers, caesarean section could significantly decreases the probability of *S. aureus* colonization in the neonate in comparison to delivery per vagina. Of significant alarming, is the increasing incidence of neonates colonized with meticillin-resistant *S. aureus* (MRSA) (Andrews *et al*., 2008).

Group *β Streptococcus*; infection by group *β streptococcus* is frequently reported as a major cause of morbidity and mortality in neonates. In this regard, colony of the maternal genital tract is considered the principal risk factor for neonatal infections (Regan,1983;Stoll *et al*.,2011). It was shown that vertical transmission of group *β streptococcus* to the neonate occurs in up to half of colonized mothers, however, early onset of group *β streptococcus* infections develops in only 2% of the colonized infants (Baker, 1997). The source primary reservoir for group *β streptococcus* in human is thought to be the gastrointestinal tract and it is suggested that gastrointestinal tract is the most probable origin for

vaginal colonization in pregnant women (Dillon *et al*., 1982). However, the vaginal colonization by group *βstreptococcus* is not consistent all the times but it might be transitory, intermittent or chronic (Anthony *et al*., 1978).

Following vaginal delivery, the first bacterial communities to colonize the neonates are those bacteria preexist in the lower genital tract. Therefore, bacterial communities colonizing the lower genital tract of the mother contribute to the establishing of the microbiota of the skin and intestine in the initial period of infancy (Dominguez *et al*., 2010).

2.8. Virulence Factors of the Pathogenic Bacteria and Yeast

2.8.1.Hemolysin

Hemolysin is a virulence factor of many bacteria such as *S .aureus.* It is a protein toxin has the ability to destroy red blood cell of human, rabbit, sheep or horses. Hemolysin production is detected by culturing the microorganism on blood ager plates . Hemolysin binds to special receptors on the membranes of red blood cells (RBCs) and making pores and thus lyses this cells (Al-Dulaymi, 2005; Kayser *et al*, 2005; Tang and Stratton, 2006). *S.aureus* produce β-hemolysin on blood agar, which surrounded by a large clear hemolytic zone (Mims *et al.*, 2004) .

Although hemolysin is not mandatory for microbial colonization in experimental urinary tract infection (UTI), but it contributesas virulence of the microorganism because it contribute to epithelial damage and bladder hemorrhage *in vivo* and may participate in causing renal damage and increasing risk for septicemia (Nielubowicz and Mobley, 2010).

2.8.2. Haemagglutination

Specific adhesion is mediated by certain adhesions which can be differentiated based on their receptor binding specificity. Fimbria mediated adherence has been proposed as an essential virulence factor in the development of urinary tract infection. Adherence of pyelonephritis *E. coli* has been correlated with their ability to cause a D-mannose resistant haemagglutination of human erythrocytes (Marrs *et al*.,2005).

2.8.3.Proteinase ,Phospholipase and Germ Tube for *Candida*

The virulence of *C. albicans* arises from the synergistic action of several aggression mechanisms (Calderoni ,2001 ; D'Eça *et al* ., 2011), such as the high production capacity of host tissue-degrading exoenzymes, morphological dimorphism (mycelium formation), and phenotypic switching. These mechanisms can lead to an altered adherence to epithelial cells, susceptibility to antifungals, fungicidal activity of neutrophils and production of toxins, which, combined with a weak host immune response, may lead to candidiasis (Tamura *et al* ., 2007; Sardi *et al* ., 2012) . Phenotypic-morphological alteration and the secretion of proteinase and phospholipase are of paramount importance in the process of host invasion

(Corrêa *et al* ., 2009 ; De Luca *et al* ., 2012) , and several typing methods, such as morphotyping, germ-tube testing and enzyme typing, have been developed to better identify and characterize *Candida* isolates, as well as to determine the virulence of the yeast (Spolidorio *et al* ., 2009; Riberio *et al* ,2010 ; Rodriguéz *et al* .,2012) .

Chapter Three
Materials and Methods

3.Materials and Methods

3.1. Materials

3.1.1 .Apparatuses and Instruments

Table (3-1): Apparatuses and instruments

Instrument	Company and Country of Origin
Autoclave	Hirayama\Japan
Benson Burner	Jenway\ Germany
Centrifuge	Hettich\ Germany
Cooling centrifuge	Hettich\ Germany
Digital camera	Sony\ Japan
Distilled	GFL\ Germany
Electrophoresis horizontal system	Cleaver Scientific Ltd\ UK
Eppendrof Centrifuge	Hermle\ Germany
Gel documentation system	Cleaver Scientific Ltd\ UK
Incubator	Fisher Scientific\ Germany
Inoculating needle	Memmert\ Germany
Inoculating loop	Loop Shandon\ England
Light Microscope	Genix\(U.S.A)
Laminar Flow Cabinet	Labtech\ Korea
Micropipettes	Slamed\ Germany
Micropipettes	Oxford\ Germany
Millipore filter unite (0.45 and 0.22) µm	Gallenkamp\ England
Oven	Memmert\ Germany

Instrument	Company and Country of Origin
PH-meter	LKB\ Sweden
Refrigerator	Ozone\ Korea
Sensitive balance	Kern\ Germany
UV transilluminator	Cleaver Scientific Ltd\ UK
Vortex shaker test tube shaker	Gemmy\ USA ,Talwan
Water bath	GFL\ Germany

3.1.2. Chemical and Biological Materials

Table (3-2): Chemical and biological materials

Chemicals and biological materials	Company and Country of origin
Alpha-naphthol, Peptone, Phenol red, Glycerol, Barium Chlorid , Methyl red, Agarose, KOH, Ethylene Diamine Tetra Acetic acid(EDTA), Gram stain: Crystal violet, Iodine, Safranin, HCl,H_2SO_4 ,$MgCl_2$.	B.D.H\ England
Alcohol (Ethanol) 99%, Kovac's reagent .	Fluka chemika\ Switzerland
Agar –Agar, Urea.	India\ Himedia
Ethidium Bromide, Hydrogen Peroxide.	Oxiod\ England
Gelatin	Merck\ Germany
NaCl	Thomas\ Baker
Primers	AccuOligo\ USA
PreMix	Bioneer, inc, CA\ USA
Tryptone	Rashmi\ India

3. 1. 3. Culture Media:

Table (3.3): culture media used in this study

Media	Company and country of origin
Blood agar	Oxoid
Brain heart infusion broth	Himidia/ India
Eosin methylene blue agar (EMB)	Oxoid
MacConkey agar	Himidia/ India
Mannitol salt agar	Himidia/ India
Nutrient agar	Himidia/ India
Nutrient broth	Himidia/ India
Peptone water	Himidia/ India
Triple sugar iron agar (TSI)	Microbiologie
Skim milk agar	Himidia/ India
Simmon citrate agar	Himidia/ India
Urea agar base	Carloerba
Phospholipase agar	Himidia/ India
Gelatin medium	Oxoid
Potato dexrose agar (PDA) & Sabouraud dexrose agar (SDA)	Himidia/ India

3.1.4. Rapid Multi Test System

Table (3- 4): Rapid multi test system

Test	Manufacture (Origin)
Api 20 E test Kit	BioMerieux (France)
McFarland(0.5) turbidity Standard	Himedia (India)

3.1.5. PCR Materials

3.1.5.1. Master Mix

Table (3-5): Master mix

***AccuPower*® PCR PreMix**	**Source**
AccuPower® PCR PreMix contains DNA polymerase, dNTPs, tracking dye and reaction buffer in a premixed format, freeze-dried into a pellet. In addition, it contains a chemical stabilizer enables the mixture to maintain activity for long time at ambient temperatures.	Bioneer Corporation, South Korea

3.1.5.2. Molecular Weight Marker

Table (3-6): Molecular Weight Marker

DNA marker	**Description**	**source**
100 bp Ladder	100-1500 bp. The ladder consists of 11 double strand DNA fragment with size of 100,200,300,400,500,600,700,800,900,1000, 1500bp. The 500bp present at triple the intensity of other Fragments and serve as a reference. All other fragments appear with equal intensity on gel.	Promega (USA)

3.1.5.3. Primers for Virulence Properties For *E.coli* .

The primers used to detect virulence properties in this study were the same primers mentioned in the study of Soto *et al*.,(2011), and were supplied by Bioneer Corporation, South Korea, and the sequences as well as the size of the products, are listed in Table (3-7).

Table (3-7):Virulence properties primers

Gene	Primer sequences(5́-3́)		Product size (bp)
papC	F	GACGGCTGTACTGCAGGGTGTGGCG	328
	R	ATATCCTTTCTGCAGGGATGCAATA	
sfa	F	CTCCGGAGAACTGGGTGCATCTTAC	410
	R	CGGAGGAGTAATTACAAACCTGGCA	
Afa	F	CGGCTTTTCTGCTGAACTGGCAGGC	672
	R	CCGTCAGCCCCCACGGCAGACC	

3.1.5.4. Primers of chuA and YjaA Genes and DNA Fragment TspE4.C2 .

The primers used to *E coli* genotypes in this study were supplied by Bioneer Corporation, South Korea, and the sequences as well as the size of the products, and the references are listed in Table (3-8).

Table (3-8) primers of phylogenic groups genes

Gene	bp Primer Sequence(5'-3')		Product Size(bp)	Reference
Chu A	F	GACGAACCAACGGTCAGGAT	279	Clermont *et al.*, 2000
	R	TGCCGCCAGTACCAAAGACA		
YjaA	F	TGAAGTGTCAGGAGACGCTG	211	Clermont *et al.*, 2000
	R	ATGGAGAATGCGTTCCTCAAC		
TspE4.C 2	F	GAGTAATGTCGGGGCATTCA	152	Bonacorsi *et al.*, 2000
	R	CGCGCCAACAAAGTATTACG		

3.2. Methods

3.2.1. Patients and Collection of Samples

All patients were attending the Kerbala Maternity Teaching hospital and showing signs of cervicitis .Patients with abnormal vaginal discharges were identified by consultant Gynecologists and referred to High Vaginal swabs.

The diagnosis of BV is based on vaginal malodorous discharge, pH, and fresh wet mount microscopy, or microscopy of Gram-stained vaginal smears (Nugent *et al.*,1991; Amsel *et al.*,1983).

The swabs were transferred to the laboratory in the same day and cultured according to standard microbiological protocols for aerobic bacteria (Niger, 2014). In addition, a detailed questionnaire was filled for each participant at the time of taking the swabs. Below is the form of questionnaire that was used for each participant.

3.2.2. Questionnaire for Vaginal Infections

Below is a sample copy of the questionnaire that was filled out for every participants in this study:

If you have a question you do not want to answer on this form please discuss it with the healthcare provider.

Is your vaginal discharge a chronic long term problem _____or a new recent problem_____?

Please describe your vaginal discharge (explain):

_____

Do you have: _____Odor _____Color _____ Itching _____Pain _____Burning with urination

Have you recently used (please check):

- Antibiotics
- Birth Control Pills
- Name
- Age
- Marriage
- Abortion
- Pregnant
- Date of sample

3.2.3.Culture Media:

Sterilization of culture media and solutions were achieved by autoclaving at 121 ^{0}C of 15 lb/inch^2 for 15 minutes, other solutions that destroy by heat were sterilized by Millipore filtration through 0.22 and 0.45µm filters.

