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Biofilm Production as a Virulence Factor in Uropathogenic Bacteria and Yeasts

A thesis

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By

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Summary

This study included isolation and identification of different uropathogene (bacteria- yeasts) from catheterized patients suffering from complicated urinary tract infections, then detection the uropathogene ability to form biofilm as virulence factor responsible for initiation of infection and relationship between plasmids and antibiotics resistance . Study had been included the following steps:

- First step: identification of uropathogene (bacteria- yeasts) obtained by swabs from 350 urine samples of catheterized patients. 339 sample (96.85%)were identified by culture as positive result while 11 sample (3.142%)were gave negative result. 221 samples (63.138%) were obtained from females and 118 sample (33.711%) from males. 339 isolate have been included 303 (89.380%)bacterial isolate and 36 (10.619%) yeast isolate. Results of biochemical tests and *api* 20 system for bacterial and yeasts isolates show that *Proteus sp.* ;*Klebseilla sp, Pseudomonas spp.*and *Candida spp.* which represent the main causative uropathiogen infect urinary system , cause complicated type of infection.

- Second step: determination of bacterial and yeasts ability to form biofilm by using test tubes method , (306) isolates which represented (90.265%) which form biofilm were difference in thickness of formed layer. *Pseudomonas spp.* give thicker biofilm then *E.coli, Candida spp.*, *Proteus spp*. and *Klebseilla spp*. respectively.

- Third step: study the resistance of bacterial isolates towards different antibiotics that have been recommended for treating those patients it was found that higher percentage of resistance to penicillin, gentamycin ,

tetracyclin, chlormphnicol , and trimethoprim- sulfmethazxol then moderate resistance to ampcillin, nalidixic acid , cephotaxime, streptomycin , cephalexin and rifampicin and less resistance to ciprofloxacin that represent the most effective antibiotic against uropathogenic bacteria.

Ketazole antibiotic was used against yeasts, which the Minimum inhibitory concentration of ketazole against 83% of *C. albicans* isolates was 128, the rest of the isolates was 64.

- **Fourth step**: isolation of bacterial and yeasts DNA (chromosome + plasmids), results of DNA isolation showed that bacterial isolates(*E.coli* (RS1), *P. mirabilis* (RS2), *K. pneumonia* (RS3) and *P. aeruginosa* (RS4) have chromosome and plasmids vary in numbers and size according to isolate

While yeasts(C13) isolate have only chromosome without plasmid .

Results of curring show that:

-Resistance to ampicillin , tetracycline, gentamycin, pencillin for all bacterial isolates carried on plasmid.

- resistance to cephalexin by RS1 isolate, resistance to cephhotaxim by RS3 isolate and resistance to chlormphnicol by RS4 isolate carried on plasmid . while resistance to the rest once were carried on chromosome .

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List of Abbreviation

Abbreviation	Meaning
SDS	Sodium Dodecyl Sulphat
EDTA	Ethelene–Dramine Tertra acetrc acid
TE	Tris-EDTA
TSI	Triple Sugar Iron agar
Tsb	Trypton soya broth
MICs	Minimum Inhibition Concentration
SDB	Sabouraud Dextrose broth
SDA	Sabouraud Dextrose Agar
UTI	Urinary tract infection
API	Analytical Profile Index
TBE	Tris – borate – EDTA
NCCLs	National Committee for Clinical Laboratory standards

E.coli	*Escherichia coli*
UPEC	Uropathogenig *E.coli*
LT	Heat labile Toxin
ST	Heat stable Toxin
STx	Shiga toxin
EHEC	Enterohemorrhagic *E.coli*
MR-VP	methyle red-Voges Proskauer

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Chapter One

Introduction

&

Literature Review

1.1 Introduction

Urinary tracts infection is an extremely common clinical problems can be defined as the presence of microorgansims in a properly collected urine samples. UTIs have been included different classification depends on the site of infection, factors trigger infection and symptoms which play important role to help physicians in diagnosis of UTIs and patients treatment (Awaness *et al*.,2000).