3.2.3.1. Preparation of Media:

MacConkey agar ,Mannitol salt agar medium,Eosin methylene blue agar (EMB),Nutrient agar ,Nutrient Broth, Peptone water ,Simmon citrate agar ,Methyl red voges proskauer (MRVP) media, Triple sugar iron agar (TSI).

The culture media were prepared according to instruction of the manufacturers and as follows:

a-Blood agar medium

It was prepared by dissolving 40g of the medium in 1 liter of distilled water, autoclaved, left to cool for 45-50 ^{o}C then whole human blood cells was added to the medium and homogenized thoroughly and

poured into Petri dishes. This medium was used for primary isolation and to detect the ability of bacteria to hemolysis red blood corpuscles (RBCs).

b- Urea agar base

It was prepared by dissolving 24g of the medium in 1liter of distilled water, boiled for complete dissolving and autoclaved, then left to cool for 45-50 ^{0}C and 50 ml of 40 % urea which sterilized by filtration was added to this medium, mixed well, poured into screw-capped test tubes, dispensed in 5 ml volume per tube, and allowed to solidify in a sloped position. This medium was used to detect the ability of bacteria to produce urease enzyme.

c- Skim milk agar

This contains: Nutrient agar, sterile 87.5 ml

Skimmed milk (Himedia/India), sterile 12.5 ml. The nutrient agar was autoclaved, cooled to 50°C, and then the skim milk was added and poured in sterile plates (Collee *et al*., 1996) .

3.2.3.2. Identification of Bacteria

A single colony was taken from each primary positive culture on blood agar, MacConckey agar, mannitol salt agar and repeat growth to gain pure culture and then it was identified depending on its morphological and cultural characteristics (blood hemolysis, lactose fermentation, mannitol fermentation, colony shape, size, colour, borders, and texture) and then it was examined under the microscope after making smear from pure colony on clean slid and stain with Gram's stain for

observation arrangement and reaction bacteria with stain (Collee *et al.*, 1996; MacFaddin, 2000).

3.2.3.2.1. Api Systems for bacteria

Api 20 E and Api staph are identification system for Enteriobacteriaceae and *Staphylococcus*, respectively. Each system contains20 standardized and miniaturized biochemical tests and database. These Api systems include a strip consist of 20 microtubes containing dehydrated substrates. These tubes are inoculated with a bacterial suspension that re-constitutes the media.

During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to the reading table and the identification is obtained by referring to the analytical profile index.

3.2.3.2.2. Biochemical Tests

The biochemical activity (biochemical tests) in bacteria are very reliable in the identification differentiation of bacteria diagnosis. All biochemical reactions (physiological characteristics) are catalyzed by enzymes, and enzyme is produced by individual genes.

a- Haemolysin Production

Haemolysis production was shown on blood agar media. the organism was inoculated at this blood agar plates and incubated again for 24 hours at 37 C˚. Any haemolysis presence showed be detected around the colonies (β-haemolysin) (De Boy, *et.al*., 1980).

b- Catalase Test

It was prepared by dissolving 3 g of H_2O_2 to 100ml of distilled water and was stored in dark container (Forbes *et al.*,2007). A colony of the organism was transferred to a drop of 3% H_2O_2 on a microscope slide. The presence of catalase was meant that the formation of gas bubbles has occurred which indicated the positive result (Collee *et al.*, 1996).

c- Oxidase Test

It was prepared by dissolving of 0.1 g of Tetra-P- paraphenylene diamine dihydrochloride in 10 ml of distilled water and stored in a dark container (Forbes *et al.*,2007). A piece of filter paper was saturated in a petri dish with oxidase reagent then a colony of organism was spread onto the filter paper. When the color around the smear turned from rose to purple, the oxidase test was positive (Collee *et al.*, 1996).

d- Coagulase Test

Several colonies of bacteria were transferred with a loop to a tube containing 0.5 ml of rabbit plasma. The tube was covered to prevent evaporation and incubated at 37°C overnight. The test was read by tilting the tube and observing the clot formation in the plasma. Negative test results in the plasma remained free-flowing with no evidence of a clot (Collee *et al.*, 1996).

e- Methyl red Test

Methyl red of 0.1 g was dissolved in 300 ml of 99% ethanol and then the volume was completed to 500 ml by distilled water (MacFaddin, 2000). The test was performed on 5 ml of MR-VP broth cultured by the organism and then it was incubated for 24 hours at 37°C. After that 6-8

drops of Methyl Red reagent were added to culture. The change of color to orange-red was a positive reaction (Collee *et al.*, 1996).

f- Voges Proskauer Reagent

According to (Collee *et al.*, 1996) this reagent consists of:

Reagent A- 5 gm of α-naphthol was dissolved in 100 ml of 99% ethanol.

Reagent B- 40 gm of KOH was dissolved in 100 ml of distilled water (Collee *et al.*, 1996). The test was performed on 5 ml of MR-VP broth cultured by the organism and then it was incubated for 24 hours at 37°C. After that 15 drops of 5% alpha naphthol (reagent A) were added followed by 10 drops of 40% KOH (reagent B) and shaken well and allowed to stand for up to 30 minutes before calling a reaction negative.

The positive culture was turning to red at the surface of the liquid, and the color was spread gradually throughout the tube (Forbes *et al.*,2007).

g- Triple Sugar Iron Agar (TSI) Test

The test was used to differentiate the *Enterobacteriaceae* according to carbohydrate fermentation and hydrogen sulfide production. The organism was grown on TSI slant by stab and streak and then it was inoculated at 37°C for 24-48 hours. The changing of the color of the media from orange-red to yellow was due to carbohydrate fermentation with or without gas formation at butt of slant. In addition, the formation of hydrogen sulfide was given a black color precipitation at butt (MacFaddin, 2000).

h- Indole Test

After that the broth inoculated with bacterial colonies and it was incubated for 48-72 hrs. at 37°C. Testing for indole production was done by adding 6-8 drops of Kovac's Reagent (p-dimethyl amino benzal dehyde in amyl alcohol). The formation of red color ring at top of broth was a positive reaction. A yellow color ring was a negative result (MacFaddin , 2000).

i- Simon Citrate Test

After the sterilization of Simon citrate slants by autoclave at 121 ^{o}C for 15 min then cooled to 50 C^{0} and inoculated with the bacterial cultures and incubated for 24-48 hrs. at 37°C. The positive result was a change of the color of media from green to blue. The unchanging of the color was a negative reaction (Benson, 1998).

j- Urease Test

The urea base agar was sterilized by autoclave at 121^{o}C for 15 minutes. After cools it to 50°C, the sterile urea substrate was added to it and was poured in sterile tubes; then inoculated by bacterial cultures and it incubated them for 24-48 hrs. at 37°C. The positive result was a deep pink color. Failure of deep pink color to develop was a negative reaction (Benson, 1998).

k- Motility Test

According to the method described by (MacFaddin, 2000). 10 ml of semisolid media were dispensed in the tubes and left to set at the vertical position ,I had inoculated with a straight wire, making a single stab down the center of the tube to about half the depth of the medium.

The tubes were incubated at 37°C and examine at 6 hours, 24 and 48 hours. Non-motile bacteria had generally confined to the stab-line and given sharply defined margins with leaving the surrounding medium clearly transparent. Motile bacteria were typically given diffuse hazy growths that spread through out the medium rendering it slightly opaque.

3.2.3.3. Culture Media Used in Yeasts Isolation and Identification:

The PDA and SDA media were prepared according to instruction of the manufacturers and as follows:

Aerobic Culture Methods

The culture swab was inoculated onto.

1- Blood agar .

2- MacConkey agar plate.

The plates were incubated at 37 ^{0}C for 24 hrs aerobically .

3.2.3.3.1. Api Systems for yeasts identification

The API yeast identification system, consists of a disposable plastic strip containing 20 microcapsules containing dehydrated substrates and/or nutrient media (Pfaller *et al.*, 1988). In this system, the yeast enzymes cleave chromogenic components from specific chromogen-attached substrates and the change of color in test wells are visually read and interpreted. The API yeast identification system has shown a rather inconsistent ability to identify clinical isolates, with an overall identification rate ranging from 55% to 60% (Salkin *et al.*, 1987;

Pfaller *et al.*, 1988). Problems with the limited database and difficulties in interpreting the test have been reported (Pfaller *et al*., 1988).

3.2. 3.3.2. Germ-Tube Test

A suspension containing 0.5 mL human serum was obtained from the isolated colony and incubated in a test tube for a maximum of 3 h at 37 ºC. During this period, a drop of suspension from each sample was collected at hourly intervals, placed between a slide and cover slip and examined under a microscope (40x objective) .The presence of germ tubes, *i.e.*, small filaments projecting from blasto conidia without any constriction at the parent cell, allows the presumptive identification of *C. albicans* (Kreeger,1984).

3.2.4. Maintenance of Isolates Medium

Microbial isolates were stored for a long period at low temperature in appropriate broth medium containing brain-heart infusion broth with 15% glycerol. This was done by adding 15% of glycerol to brain-heart infusion broth, then put in tube and sterilize with autoclave (Collee *et al.*, 1996).

3.2.5. McFarland Solution (No. 0.5)

Consisting of barium chloride $BaCl_2$ (1%) 0.6 ml with 99.4 ml from H_2SO_4 (1%). The mixture was shacked well and placed in a screw capped test tube and kept in dark at 4°C. The solution was mixed well before use. The solution must give a bacterial turbidity equal to 1.5 x 10^8 bacteria/ml. (Cruickshank *et al*., 1975).

3.2.6. Normal Saline Solution

This solution was prepared by dissolved 0.85g of NaCl in 100 ml of distilled water; pH was adjusted to 7.2, and then sterilized by the autoclave.

3.2.7. Phosphate Buffer Solution (PBS) (pH 7.3)

Eighteen g of NaCl, 0.34 g of KH_2PO_4 and 1.12 g of K_2 HPO_4 were all dissolved in 1000 ml of D.W; the solution was autoclaved at 121C°,1.5b for 20 min. Then pH was adjusted at 7.3. It was used in washing and preserving human and sheep RBCs used in the haemagglutination test (Forbes *et al*., 2007).

3.2.8. Detection of Bacteria Virulence Factors

3.2.8.1. Protease production

Skim milk agar plates were inoculated by streaking and incubated at 37ºC for 48 hours. The clear zone exists adjacent the positive result. That indicated the production of protease enzyme (Collee *et al*., 1996; Benson, 1998).

3.2.9. Detection of *E.coli* virulence phenotypes

3.2.9.1. Haemagglutination Assay Procedure

For the haemagglutination (HA) assay, bacteria were grown on various soild and liquid media at 37°C to determine the conditions for optimal expression of haemagglutinating activity, those media included Tryptose soy agar (TSA) and broth and brain heart infusion agar (BHIA) and broth. Bacteria were harvested and washed twice in phosphate-buffered saline (PBS; pH7.2) and the cells were suspended in the same

buffer to adencity of approximately 10 bacteria per ml in comperision with McFarland turbidity standards (0.5tube). Erythrocytes were obtaind from various species including human blood group A, Sheep and Chicken ,before use blood cells were washed two time in PBS (pH7.2) and a 1% (vol /vol) erythrocyte suspension was prepared (Jane *et al.*, 2004).

Samples for HA were titrated in a 96-well U-bottom microtiter plate, serial doubling dilutions of the bacterial suspension in PBS were made in 50 µl volumes, after which an equal volume of 1% suspension of erythrocytes in PBS was added to each well . The plate was incubated at 4°C for 2 hrs., and the result was read. The HA unit was defind as the reciprocal of the highest dilution of the bacterial suspension causing visible agglutination of the erythrocytes, reaction was compared with negative control (50 ul of PBS and 50 µl of blood cell) (Jane *et al*., 2004).

3.2.10. Detection of Yeast Virulence Factors

3.2.10.1.Protease production

Skim milk agar plates were inoculated by streaking and incubated at 37ºC for 48 hours. The clear zone exists adjacent the positive result. That indicated the production of protease enzyme(Collee *et al*., 1996; Benson, 2001).

3.2.10.2. Phospholipase Production

The nutrient agar was autoclaved, cooled to 50°C. and then the two egg yolk was added poured in sterile plates ,were inoculated by streaking and incubated at 37ºC for 48 hours. The clear zone exists adjacent the

positive result indicated the production of phosphlipase enzyme(Collee *et al*., 1996; Benson, 2001).

3.2.11. Buffers used in DNA extraction

3.2.11.1. Tris-EDTA buffer (TE buffer) pH 8.0

It was prepared as 10X buffer by mixing 10 ml of 1M Tris- HCl buffer with 50ml of D.W and then adding 0.37 gm of EDTA, the pH was adjusted to 8, then the volume was completed to 100ml with D.W. The final concentration was 10X, then autoclaved at 121˚C for 20 minutes, and stored at 4˚C until use. Working solution of 1X TE buffer (pH 8.0) was prepared by diluting the stock solution 10X (Sambrook and Rusell, 2001).

3.2.12. Buffers used for Agarose Gel Electrophoresis

3.2.12.1. Working solution of Tris-Borate-EDTA (TBE buffer)

Tris-borate-EDTA buffer was used at dilution of 0.5X (5:45 dilution of the concentration stock). The stock solution was diluted by D.W. and stored at room temperature (Sambrook and Rusell, 2001).

3.2.12.2. Ethidium Bromide

It was (5mg/ml) prepared by dissolving 0.05 gm of ethidium bromide in 10 ml of D.W and stored in dark reagent bottle(Sambrook and Rusell, 2001).

3.2.13. Polymerase Chain Reaction Assay for Detection of *E. coli* Virulence and Phylogenic Groups Genes

3.2.13.1. Preparation of Primers

The DNA primers were prepared depending on manufacturer instruction. At first, stock solution (100 pmol/µl)for each primers was prepared by dissolving the lyophilized primer into 1X TE buffer (pH 8.0) in a volume that was recommended by the manufactures, from this stock, a working solution (20 pmol/µl) was prepared by diluting the stock solution using 10X TE buffer (pH 8.0). The final picomoles depended on the procedure of each primer.

3.2.13.2. DNA Extraction

Total DNA was extracted by boiling method,5colonies of overnight bacterial culture on tryptose soy agar (TSA) were suspended into 100 µl sterile distilled water and treated at 94˚C (by using water bath) for 10 minutes. Followed by centrifugation at 12000 rpm for one minute, and the supernatant used as DNA template in PCR (Browne *et al.*, 2004; Yazdi *et al.*, 2011). Long- term storage of extracted DNA was done by using 10X TE buffer (pH 8.0) to obtain a DNA suspension in 1X TE buffer (pH 8.0). Then the DNA samples were stored at -20˚C until use.

3.2.13.3. PCR Supplies Assembling

Escherichia coli DNA templates were subjected to multiplex PCR (MPCR) using 6 sets (F and R) of primers targeting two groups of genes: the first group listed in Table (3.7) to detect the virulence genes and the second group listed in Table (3.8) to determine phylogenic groups' genes. The AccuPower® PCR PreMix which is premixed ready-to-use solution

containing DNA polymerase dNTP, $MgCl_2$ and reaction buffers at optimal concentrations and its recommended for any amplification reaction that to visualized by agarose gel electrophoresis and ethidium bromide staining.

Assembling MPCR materials were done using MPCR reaction mixtures prepared in 0.2 ml eppendorf tube with 20µl reaction volumes (for virulence genes) which contain: 6µl of primers (1µl of each F and R, the final volume of 3 primers was equal to 6 µl), 5µl of DNA template and 9µl nuclease free water. According to the phylogenic groups, the reaction mixtures were 20 µl, which contain: 6µl of primers (1µl of each F and R, the final volume of 3 primers was equal to 6 µl), 5µl of DNA template and 9µl nuclease free water. All the addition was done in laminar flow on ice.

3.2.13.4. PCR Cycling Profiles

Multiplex PCR was carried out in 20 µl reaction volumes, according to virulence genes, the samples were amplified under the following conditions: denaturation for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and a final extension step of 7 min at72°C.

For phylogenic groups genes, the samples were amplified for 35 cycles and each cycle consisted of 10 minute at 94˚C (for initial denaturation) 2 minute at 94˚C (for denaturation) 30 second at 65˚C (for annealing) 1 minute at 72˚C (for extension) and final extension at 72˚C for 10 min (Clermont *et al.*, 2000; Soto *et al.*, 2011).

3.2.13.5. Detection of PCR Amplicons

3.2.13.5.1. Preparation of Agarose Gel

Agarose gel was prepared by dissolving 1.5 g of agarose powder in 100 ml of 0.5X TBE buffer (pH 8), the bottle was placed in boiling water bath until it become clear, allowed to cool to 50˚C, then1 µl ethidium bromide (at concentration of 0.5 mg/ml) was added. A tape was placed across the end of the gel tray, the comb was fixed at one end of the tray for making wells used for loading DNA samples. The agarose was poured gently in to the tray, and allowed to solidify at room temperature for 30 minutes. Then the comb was removed gently from the tray and the tap was also removed from the ends of the tray. The latter was fixed in electrophoresis chamber which was filled with TBE buffer has covered the surface of the gel.

3.2.13.5.2. Agarose Gel Electrophoresis

The amplified PCR products were detected by agarose gel electrophoresis and visualized by staining with ethidium bromide. PCR products were loaded to the agarose gel wells: 3µl from 100 bp ladder to one of the wells in the row, followed by 8 µl of reaction mixture of each sample. The gel tray was fixed in electrophoresis chamber. 0.5X TBE buffer was added to the chamber until covered the surface of the gel. The electric current was performed at 70 volt for 1hour.

3.2.14. Analysis of Results

The electrophoresis result was detected by using gel documentation. The base pair of DNA bands were measured according to the ladder. The positive results were distinguished when there was DNA

band equal to the target product size. Finally, the gel was photographed using gel documentation saving picture.

3.2.15. Data Analysis

Data analysis was performed using SPSS version 16.0 (SPSS Inc, Chicago, Illinois, USA). The Chi square test was used to compare between different groups, whereas Pearson's correlation coefficient was used to find out if positive or negative correlation could be found between the studied variables. P value less than or equal to 0.05 considered as statistical significant for all analyses.

Chapter Four

Results and Discussion

4.1. Characteristics of Patients

A total of 100 female patients were enrolled in this study. The study conducted in the period from December 2013 to January 2015. All patients were attendants of the Gynecology and Obstetric Teaching Hospital in Kerbala, holy Kerbala, Iraq. The study was ethically approved by the ethical committee of the health directorate in Holy Kerbala Province.

As shown in Table (4-1), the majority of the patients with vaginal discharge (73%) were below 35 years old and 93% were equal or in the fertility age (14-45 years). In addition, only 7 patients (7%) were above 45 years old that is the most probable age of menopause. This may indicate that vaginal discharges are more likely to occur in ages associated with frequent hormonal changes (represented by menstrual cycle), higher sexual activity and/or pregnancy.

Data on signs and symptoms associated with the vaginal discharges (such as burning at urination) and physical appearance of the discharge (color and odor) were available for 99 patients (data on one patient was missing). Discolorations of the discharges were noticed in 43 patients (43.4%), whereas bad odor was detected in 69 patients (69.7%). A 62 patient (62.6%) had dysuria (burning at urination).

Regarding the duration of symptoms, around half of the patients (49%) were chronic (symptoms persisted for more than 1 month). Furthermore, recurrent infections were reported in 37% of the patients.

42 patients were pregnant (42%), 15 patients (15%) mentioned that their partners were using condoms. Most of the patients (no= 59) reported a history of taking birth control pills.

Table (4-1): Patients' characteristics

Parameters		Frequency	Percent	Cumulative Percent
Age	14-24 years	41	41.0	41.0
	25-34 years	32	32.0	73.0
	35-44 years	20	20.0	93.0
	45-54 years	7	7.0	100.0
	Total	100	100.0	
Color of Vaginal Discharges	No change (white color)	56	56.6	56.6
	Discoloration	43	43.4	100.0
	Total	99	100	
Odor of Vaginal Discharges	No odor	30	30.3	30.3
	Bad odor	69	69.7	100.0
	Total	99	99.0	
Burning at Urination	No Burning	37	37.4	36.3
	Burning	62	62.6	100.0
	Total	99	99.0	
Duration of Symptoms	Acute	14	14.0	63.0
	Chronic	49	49.0	49.0
	Recurrent	37	37.0	100.0
	Total	100	100.0	
Pregnancy	Aborted	19	19.0	19.0
	Pregnant	42	42.0	61.0
	Non-pregnant non-aborted	39	39.0	100.0
	Total	100	100.0	
Use of Condoms by the partner	Not used	85	85.0	85.0
	Used	15	15.0	100.0
	Total	100	100	
History of Use of Contraception	None used	28	28.0	28.0
	Birth control pills	59	59.0	87.0
	Mixed	13	13.0	100.0
	Total	100	100.0	

4.2. Microorganisms Isolated from High Vaginal Swabs

Standard microbiological methods were used for the cultivation and identification of microorganisms from the high vaginal swabs including Gram's staining, primary cultures on blood agar and MacConcky agar followed by culture on specific media such as mannitol salt agar..etc. and biochemical tests and/or API20 systems.

4 .2.1. Microbial Isolation and Identification

In this study, several bacterial species were isolated from female patients. The growth characteristics and laboratory identification are mentioned below:

4.2.1.1. Morphological and Cultural Characteristics

A preliminary diagnosis of bacteria was made based on growth characteristics and colony morphology on blood agar and MacConkey agar. Colony morphology was of value in preliminary identification of mixed growths. A total of 54 isolates were recovered from mixed culture from 100 patients.

On blood agar, *S. aureus* showed hemolysis on these media because these bacteria had the ability to release hemolysin (lyses of red blood cells) (Collee *et al.*, 1996; Todar, 2001)

On MacConcky agar *K. pneumoniae,* showed circle, large, mucoid and pink colonies. When Gram-negative bacilli ferment lactose, mixed acid by products are formed. These acids cause a localized decrease in pH, shown by neutral red, producing pink-red colonies. Some lactose fermenters, such as *E. coli*, *K. pneumonia*e also produce a zone of precipitated bile salts around the colonies (Koneman *et al.*, 1983).