The most common uropathogenes which responsible for properties of UTIs : members of *Enterobacteriacea* , *Staph spp.* and *Candida spp*. because they have different virulence factors enable them to invade urinary system and cause infection (John, 2001).

There are many factors increase the chance of UTIs like pregnancy, sexual intercourse , prostate inflammation , narrowed urethra, kidney stone and catheterization. Catheter although an essential component of medical care are also leading to cause nosocomial infection because catheter provided the bacteria with main route to entrance the urinary tracts and form biofilm.

Biofilm is a complex aggregation of microorgansim marked by the excretion and adhesive matrix which often characterized by surface attachment, structural heterogeneity, genetic diversity, complex community interactions and an extracellular matrix of polymeric substances. Biofilm decrease the susceptibility to antibiotics by physical impairment of antibiotic diffusion and local alteration of microenvironment (Stewart and Costerton, 2001; Parsek and Singh , 2003).

The resistance of bacteria to particular antibiotic may result from mutation which change the components of bacterial cell or the bacteria may have the plasmid carrying genes encoded for these resistance or by transposons that encoded for resistance . Resistance of members of enterobacteriacea frequently contain multiple plasmids which carry genes that confer resistance to numerous antibiotics, the larger of which can carry for resistance to 10 or more antimicrobial agents. Environmentally, a cell containing such plasmids had a better chance to survive (Hardy,1986; Jawetz *et al*., 1998).

Aims of the study

1- Isolation and identification of most common bacteria and yeast from the catheterized patients which have urinary tract infection.
2- Detection the ability of isolates for biofilm formation as virulence factors.
3- Detection the resistance pattern of isolates to antimicrobial agents.
4- Study plasmid profile of isolates and their relationships with antimicrobial resistance.

1.2 Literature review

1.2.1Urinary Tract Infection

Urinary tract infection (UTI) is an extremely common clinical problem. It is important because it may involve the urethra, bladder, uterus, and kidney. UTI affects all age groups, but women are more susceptible than men (Awaness *et al.*, 2000).

A urinary tract infection (UTI) can be defined as the presence of microorganisms in a properly collected specimen of urine (bacteriurea ;more than 10^5 bacteria /ml of urine). There are many risk factors that increase the chances of getting a UTI: Pregnancy and menopause, kidney stones, sexual intercourse, prostate inflammation or enlargement, narrowed urethra, immobility, not drinking enough fluids, bowel incontinence, catheterization(Pingle,1984;Glauser, 1986; Johnson, 2000).

The effects of the infection depend on the interaction between the bacteria and the host's defence mechanisms. If the brunt of the infection is situated in the bladder, the symptoms tend to be of a local nature and the disease is called cystitis, or a lower urinary tract infection. Infection involving mainly the kidney is called pyelonephritis, or an upper urinary tract infection. In pyelonephritis the symptoms tend division between upper and lower urinary tract infection, since the infection enters the system by an ascending route via the urethra (the tube connecting the bladder to the outside) in both cases to be of a more systemic nature, such as fever, chills and a fast heart rate (Stamm and Schaeffer, 2002).

Urinary tracts infections can be classify according to the complexity which are uncomplicated UTI that refer to UTIs seen

in patients with normal anatomic structure and function of the urinary tract. Cystitis is the most common urinary tract infection and is sometimes referred to as acute uncomplicated UTI. It occurs in the lower urinary tract (the bladder and urethra) and nearly always in women. In most cases, the infection is brief and acute and only the surface of the bladder is infected. Deeper layers of the bladder may be harmed if the infection becomes persistent, or chronic, or if the urinary tract is structurally abnormal. When infection spreads to the upper tract (the ureters and kidneys) it is called pyelonephritis, or more commonly, kidney infection. As many as half of all women with cystitis may have infections of the upper urinary tract at the same time as cystitis (Stamey, 1975; Bent *et al*., 2002).