Mannitol salt agar utilized as selective media for Gram positive bacteria; *S. aureus* appeared to be yellow, circle and large colonies. *S. aureus* has the ability to ferment mannitol with production acid in media (Brooks *et al.*, 2001).

The results of microscopic examination show that the Gram negative bacteria produced rod shapes and Gram positive bacteria produced cluster-like shapes.

4.2.1.2. Biochemical Tests

Table (4-2) showed the results of the biochemical tests used for the identification of Gram- negative bacterial isolates. All Gram-negative bacteria were positive to citrate and catalase tests and negative to H_2S production, whereas only *E.coli* was positive to indole test. All Gram- negative bacteria were negative to oxidase and hemolysis tests except *P. aeruginosa* that was positive. Gram negative bacteria displayed variable results to motility, methyl red, voges-proskauer, urease.

Table (4-2): Biochemical tests for identification of Gram negative bacteria

Test	*E. coli*	*Klebsiella puenmoniae*	*Proteus mirabilis*
Indole	+	-	-
MR	+	-	+
VP	-	+	variable
Citrate	-	+	+
Urease	- Yellow	The slow \ + Pink	+ Pink
Lactose fermenter	+	+	-
Motility	variable	-	+
EMB	Metalic	Gentrally dark	
TSI	+ A/A / H_2S -	+A/A /H_2S -	+ K\A / H_2S +

Regarding the Gram positive bacteria, as shown in Table (4-3) all isolates showed negative results to motility and positive to catalase. The coagulase test

revealed that all *S. aureus* were positive to this test. *S. aureus* has the capability to coagulate plasma by the production of fibrin (Collee *et al.*, 1996; Brooks *et al.*, 2001; Todar, 2001).

Table (4-3):The biochemical tests for identification of *S. aureus*

Tests	*S.aureus*
Catalase	+
Coagulase	+
Hemolysin	+
Mannitol fermenter	+
Motility	-

4.2.2. Types of Microorganisms Isolated from High Vaginal Swabs

Table (4-4) presents the type of isolated microorganisms from the high vaginal swabs in addition to their frequencies. A total of 174 microbial isolates were recovered from the 100 patients. The most predominant bacterial type was *E.coli* (n= 65, 37%) followed by *P.maribilis* (n=37, 21 %), Coagulase-negative staphylococci (CoN) (n= 33, 19 %), *K.puenmoniae* (n=13, 7%), as well as other microorganisms in low frequencies.

E coli was isolated from 60 patients. In addition, 5 patients were found to harbor 2 different type (strains) of *E. coli* (that are different on API20E system and genotyping by PCR; see later). Therefore, the total *E. coli* isolates in this study was 65 isolated from 60 patients.

The high prevalence of *E. coli* in high vaginal swabs may be explained on the basis of the known virulence factors of this microorganism. For example, *E.coli* is a motile bacteria thus it has the ability to cause ascending infections (Conrad *et*

al. 2012), in addition it has attachment virulence factors such as certain specific adherence factors that allow this pathogen to colonize in specific position. In addition, *E .coli* possesses definite morphological structure called fimbriae or pili that play important role in adhesion these bacteria mucous layers.(Chromek, 2006), these factors allow the bacteria to adhere to the high vaginal wall and resist the washing forces and thus facilitate colonization. In addition, because of *E coli*, is present normally in feces and may contaminate the vagina in different occasion .

The secondly ranked microorganism isolated was *P. mirabilis*, isolated from 37 patients. This high incidence of *P. mirabilis* in the vaginal region could be attributed to the specific properties of this organism that aid in ascending and colonization of the vaginal such as being highly motile and possessing attachment factors. Motility in *P. mirabilis* is highly complex as they engage in several different kinds of movement depending on the specific environment they are inhabiting. Most of these movements are directly tied to the differential expression of flagella and other factors. When in liquid environments, normal movement is facilitated by swimming. However, in more viscous and solid environments, *P. mirabilis* have the ability to differentiate in elongated, multinucleated, highly flagellated cells, which then allows them to move together over solid surfaces at very high rates (Carey *et al*., 2013). This activity, known as swarming, is a primary factor in the success of *P. mirabilis* in causing complicated uriniary tract infections and other more serious bladder and kidney infections (Burall *et al.*, 2004). The bacteria's ability to swarm over surfaces allows them to ascend up the urethra, eventually invading the bladder and kidneys (Bacheller and Bernstein, 1997). *P. mirabilis* infection then leads to more complicated problems, such as bladder/kidney stones.

A total of 33 Coagulase-negative staphylococci were detected in swabs from

29 patients. It was found that 4 patients were harboring 2 different CoN species. These microorganisms are frequently isolated from UTI (Kumari *et al*., 2001) and, therefore, urinary tract could be the source from which those bacteria had the opportunity ascends.

K. pneumonia was isolated from swabs of 13 patients. One or more virulence factors may contribute to pathogenicity of *Klebsiella pneumoniae* in humans. Three factors that may mediate virulence: cell wall receptors, capsular polysaccharide, and endotoxin. First, the presence of cell wall receptors enables *K. pneumoniae* to attach to the host cell, thereby altering the bacterial surface so that phagocytosis by polymorphonuclear leukocytes and macrophages is impaired and invasion of the non-phagocytic host cell is facilitated(Cleggs, 2007). Second, invasion of the host cell is also facilitated by the large polysaccharide capsule surrounding the bacterial cell; in addition this capsule acts as a barrier and protects the bacteria from phagocytosis(Kabha K, 1997). Third, *K. pneumoniae* produces an endotoxin that appears to be independent of factors that determine receptors and capsular characteristics(Gilchrist, 1995). Marked interspecies differences in endotoxin production may correlate with virulence. Although some or all of these factors may ultimately determine virulence, the interaction of these factors in vivo has made it difficult to assess the relative contribution of any one of these virulence factors. The pathogenic mechanisms of *K. pneumoniae* that ultimately determine virulence remain unclear and will require further study.

Polymicrobial colonization were reported in more than half of the patients (n=54). This result may be expected because the lower parts of the genito-urinary tract are frequently colonized by different microbes, in addition to being exposed for frequent contamination from faces and other sources. Organisms causing UTI are derived primarily from the aerobic members of the fecal flora. An

overwhelming majority of uncomplicated urinary tract infections (95%) are caused by a single organism. In contrast, infections among hospitalized patients, patients with urinary catheters, or individuals with structural abnormalities of the urinary tract may be polymicrobial. The most common pathogens are Gram-negative rods. *E.coli* causes about 80% of acute infections in patients without urinary tract abnormalities. Other Gram-negative organisms include *P. mirabilis* and *K. pneumoniae*, organisms which colonize the enteric tract. *Enterobacter*, *Serratia*, and *Pseudomonas* are infrequent in the outpatient population, but they are more frequent in patients with complicated UTI. They are important pathogens in individuals with structural abnormalities of the urinary tract and in individuals with urinary tracts that have been instrumented. *S. saprophyticus*, a Gram- positive coagulase negative *Staphylococcus*, causes about 10% of infections among young, sexually active women.

Table (4-4) Type and frequency of isolated microorganisms

Types of bacteria &yeasts	Number of isolates	Percentage from all isolates	Number of patients infected by this microorganism	% from all patients
E.coli	**65***	**37%**	**60**	**60**
P.maribilis	**37**	**21%**	**37**	**37**
Coagulase Negative staphylococcus	**33***	**19%**	**29**	**29**
Klb.puenmoniae	**13**	**7%**	**13**	**13**
Staphylococcus aureus	**5**	**3%**	**5**	**5**
Bacillus	**4**	**2%**	**4**	**4**
Streptococcus	**3**	**2%**	**3**	**3**
candidia albicans	**2**	**1%**	**2**	**2**
C.tropicalis	**1**	**1%**	**1**	**1**
Entero fecalis	**1**	**1%**	**1**	**1**
Moraxella	**1**	**1%**	**1**	**1**
Listeria	**1**	**1%**	**1**	**1**
Citro freundii	**2**	**1%**	**2**	**2**

Types of bacteria &yeasts	Number of isolates	Percentage from all isolates	Number of patients infected by this microorganism	% from all patients
Citro kos,farmeri	**1**	**1%**	**1**	**1**
Enterbacter.crota	**2**	**1%**	**2**	**2**
Burkhol.cepacia	**1**	**1%**	**1**	**1**
Aci baunannii\calco	**1**	**1%**	**1**	**1**
Raou.ornithinolytica	**1**	**1%**	**1**	**1**
Total	**174**	**100%**	**165**	**165**
Polymicrobial infection	**54**	**31%**	**54**	**54**

***Five patients were found to be infected by 2 types of *E. coli* strains and other 4 patients were infected by 2 types of coagulase-negative staphylococci.**

Candida spp. was detected in 3 patients only. The incidence may be considered low because of the high incidence of these microorganisms in the lower parts of the genito-urinary tract. However, this low incidence may reflect inability of the *Candida* spp. to ascend and colonize vaginal-epithelium. *Candida* spp is proved to be one of the causative agent of UTIs in human particularly in elderly women patients and it colonises the lower parts of the gental tract (Jacqueline and Bettina, 2010).

4.3. Comparing Type of Isolates with Patients Characteristics

Detailed comparisons were performed between type of isolated microorganisms and the different characteristics and results are summarized in Table (4-5).

In these comparisons each type of isolate was compared to the rest of isolates in respect to patient's' characteristics. The purpose of performing these kinds of comparisons was to find out if any type of isolates was independently linked to any of the patient's characteristics.

No correlation was found between age of the patients and type of isolates (Table 4-5). This may indicates that all types of microorganisms in this study were able to cause infection at different age groups.

Regarding the color of the high vaginal discharge, a negative correlation was found between discoloration of the discharge and presence of polymicrobial infections (R=-0.233, p= 0.020), however, no correlation was found with any single-isolate.

Concerning odor of the discharges, although the majority of infections were associated with bad odor, no specific type of isolates was statistically linked to the bad odor. All *S. aureus* isolates (n=5) were isolated from patients suffering from dysuria (burning at urination).

Regarding the duration of symptoms, no significant correlation was noted with the type of isolates. This may indicate that all types of isolates may cause acute, chronic and recurrent infections.

It's worth to noting that all *S. aureus* isolates were recovered from either pregnant (n=3) or aborted females (n=2) and no isolates could be isolated from non-pregnant-non aborted females. These results may indicate that pregnancy and abortion may predispose for infection by *S. aureus*.

A statistically significant positive correlation (R= 0.292, p= 0.003) was found between history of taking contraceptives and infection by *K. pneumoniae*. Among 13 patients infected by *K. pneumonia*, 11 (84.6%) were taking contraceptives). Cervical discharge and cervicitis are known to be associated with oral contraceptives (Willmott,1988).

Table(4-5):Comparison between microbial isolates in respect to patient's characteristics

Parameter		*E. coli*		*P.mirabilis*		*K.pneumoniae*		*CoN-staphylococci*		*S.aureus*		*Candida* spp		*Polymicrobial infection*	
		Others	Positive	Others	Positive	Others	Positive	Others	Positive	others	Positive	others	Positive	others	positive
Age	14-24	13	28	24	17	36	5	30	11	39	2	39	2	16	25
	25-34	15	17	20	12	29	3	21	11	31	1	31	1	19	13
	35-44	10	10	13	7	16	4	14	6	19	1	20	0	12	8
	45-54	2	5	6	1	6	1	6	1	6	1	7	0	3	4
	Total	40	60	63	37	87	13	71	29	95	5	97	3	50	50
	correlation	R= - 0.082-, p=0.415		R=- 0.119- P=0.238[c]		R= 0.060 P=0.551[c]		R=- 0.023- P=0.822[c]		R= 0.066 P=0.515[c]		R=- 0.112- P= 0.269[c]		R=- 0.0117 P=0.247	
Color of the discharge	No change (white color)	19	37	33	23	48	8	40	16	52	4	54	2	22	34
	Discoloration	21	22	29	14	38	5	30	13	42	1	42	1	27	16
	Total	40	59	62	37	86	13	70	29	94	5	96	3	49	50
	Correlation	R= -0.151-, p= 0.137		R=-0.087- P=0.391[c]		R=-0.039- P=0.701[c]		R=0.018 P=0.859[c]		R=-0.109- P=0.283[c]		R=-0.036- P=0.723[c]		R=- 0.233 P=0.020	
Odor	No-odor	12	18	21	9	26	4	22	8	29	1	29	1	17	13
	Bad odor	28	41	41	28	60	9	48	21	65	4	67	2	32	37
	Total	40	59	62	37	86	13	70	29	94	5	96	3	49	50
	Correlation	R= -0.005-, p= 0.957		R=0.100 P=0.322[c]		R=-0.004- P=0.969[c]		R=0.038 P=0.708[c]		R=0.052 P=0.611[c]		R=-0.012- P=0.909[c]		R=0.095 P=0.352	
Burning at Urination	No burning	13	24	22	15	32	5	28	9	37	0	36	1	18	19
	Burning	27	35	40	22	54	8	42	20	57	5	60	2	31	31
	Total	40	59	62	37	86	13	70	29	94	5	96	3	49	50
	Correlation	R= -0.083-, p= 0.414		R=-0.051- P=0.619[c]		R=-0.009- P=0.932[c]		R=0.084 P=0.407[c]		R=0.178 P=0.078[c]		R=0.015 P=0.885[c]		R=- 0.013 P=0.898	
Duration of symptoms	chronic	21	28	30	19	44	5	33	16	46	3	48	1	24	25
	Acute	3	11	11	3	13	1	10	4	13	1	13	1	8	6
	Recurrent	16	21	22	15	30	7	28	9	36	1	36	1	18	19
	Total	40	60	63	37	87	13	71	29	95	5	97	3	50	50
	Correlations	R= 0.004, p= 0.965		R=0.010 P=0.922[c]		R=0.115 P=0.254[c]		R=-0.084- P=0.404[c]		R=-0.070- P=0.490[c]		R=0.023 P=0.821[c]		R=0.000 P=1.000	
Pregnancy	aborted	9	10	12	7	17	2	16	3	17	2	18	1	12	7
	preganant	17	25	24	18	38	4	29	13	39	3	40	2	17	25

Parameter		*E. coli*		*P.mirabilis*		*K.pneumoniae*		*CoN-staphylococci*		*S.aureus*		*Candida* spp		*Polymicrobial infection*	
		Others	Positive	Others	Positive	Others	Positive	Others	Positive	others	Positive	others	Positive	others	positive
	non preganant non aborted	14	25	27	12	32	7	26	13	39	0	39	0	21	18
	Total	40	60	63	37	87	13	71	29	95	5	97	3	50	50
	Correlation	R= 0.083, p= 0.410		R=-0.068- P=.504[c]		R=0.097 P=0.336[c]		R=0.126 P=0.212[c]		R=-0.187- P=0.062[c]		R=-0.128- P=0.206[c]		R=0.027 P=0.788	
Use of condoms by partners	none	35	50	52	33	73	12	61	24	81	4	84	1	44	41
	used	5	10	11	4	14	1	10	5	14	1	13	2	6	9
	Total	40	60	63	37	87	13	71	29	95	5	97	3	50	50
	Correlation	R= 0.057, p= 0.572		R=-0.090- P=0.374[c]		R=-0.079- P=0.434[c]		R=0.040 P=0.692[c]		R=0.032 P=0.751[c]		R=0.254 P=0.011[c]		R=0.084 P=0.406	
Contraception	none	9	19	14	14	26	2	22	6	26	2	27	1	10	18
	Birth Control Pills	24	35	41	18	53	6	38	21	58	1	57	2	34	25
	mixed	7	6	8	5	8	5	11	2	11	2	13	0	6	7
	Total	40	60	63	37	87	13	71	29	95	5	97	3	50	50
	Correlations	R= -0.126-, p=0.210		R=-0.039- P=0.698[c]		R=0.292 P=0.003[c]		R=-0.072- P=0.476[c]		R=0.145 P=0.150[c]		R=-0.066- P=0.511[c]		R=- 0.026 P= 0.795	

*c: Based on normal approximation

4.4. Associations among the Isolated Microorganisms

Microbial synergism and antagonisms are biological phenomen a that result from symbiosis between organisms. Bacterial synergy can be defined as the cooperative interaction of two or more bacterial species to produce results not achieved by the individual bacterium acting alone .

In the context of interaction, this synergistic result is an increase in virulence, as poly microbial infections have been shown to be more virulent there infections caused by single organism in both human and animals (Dalton *et al*., 2011).

In this study was analyzed by constructing a correlation matrix using pearson's correlation test. The purpose of those analyses was to find out the among these organisms. Table(4-6), shows the correlation matrix among the different types of microbial isolates.

Highly significant negative correlation was found between the presence of *E. coli* and *K. pneumoniae* (R= -0.291, *p*= 0.003) where most of *E. coli* isolates (no= 56) were not associated with *K. pneumoniae* infection. Furthermore, all *Candida spp*. isolates were recovered from patients not infected by *E. coli* .On the other hand, a highly significant positive correlation (R=0.327, p=0.001) between *E. coli* and polymicrobial infections.

P. mirabilis was negatively correlated with CoN staphylococci (R=-0.216, p=0.031), however, it was positively correlated with polymicrobial infections (R=0.394, p= 0.000).

Table (4-6): Correlation matrix among the microbial isolates

Parameter		*E. coli*		*P.mirabilis*		*K.pneumoniae*		*CoN-Staphylococci*		*S.aureus*		*Candida spp*	
		-	+	-	+	-	+	-	+	-	+	-	+
P. mirabilis	Negative	24	39										
	Positive	16	21										
	Total	40	60										
	Correlation	R= -0.051-, p=0.616											
K. pneumoniae	Negative	30	57	55	32								
	Positive	10	3	8	5								
	Total	40	60	63	37								
	Correlation	R= -0.291-, p= 0.003		R=0.012 P=0.908^0									
CoN-Staphylococci	Negative	25	46	40	31	60	11						
	Positive	15	14	23	6	27	2						

Parameter		*E. coli*		*P.mirabilis*		*K.pneumoniae*		*CoN-Staphylococci*		*S.aureus*		*Candida spp*	
		-	+	-	+	-	+	-	+	-	+	-	+
	Total	**40**	**60**	**63**	**37**	**87**	**13**						
	Correlation	**R= -.153-, p= 0.129**		**R=-0.216- P=0.031[c]**		**R=-0.116- P=0.250[c]**							
S.aureus	**Negative**	**38**	**57**	**59**	**36**	**82**	**13**	**66**	**29**				
	Positive	**2**	**3**	**4**	**1**	**5**	**0**	**5**	**0**				
	Total	**40**	**60**	**63**	**37**	**87**	**13**	**71**	**29**				
	Correlation	**Not calculated**		**R=-0.081- P=0.424[c]**		**R=-0.089- P=0.380[c]**		**R=-0.147- P=0.145[c]**					
Candida spp.	**Negative**	**37**	**60**	**60**	**37**	**84**	**13**	**68**	**29**	**93**	**4**		
	Positive	**3**	**0**	**3**	**0**	**3**	**0**	**3**	**0**	**2**	**1**		
	Total	**40**	**60**	**63**	**37**	**87**	**13**	**71**	**29**	**95**	**5**		
	Correlation	**Not calculated**		**R=0-.135- P=0.181[c]**		**R=-0.068- P=0.502[c]**		**R=-0.112- P=0.266[c]**		**R=0.229 P=0.022[c]**			
Mixed bacteria growth	**Single microbial infection**	**28**	**22**	**41**	**9**	**46**	**4**	**40**	**10**	**49**	**1**	**49**	**1**
	Mixed microbial infection	**12**	**38**	**22**	**28**	**41**	**9**	**31**	**19**	**46**	**4**	**48**	**2**
	Total	**40**	**60**	**63**	**37**	**87**	**13**	**71**	**29**	**95**	**5**	**97**	**3**
	Correlation	**R= 0.327, p= 0. .001**		**R=0.394 P=0.000[c]**		**R=0.149 P=0.140[c]**		**R=0.198 P=0.048[c]**		**R=0.138 P=0.172[c]**		**R=0.059 P=0.562[c]**	

4.5. Evaluation of the Virulence Factors of Isolated Microorganisms

In order to confirm the pathogenicity of the isolated microorganisms, a number of virulence assays were performed and as follows:

4.5.1. Proteinase Production Assay

Proteinase production was tested for *P. mirabilis*, *K. pneumoniae*, *Staphylococci* spp. and *Candida* spp.

Proteinases are crucial virulence factors produced by many pathogens, which can cause the degradation of host proteins for essential nutrients but they

can also protect the bacteria from the host's defenses such as the complement system (Potemp ,2009) .

All tested isolates in this study were found to be proteinase producers and, thus, may consider being virulent.

4.5.2. Present of Pili

This test was performed for all *E. coli* isolates

Virulence factors of the *E. coli* isolates evaluated in this study included haemagglutination (HA). Out of 65 cases, 49 isolates (75.38%) were HA positive ; while,16 isolates (24.61%) were negative HA. The is finding is corresponding with other studies findings in which show similar results to this study (Sharma *et al*., 2007; Fatima *et al*., 2012; Mohajeri *et al.*, 2014) while an Indian study demonstrates opposite findings to this study, 66%, 74.8% were HA and 76%, 61% were CSH (Desai *et al*., 2013; Mittal *et al*., 2014) respectively.

4.5.3. Virulence factors of *Candida* spp.

For *Candida* spp. virulence tests were phospholipase production and production of proteinase.

All isolates of *Candida* spp in this study were positive for phospholipase production. Phospholipases are enzymes that hydrolyze ester linkages of glycol phospholipids and hence facilitate tissue invasiveness to *Candida* cells. A study conducted by Ibrahim *et al*.(1995) revealed an increased level of phospholipase production in blood isolates compared to commensal isolates.

All *Candida* spp were position for proteinase production. Secretion of proteinases by pathogen is mandatory in order to degrade the tissue barriers and obtain nutrition at the infection site. Secreted aspartyl proteinases (SAPs) from

Candida have been reported that hydrolyze many proteins such as albumin, hemoglobin, keratin, collagen, laminin, fibronectin, mucin, salivary lactoferin, interleukin1b, cystatin A, and Immunoglobulin A (Hube *et al*. 1998). In vivo studies have confirmed the role of SAPs in colonization, increased adhesion and tissue penetration (Hube and Naglik 2001;Naglik *et al*. 2004). Expression of SAPs has been found to be correlated with other virulence determinants to enhance the pathogenicity of *C. albicans*.