Approximately 80% of acute uncomplicated UTIs are caused by *Escherichia coli*, 10-20 % are caused by coagulase-negative *Staphylococcus saprophyticus* (the second most common cause in young sexually active women), and 5 % or less are caused by other enterobacteriaceae such as *Proteus spp.* and *Klebsiella spp*. or by *Enterococcus* species (Alraek *et al*., 2002).

Complicated UTIs are resulting from anatomic obstructions of the urinary tract or catheterization. These abnormalities increase the volume of residual urine and interfere with the normal clearance of bacteria by urination. Such factors include prostate enlargement, sagging uterus, expansion of the uterus during pregnancy and catheterization. The most common causes of complicated UTIs are *Proteus spp. Klebsiella spp.,* and *Pseudomonas spp.*(Marx *et al.*,2000).

UTIs are classified as primary or recurrent, depending on whether they are the first infection or whether they are repeat events. Recurrence is common after both complicated and uncomplicated UTIs (Foxman, 1990).

After single uncomplicated acute urinary tract infection recurrence occur in approximately 27% to 48% of women, infection usually recur a few months a part. The risk after a complicated UTI is even higher between 50% and 60% of individual will have recurrent infection by 4 to 6 weeks following treatment if the underlying problems is not corrected recurrence is often defined as either reinfection or relapse (Johnson, 2003).

About 80% of recurring UTIs are reinfection. A reinfection occur several weeks after antibiotic treatment has cleared up the initial episode and is caused by a different organism from the one that caused the original episode. The infecting agent is usually introduced though the rectal region from fecal matter and move up through the urinary tract. It should be noted that the original infecting organism frequently persists so it is often difficult to distinguish a reinfetcion from a relapse (Shahid, 2003; Josip, 2006).

Relapse is the less common form of recurrent urinary tract infection . It is diagnosed when a UTI recurs within two weeks of treatment of the first episode and is caused by the same organism .Relapse usually occur in (Pyelonephritis) or is associated with obstruction such as kidney stones, structural abnormalities or in men, chronic prostatitis (Le *et al.*, 2004).

1.2.2 Pathogenesis of UTIs

UTIs occur as a result of the interaction of bacterial virulence factors, host biological and behavioural factors as opposed to highly efficient host defence mechanisms. There are three possible routes by which bacteria can invade and spread within the urinary tract and these routs include:

1.2.2.1 The Ascending Route

The urethra is usually colonized with bacteria. There are some conditions such as sexual intercourse and using of catheterization may result in the ascending of these bacteria to the bladder , and thus causing the urinary tract infection (Lohr *et al.*,1993; Khalili *et al.*,2000).

1.2.2.2 Hematogenous Route

Infection of the renal parenchyma by blood-borne organisms clearly occurs in humans. The kidney is frequently the site of abscesses in patients with staphylococcal bacteraemia or endocarditic, or both. Experimental pyelonephritis can be produced by the intravenous injection of several species of bacteria and *Candida*. However, the production of experimental pyelonephritis by intravenous route with gram-negative enteric bacilli, the common pathogens in urinary tract infection, is difficult. Additional manipulations such as the creation of urethral obstruction are often necessary. It would appear that in humans, infection of the kidney with gram-negative bacilli rarely occurs by the haematogenous route (Walter *et al.*,1990).

1.2.2.3 Lymphatic Route

Evidence for a significant role for renal lymphatic in the pathogenesis of pyelonephritis is unimpressive and consists of the demonstration of lymphatic connections between the ureters and kidneys in animals and the fact that increased pressure in the bladder can cause lymphatic flow to be directed toward the kidney. Thus, it would seem that the ascending pathway of infection is the most important (Mandell *et al.*, 2000).