4.6. Molecular characterization of *E. coli* isolates

Because *E. coli* was the most prevalent microorganism in this study, further molecular characterizations of the isolates were performed. Two types of molecular test were done and as follows:

4.6.1. Phylogeny

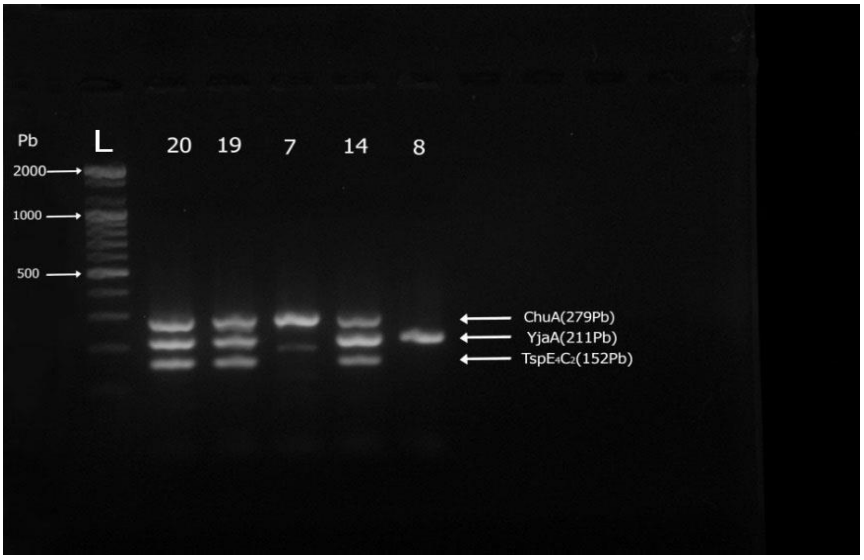
4.6.1.1. Molecular Detection of Phylogenic Groups

A phylogenetic analysis using PCR was performed to determine which genotype is most prevalent among *E. coli* isolates and to find if there is a specific genotype is associated with any of the studied parameters.

The phylogenetic grouping of *E.coli* isolates was made on the basis of the presence of specific PCR-amplified fragments as follows (Clermont *et al.*, 2000):

- (*chu* A - , *yja* A +/-, *TspE4C2* -) ⟶ group A
- (*chu* A -, *yja* A+/-, *TspE4C2* +) ⟶ group B1
- (*chu* A+, *yja* A +, *TspE4C2* +/–) ⟶ group B2
- (*chu* A+, *yja* A -, *TspE4C2* -/+) ⟶ group D

The phylogenic group genes of *E.coli* isolates such as *chuA, YjaA* and *TspE4.C2* were detected by Agarose-electrophoreses technique; shown in Figure(4.1)



Figure(4-1): Agarose gel electrophoresis of *E.coli* phylogenic group genes (*chu*A, *yja*A and DNA fragment *TSPE4.C2*) detected by multiplex PCR in 65 isolates of *E. coli* isolated from female with vaginal infection . **Lane** (L), DNA molecular size marker (100-bp ladder). **Lanes** (7) group D isolates showing amplication products of *Chu*A(279 bp) and negative result with all products of phylogenic groups respectively.**Lanes** (8) group A isolates showing amplification product of *yja*A (211bp) and negative result with all products of phylogenic groups respectively. **Lanes** (20),(19) and (14) group B2 isolates showing amplification products of *Chu*A and *Yja*A and *Chu*A, *Yja*A and *TspE4.C2* (279 bp, 211 bp and 152bp) respectively.

Agarose gel electrophoresis is used for study and detection of phylogenic group genes in local isolates of *E. coli* isolated from patients, and grouping these isolates into major standard groups of genotyping (genes: *chu*A, *yja*A and DNA

fragment *TSPE4.C2*).

In the present study among of 65 *E .coli* isolates frequencies of (A, B1, B2 and D) were 22 (33.84%), 2 (3.07%), 40 (61.53%) and 1 (1.53%) respectively in patients. Each group showed two different patterns as shown in Table(4-7):

Table(4-7): Phylogenetic distribution of *E. coli* isolates

Phylogenetic Groups	*ChuA gene*	*YjaA gene*	TspE4.C2 Fragment	Number of profiles within Phylogenetic group	No. of *E. coli* isolate n=65 (%)	Number of patients infected n=60(%)
Group A	-	+	-	9	22(33.84)	21(35.00)
	-	-	-	13		
Group B1	-	-	+	0	2(3.07)	2(3.33)
	-	+	+	2		
Group B2	+	+	+	38	40(61.53)	36(60.00)
	+	+	-	2		
Group D	+	-	-	1	1 (1.53)	1(1.66)
	+	-	+	0		

As shown in Table (4-7), the most frequent Phylogenetic group of *E. coli* was " B2" which comprised 60% (no= 36) followed by " A" that comprised 35.00% (no= 21).

Previous studies have shown that phylogenetic groups B2 and D are virulent because these groups are associated with the presence of several virulence factors (Soto *et al.*,2008). Regarding this study, the majority of patients were infected by *E. coli* isolates belong to B2 group (65%),and among the total number of isolates (n=65),40 (61.5%) were belong to this phylogenetic groups. In addition, most of the *E. coli* isolates in this study were belong to B2 group (61.5%).

4.6.1.2.Distribution of *E. coli* Phylogenetic Groups According to Patients Charcateristics

Because that certain phylogentic groups were reported to be more virulent than others, we analyzed the distribution of these groups according to the different characteristics of the patients. Table (4-8) summarizes the association of *E. coli* phylogentic groups A and B2 with different parameters.

Regarding the use of contraception , no significant differences were seen in the distribution of phylogentic groups A and B2 of *E. coli* isolates (p= 0.118).

Table (4-8): Association of *E. coli* phylogentic groups A and B2 with different parameters

Parameter		Genotype		Total	P-value
		Genotype A	Genotype B2		
Contraception	none	6	12	18	Chi-square= 0.118
	Pills	8	25	33	
	Mixed	4	2	6	
Pregnancy Abortion	aborted	5	5	10	Chi-square = 0.356
	preganant	7	16	23	
	non preganant non aborted	6	18	24	
Condems	none	17	30	47	Chi-square = 0.106
	used	1	9	10	Fisher s Exact Test = 0.103
Age	14-24	7	19	26	Chi-square = 0.003
	25-34	2	14	16	
	35-44	8	2	10	
	45-54	1	4	5	
Burning Urination	.00	6	18	24	Chi-square = 0.322
	1.00	12	20	32	Fisher s Exact Test = 0.243
Color Change	No change (white color)	11	23	34	Chi-square = 0.967
	Discoloration	7	15	22	
Poly microbial infection	single organism	7	14	21	Chi-square =

Parameter		Genotype		Total	P-value
		Genotype A	Genotype B2		
	infection				0.828
	mixed infection	11	25	36	Fisher s Exact Test = 0.527
Disese Duration	1.00	12	14	26	Chi-square = 0.063
	2.00	1	10	11	
	3.00	5	15	20	
Odor	No odor	7	10	17	Chi-square = 0.339
	Bad odor	11	28	39	Fisher s Exact Test = 0.257

On the other hand no significant differences were detected in the distribution of the *E. coli* phylogentic groups according to pregnancy (p= 0.356) ,whereas phylogentic group B2was more prevalent among pregnant (16 versus 7) and non-pregnant non aborted (18 versus 6) .

Concerning the use of condoms , very interesting results were seen, where 9 out of ten women isolates of women whose husband using condoms were shown to be of B2 phylogentic group. However due to the low number of isolates in this category static assay could not confirm this result . No differences were seen with other parameters.

The findings of phylogenic groups in present study are in agreement with several previous investigations have indicated that most of the isolates belonged to B2 followed by D and B1 groups(Kawamura-Sato *et al*., 2010, Choi *et al*., 2012) . In contrast, the study by Abdul-Razzaq and Abdul-Lateef (2011) in Hilla, Iraq who reported A and B1 phylogenetic groups were the most prevalent in the different clinical isolates so Piatti *et al* (2008) found the same results that phylogenetic groups A and B1 were the most prevalent in the isolates, respectively. In other studies the results were B2 (50%), D (12%) and (19%) each of A and B1

(Bashir, 2012), while in Mexic study,Molina-López (2011) showed 36% of studied isolates belonged to B2, 28.7% to A, 27.8% to D, and 8.4% to B1. Significantly, strains responsible for extra-intestinal infection were far more likely to be members of phylogentic groups B2 or D than A or B1 (Johnson, 2000). The clinical significance of these observations suggested that a simple method of assigning isolates to a phylogentic group would be of value. This led to the development and validation of a PCR assay to detect the genes *Chu*A and *Yja*A and a DNA fragment *TspE4.C2* (Gordon, 2008). This triplex PCR phylo-group assignment has been used extensively as a simple and inexpensive method for assigning an *E. coli* isolate to a phylo-group and has provided further evidence that strains of the various phylo-groups differ in their phenotypic and genotypic characteristics, their ecological niche, life history traits and ability to cause disease (Tenaillon, 2010, Alm *et al*., 2011).

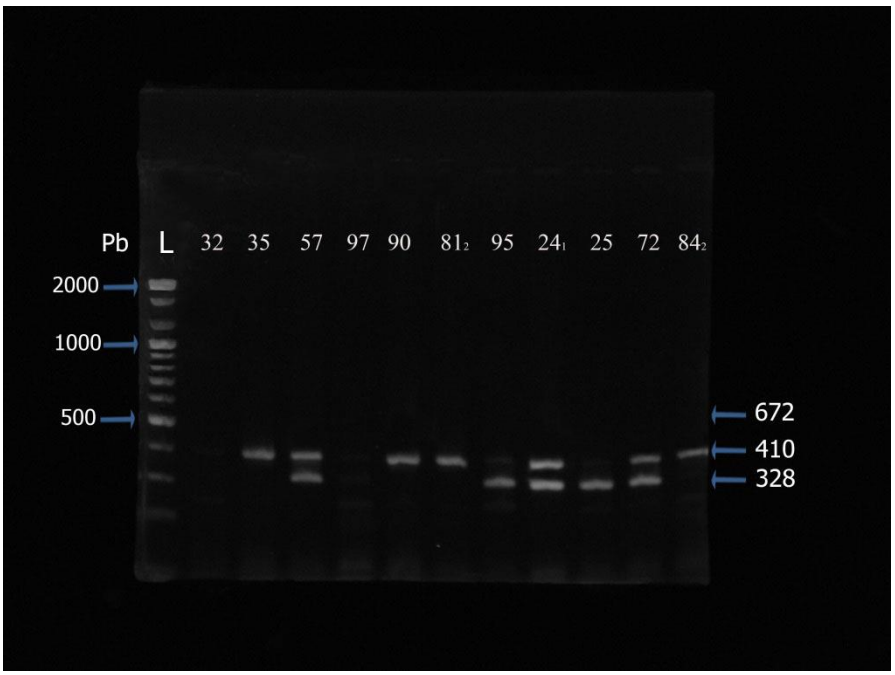
4.6.2.Molecular Detection of *E. coli* Virulence Genes

Data on the features and virulence factors of *E. coli* colonizing the cervico-vaginal epithelium are very limited. The study of the virulence factors of *E. coli* strains is necessary to understand the potential risk factors for vertical transmission of neonatal infections by pregnant women and to design interventions to address such risk factors adequately. Previous study has shown that neonatal sepsis by *E. coli* is related to a limited number of phylogenetic groups B2 and D, both considered as virulent. The pathogenicity of these groups is associated with the presence of several virulence factors (Soto *et al*.,2008).

Virulence genes of adhesion factors in *E.coli* isolates such as *Pap*C, *Afa* and *Sfa* were analyzed by the multiplex PCR assay. The results of this study showed that; frequencies of *Pap, Sfa* and *Afa,* among *E.coli* isolates were 14, 42 and 5,

respectively, 48 isolates had at least one virulence gene and 17 isolates had none of the studied genes. Furthermore, 34 isolates carrying just one virulence factor, and 14 isolates carrying 2 genes, however, none of the isolate out has found to carry all the three genes.

In Present results, the virulence genes of *E.coli* adhesion factors such as *PapC*, *Afa* and *Sfa* were detected by Agarose-Electrophoresis technique; shown in Figure(4-2):



Figure(4-2): Agarose gel electrophoresis of *E.coli* virulence genes (*PapC*, *Sfa* and *Afa*) genes detected by multiplex PCR in 65 isolates of *E. coli* isolated from female with cervicitis. **Lane** (L), DNA molecular size marker (100-bp ladder). **Lanes** (57), (24_1) , and (72) show positive results with *PapC* and *Sfa* virulence factors genes. **Lanes** (32) and (97) show negative results with all virulence genes. All **Lane** *E. coli* shows negative result with *Afa* gene (672bp) only.

4.6.3.Relationship Between *E. coli* Phylogenetic and Virulence Factors

When the relationship of phylogenicity and virulence was studied, the prevalence of virulence genes was showed in Table(4-9). *Pap*C was found higher in phylogenetic group B2 isolates. *Sfa* gene was prevalent in phylogenetic group B2 isolates, followed in group A .

Table(4-9): Prevalence of virulence related genes in various phylogenetic groups of *E. coli* isolates

Phylogenic groups	Distribution of virulence genes		
	PapC	***Sfa***	***Afa***
Group A	1	8	2
Group B2	12	30	3
Chi-square	0.035	0.016	Nc*
Fisher s Exact Test	0.032	0.018	

*Not calculated

Conclusions

&

Recommendations

Conclusions

1- *E. coli* was the most Dissemination microorganism colonizing the vaginal epithelium, in addition to other potential pathogens.

2- polymicrobial colonization is quite common among the studied cases.

3- Molecular analyses showed that most of the *E. coli* isolates belong to the virulent group “B2” and that those isolates harbor several virulence factors.

4- *PapC* and *Sfa* adhesivs genes were prevalent among the *E. coli* isolates.

Recommendations

In view of our results we recommend the following ;

1- In this this study, it is recommended that further studies be conducted to investigate the actual causes for the high prevalence of *E.coli* and to document the sources,if its external or endogenous the actual role of urinary system in *E. coli* transmission the vaginal epithelium.

2- The use of PCR- established analyses for fast identification in routine work Labs.

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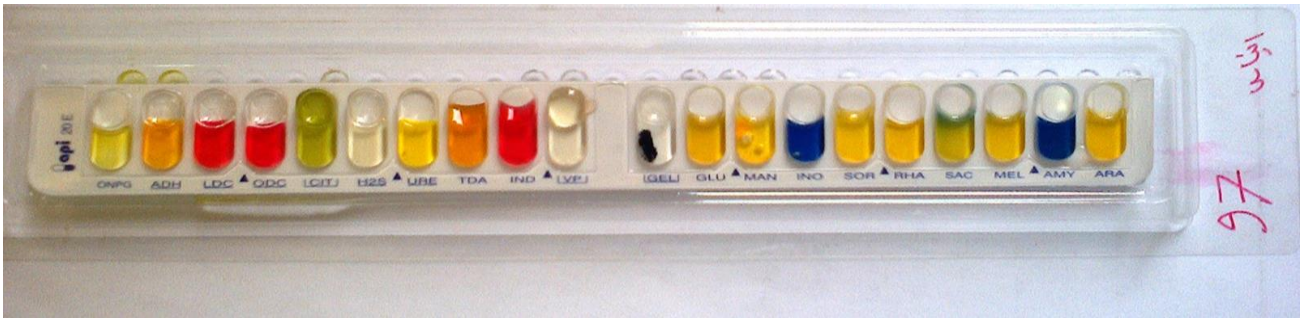
Appendix

Analytical Profile index (API)

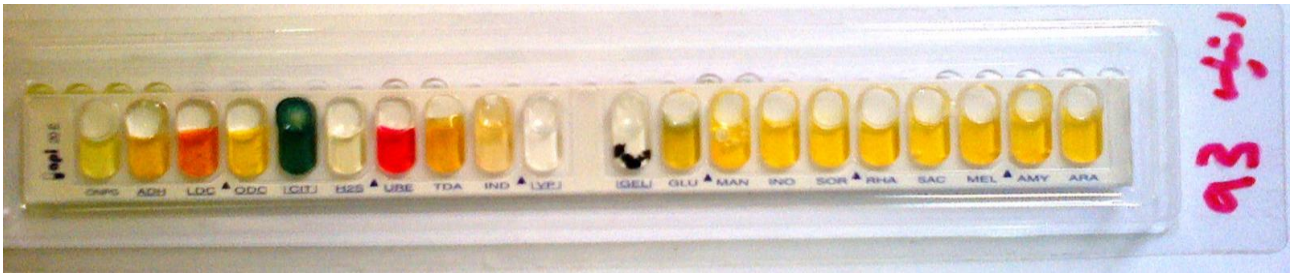
Results of ***Candida albicans*** as diagnosed by using API *Candida* system strips.

Results of ***Candida tropicalis*** as diagnosed by using API *Candida* system strips.



Results of ***E.coli 1*** as diagnosed by using API 20E system strips.

Results of ***E.coli 2*** as diagnosed by using API 20E system strips.



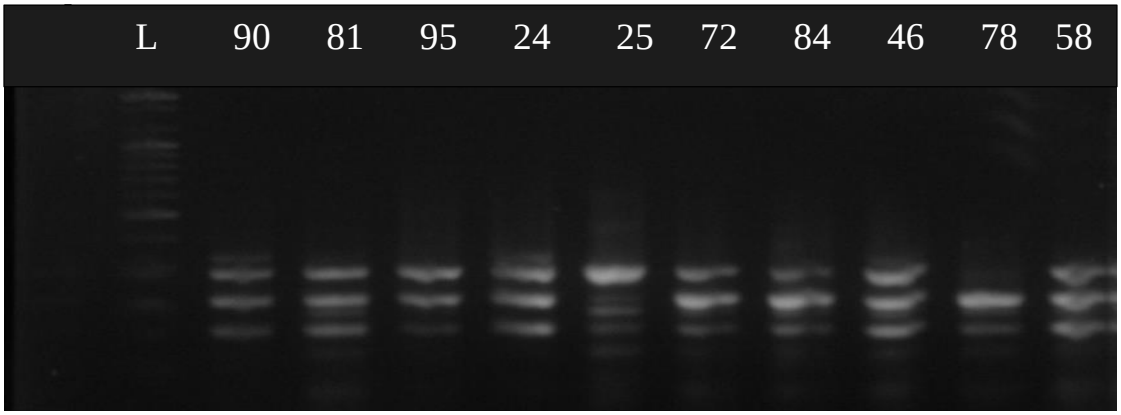
Results of ***K. puenmoniae*** as diagnosed by using API 20E system strips.

Results of ***P.mirabilis*** as diagnosed by using API 20E system strips.

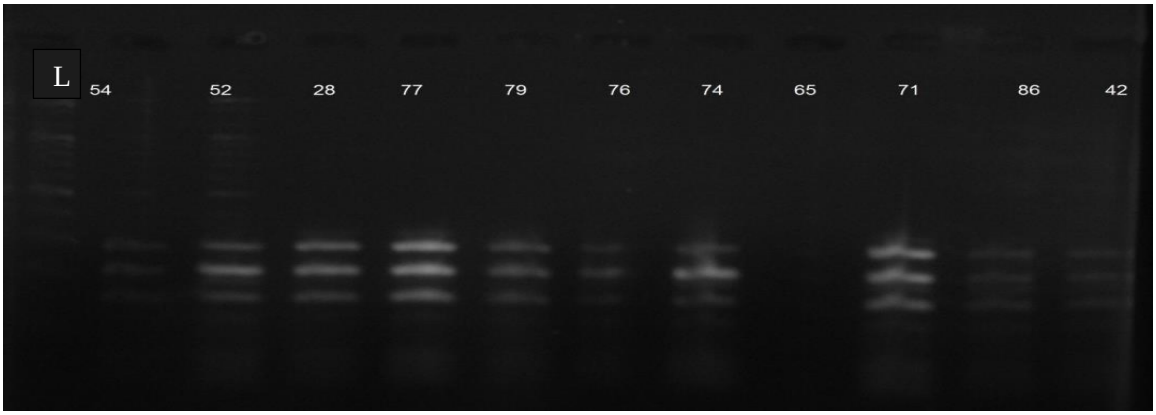
Results of ***staphylococcus*** as diagnosed by using API stap system strips.

Molecular characterization of *E. coli* isolates

a- Molecular Detection of Phylogenic Groups

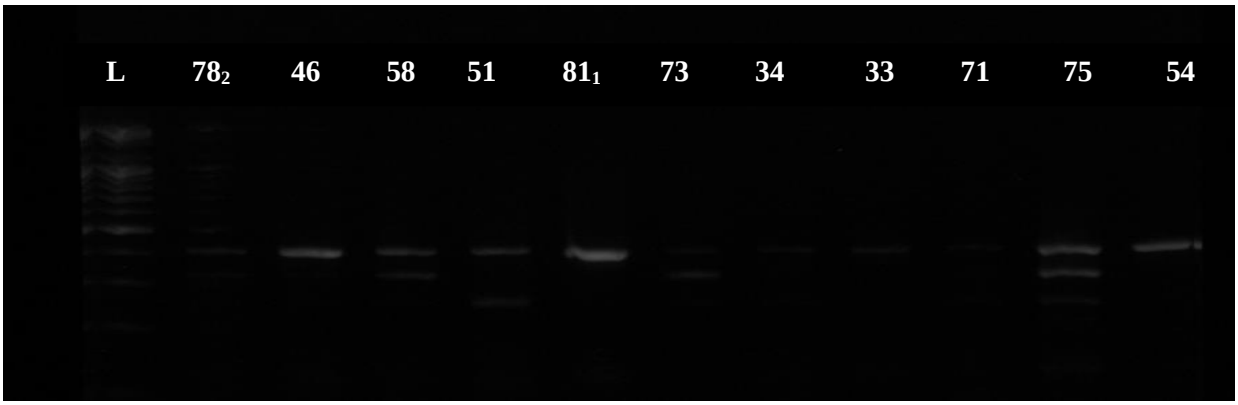


This picture show : Agarose gel electrophoresis of *E.coli* phylogenic group genes (*chu*A, *yja*A and DNA fragment *TSPE4.C2*) detected by multiplex PCR in 65 isolates of *E. coli* isolated from female with vaginal infection . **Lane** (L), DNA molecular size marker (100-bp ladder). **Lanes** (90,81,95,24,25,72,84,46,58) group B2 isolates showing amplification products of *Chu*A and *Yja*A and *Chu*A, *Yja*A and *TspE4.C2* (279 bp, 211 bp and 152bp) respectively. **Lanes** (78) group B1 isolates showing amplification product of *yja*A (211bp) and *TspE4.C2*(152bp) and negative result with *Chu*A products of phylogenic groups respectively.