1.2.3 Catheter-Associated Urinary Tract Infections

Catheter-associated urinary tract infection (CAUTI) is an infection from using tubes (catheters) that drain urine from the body. The diagnosis of catheter-associated urinary tract infection can be made when the urine culture shows 100 or more CFU per ml of urine from a catheterized patient. The microbiology of catheter-associated urinary tract infections includes different members *of Enterobacteriacea , Staph spp. , Pseudomonas spp. amd Candida spp*. The bacterial distribution reflects the nosocomial origin of the infections because so many of the uropathogens are acquired exogenously via manipulation of the catheter and drainage device. (Stamm,1999; Lemon and Burke, 2000).

Symptomatic bacteriuria in a patient with catheter should be treated with antibiotics that cover potential nosocomial uropathogens usually for 10 to 14 days. Parenteral antibiotic therapy may be necessary in patients with severe infections or patients who are unable to tolerate oral medications. The recommended duration of therapy for severe infections is 14 to 21 days (Warren, 1997; Hardyck and Petrinovich, 1998).

Catheters, although an essential component of modern medical care, are also the leading cause of nosocomial infections in both acute and chronic care facilities. Complications of catheter use may include: urinary tract or kidney infections, blood infections (septicemia), urethral injury, skin breakdown, bladder stones, and blood in the urine (hematuria)(Verleyen *et al*.,1999; Turck *et al*.,1999).

In an attempt to prevent infection, some catheters have been coated with antibiotics but there is debate if these catheters have any effect on decreasing infection. (Saint *et al*.,1998; Oni *et al*.,2003).

Catheters provide microorganisms with direct access to the normally sterile urinary tract, thereby predisposing to both bacteriuria and funguria (Tambyah and Maki, 2000).

Catheter associated urinary tract infections are the commonest nosocomial infections worldwide. While they are often asymptomatic and frequently cost less than nosocomial surgical site infections or nosocomial pneumonia, they are major reservoirs of antimicrobial resistant pathogens. Numerous strategies have been devised in an attempt to reduce the incidence of CAUTI but few have proven effective. Novel technologies such as the potential use of antiseptic or antimicrobial coatings on catheters hold promise for possibly reducing these infections in the fight against antimicrobial resistance (Turck *et al*.,1999).

The appearance of *Candida* in the urine is an increasingly common complication of catheterization, particularly for patients in the intensive care unit, on broad-spectrum antimicrobials, or with underlying diabetes mellitus. *C. albicans* is still the most common isolate, although *C. glabrata* and other non-*albicans* species are also frequently isolated. The clinical presentation can vary from an asymptomatic laboratory finding to sepsis. In asymptomatic patients, removal of the urethral catheter results

in resolution of the candiduria in as many as one third of cases. For patients with symptomatic candiduria (Jarvis and Martone, 1998).

Treatment of catheter-associated UTI depends on the clinical circumstances. Symptomatic patients (e.g., those with fever, chills, dyspnea, and hypotension) require immediate antibiotic therapy. In addition, it may be useful to remove and replace the urinary catheter if it has been in place for a week or longer. This eliminates difficult-to-eradicate organisms in the biofilm on the catheter. In an asymptomatic patient, therapy should be postponed until the catheter can be removed. Patients with persistent asymptomatic bacteriuria and those with lower urinary tract symptoms who have had the catheter removed respond well to short-course therapy (Wazait *et al.*, 2000).

Fig (1-1) The Catheter .

1.2.4 Symptoms of UTI - associated with a catheter

Symptoms of UTI usually begin with abnormal urine color (cloudy urine), blood in the urine (hematuria), strong urine odor, leakage of urine around the catheter ,pressure in the lower pelvis. Additional symptoms that may be associated with this disease: flank pain, fatigue, fever, chills, vomiting, mental changes or confusion .Often in an elderly

person, mental changes, or confusion are the only signs of a possible urinary tract infection (Kalsi *et al*., 2003; Walsh, 2003).