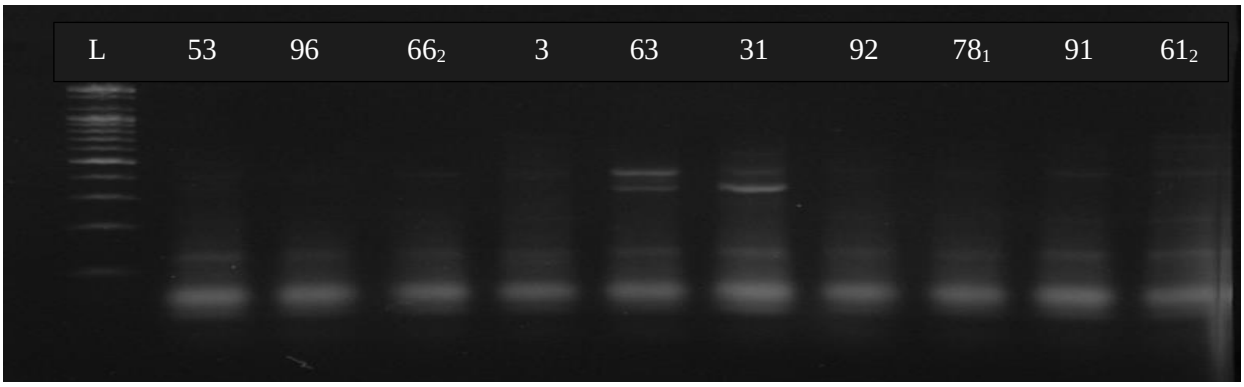


This picture show : Agarose gel electrophoresis of *E.coli* phylogenic group genes (*chu*A, *yja*A and DNA fragment *TSPE4.C2*) detected by multiplex PCR in 65 isolates of *E. coli* isolated from female with vaginal infection . **Lane** (L), DNA molecular size marker (100-bp ladder). **Lanes** (54,52,28,77,79,76,74,71,86,42) group B2 isolates showing amplification products of *Chu*A and *Yja*A and *Chu*A, *Yja*A and *TspE4.C2* (279 bp, 211 bp and 152bp) respectively. **Lanes** (65) negative result with all products of phylogenic groups respectively.

b- Molecular Detection of *E. coli* Virulence Genes



This picture show : Agarose gel electrophoresis of *E.coli* virulence genes (*PapC*, *Sfa* and *Afa*) genes detected by multiplex PCR in 65 isolates of *E. coli* isolated from female with cervicitis. **Lane** (L), DNA molecular size marker (100-bp ladder). **Lanes** (58,75) show positive results with *PapC* virulence factors genes . All **Lanes** show positive results with *Sfa* virulence factors genes. All **Lane** *E. coli* shows negative result with *Afa* gene (672bp) only.



This picture show : Agarose gel electrophoresis of *E.coli* virulence genes (*PapC*, *Sfa* and *Afa*) genes detected by multiplex PCR in 65 isolates of *E. coli* isolated from female with cervicitis. **Lane** (L), DNA molecular size marker (100-bp ladder). **Lanes** (31) show positive results with *PapC* virulence factors genes. **Lanes** (63) show positive results with *Sfa* virulence factors genes. **Lane** ($53,96,66_2,3,92,78_1,91,61_2$) shows negative result with Pap C , Sfa and *Afa* gene .

الخلاصة

اشتملت هذه الدراسة على 100 مريضة من الإناث. وقد أجريت هذه الدراسة في الفترة من كانون الأول 2013 إلى كانون الثاني 2015. جميع المرضى حضروا الى مستشفى النسائية والتوليد التعليمي في كربلاء المقدسة .العراق.

كانت الغالبية العظمى من المرضى الذين يعانون من إفرازات مهبلية (73٪) أقل من 35 سنة، وكانت 93٪ في سن البلوغ (14-45 سنة) . وقد لوحظ تغيير الألوان في الإفرازات في 43 مريضة (43.4٪)، في حين تم الكشف عن رائحة سيئة في 69 مريضة (69.7٪). ولم يكن لدى 62 مريض (62.6٪) عسر البول (حرق في التبول). وفيما يتعلق بمدة الأعراض، حوالي نصف المرضى (49٪) كانت مزمنة (استمرت الأعراض لأكثر من شهر) . تم الكشف عن الالتهابات المتكررة في 37٪ من المرضى. وكان 42 من المرضى الحوامل (42٪)، 15 مريضة (15٪) ذكرت بأن ازواجهم كانوا يستخدمون الواقي الذكري. معظم المرضى (59) ذكرت تاريخ أخذ حبوب منع الحمل .

تم الحصول على 174 عزلة جرثومية من مجموعه 100 مريضة. كان نوع البكتيريا الأكثر شيوعاً *E.coli* 37٪ (65) ، يليه *P.maribilis* 21٪ (37) ، Coagulase-negative staphylococci 19٪ (33) ، *K. puenmoniae* 7٪ (13) ، بالأضافة الى اعداد قليلة من الكائنات الحية الدقيقة الأخرى(26).

بالأضافة الى ذلك، تم الحصول إصابات متعدد المكروبات polymicrobial في أكثر من نصف المرضى (54). وبالإضافة إلى ذلك، تم الكشف عن *Candida* spp في 3 مرضيات فقط. ولم يعثر على أي علاقة بين عمر المريضات ونوع العزلات . تم العثور على وجود علاقة سلبية بين تلون الافرازات و وجود اصابات متعدد المكروبات (R = -0.233، P = 0.020)، لا يوجد ارتباط مع أي عزل منفردة. وارتبطت غالبية الاصابات مع رائحة سيئة، مع ذلك، لا يوجد نوع معين من العزلات ارتبط إحصائيا مع رائحة سيئة. يمكن أن يتم الكشف عن عدم وجود ارتباط كبير بين استخدام الواقي الذكري من قبل ازواج المريضات ونوع العزلة، مع ذلك، من بين 15 مريضة مرتبطة باستخدام الواقي الذكري، يوجد 10 اصابات بـ *E.coli* .علاوة على ذلك، تم العثور على وجود علاقة إحصائية موجبة (R = 0.292، P = 0.003) بين تاريخ اخذ حبوب منع الحمل والإصابة بـ *K.pneumoniae* .

في هذه الدراسة، توجد ارتباط سلبي كبير للغاية بين وجود *E. coli* و*K .pneumoniae* (R = -0.291، P = 0.003)، حيث ان معظم *E.coli* (56) لم تكن مرتبطة بالإصابة بـ *K .pneumoniae*.

الخلاصة

بالإضافة الى ذلك، جميع عزلات *Candida* spp من المرضى غير مصابين بـ *E. coli* . من ناحية أخرى، وجود علاقة إيجابية عالية (R = 0.327، P = 0.001) بين *E. coli* و الاصابات متعدد المكروبات polymicrobial. بالإضافة إلى ذلك، توجد علاقة سلبية بين *P.mirabilis* و Coagulase-negative staphylococci (R = -0.216، P = 0.031) ومع ذلك، فقد ارتبط بشكل إيجابي مع الاصابات متعدد المكروبات (R = 0.394، P = 0.000). لأن *E.coli* كانت الكائنات الحية الدقيقة الأكثر انتشارا في هذه الدراسة، تم اجراء دراسة جزيئية لها. وأجري تحليل باستخدام تقنية PCR لتحديد النمط الجيني الأكثر انتشارا بين عزلات *E. coli*،و كان النمط الجيني الأكثر شيوعا لـ *E. coli* " B2" الذي شكل 60٪ (36)، يليه "A" الذي شكل 35.00 ٪ (21). الجينات الضارة من عوامل التصاق في عزلات *E.coli* مثل *PapC*، *Afa* و*Sfa* حللت بواسطة PCR. وأظهرت نتائج هذه الدراسة تكرار *PapC*، *Afa* و*Sfa* ، بين عزلات *E.coli* حيث 14 و 42 و 5 على التوالي . في هذه الدراسة من مجموع 65 عزلة كانت 48 عزلة على الاقل تحمل جينات ضراوة (34 عزلة تحمل عامل ضراوة واحد فقط و 14عزلة تحمل 2 من الجينات) و 17 عزلة لا تحمل أي من الجينات التي شملتها الدراسة. ومع ذلك، لا توجد عزلة تحمل كل الجينات الثلاثة. كان النمط الجيني B2 أكثر انتشارا بين الحوامل (16 مقابل 7) وغير الحوامل و غير المجهضة (18 مقابل 6). ومن المثير للاهتمام، 9 من أصل عشر نساء عزل منهن النمط الجيني B2 كان ازواجهم يستخدمون الواقي الذكري. *papC* و*Sfa* أكثر تواجد مع النمط الجيني B2 ، هذا إضافة إلى أدلة أن B2 هي المجموعة المسببة للأمراض. في الختام، *E.coli* كانت الكائنات الحية الدقيقة الأكثر انتشاراً في استعمار البطانة الظهارية لعنق المهبل ، بالإضافة إلى المسببات المرضية المحتملة الأخرى. علاوة على ذلك، الاصابات متعدد المكروبات هو أمر شائع جدا بين الحالات التي تمت دراستها. وأظهرت التحليلات الجزيئية أن معظم عزلات *E.coli* كانت تنتمي إلى مجموعة ضراوة "B2"، وأن تلك العزلات تحمل عدة عوامل ضراوة. وبالإضافة إلى ذلك، كانت جينات الالتصاق *PapC* و*Sfa* سائدة بين عزلات *E.coli* ، وخاصة تلك المعزولة من النساء الحوامل، وبالتالي، قد ينطوي على مخاطر كبيرة لانتقال الاصابة إلى الجنين والاغشية الجنينة الذي بدوره قد يكون له عواقب سيئة على الأم والجنين.

ووفقا لنتائج هذه الدراسة، فمن المستحسن إجراء مزيد من الدراسة لتحقيق في الأسباب الفعلية لارتفاع معدل انتشار *E.coli* وتوثيق المصادر،وإذا كان الدور الفعلي خارجي أو داخلي المنشأ المتمثلة في الجهاز البولي في انتقال *E.coli* للبطانة الظهارية لعنق المهبل.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء
كلية العلوم

دراسة مايكروبية وجزيئية لخمج اعلى المهبل للنساء المرضى في كربلاء المقدسة

رسالة مقدمة إلى
مجلس كلية العلوم / جامعة كربلاء وهي جزء من متطلبات
نيل درجة الماجستير في علوم الحياة
من قبل

ايناس خالد كاظم الخالدي
بكالوريوس علوم حياة (2011)

بأشراف

ا.م.د .زهير حميد عبود
ا.م.د .مهند محسن احمد

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