1.2.5 Types of Microorganisms which causes UTI

Urine is an excellent culture medium for the common pathogens of the urinary tract, and when bacteria are deposited in urine, they multiply readily often exceeding one million / ml. Bacteria infection that affects any part of the urinary tract. Although urine contains a variety of fluids, salts and waste product, it usually does not have bacteria in it (Ehrlich *et al.*, 2005).

When bacteria get into the bladder or kidney and multiply in the urine they cause urinary tract infection, the culture of urine sample obtained from patient represent the main way to identify the type of causative agent of urinary tract infection. The most common causative agents of UTIs are:-*Escherichia coli, Staphylococcus saprophytics , Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeruginosa and Candida sp*. (Chomart, 2000; Katz, 2003).

The less common causative microorganisms are :- *Citroacter spp. , Pantoea spp., Streptococcus spp.* and *Salmonella spp*. while the rare causative agents are:- *Neisseria gonorhoea, Leptospira, Chlamydia* and *Mycoplasma* (Haward,1997; Mobley,2000).

Chlamydia and *Mycoplasma* may also cause UTIs in both men and women, but these infections tend to remain limited to the urethra and reproductive system. Unlike *E.coli*, *Chlamydia* and *Mycoplasma* may be sexually transmitted .Viruses, fungi , and parasites can cause UTIs (Allen *et al.*,1999; Mobley,2000).

1.2.6 The Most Common Bacteria and Yeast that Cause CAUTI

1.2.6.1 *Escherichia coli*

Escherichia coli and it's relative are known to microbiologists as "enteric bacteria" because they live in the intestinal tract of human and animals as normal flora, this bacteria grow well on the usual laboratory media in both the presence and absence of oxygen, which grow in colonies called coliform, and metabolism can either by respiration or fermentation.This bacteria replicate by binary fission , double in number every 20 minutes (Kaper *et al.*,2004; Berghoward,2004).

1.2.6.1.1 Virulence Factors of *E.coli*

UPEC are the most important group of microorganism responsible for UTI that differ from non- pathogenic *E.coli* and other *E.coli* pathotype by it's production of specific virulence factors (Pass *et al.*, 2000; Gehua *et al.*, 2002).

There are different virulence factors which include:-

A. Adhesin

Adhesin which come into play during establishment of UTI, besides their primary function as adhesin molecule several other additional function may also function as invasins, promote biofilm formation and transmit signals to epithelial cells resulting in the inflemation (Oelschlaerger *et al.*, 2002).

E.coli has different type of adhesin like :-

a- Fimbrial adhesin which include type I fimbria , P–fimbria and S-fimbria , the genes encoding for fimbrial adhesion are closely linked on the chromosome of different types of pathogenic *E.coli* (Robert *et al.*, 2004).

b- Non –fimbrial adhesin (Intimin):- It is outer membrane protein which responsible for attachment and effacing of host intestinal or urothelial cells(intimate adherence between bacteria and enterocyte membrane)(Monica *et al.*, 2000; Peterfeng *et al.*, 2001).

B. Hemolysin

hemolysin is pore forming toxin, which lyses the cells by creation of pores in the target cell membrane and affect erythrocyte, leukocytes and renal tubular cells. There are three types of hemolysin produced by *E.coli*: extracellular free hemolysin called alpha, cell associated hemolysin called beta and gamma hemolysin produced by strain have the resistance for nalidexic acid antibiotic (Russo *et al.,* 2005).

C. Enterotoxins

Enterotoxins which produced by non invasive enterotoxogenic *E.coli* which has the genes carried on the plasmid encoded for these toxins that include two types heat labile enterotoxins (LT)and heat stable enterotoxins (ST) (Escobar-Paramo *et al.,* 2004).

D. Verotoxins or shiga like toxins

This term is based on the reaction of toxins on vero cells, at least two families of these toxins ST X1 and ST X2, which produced by EHEC strains that have the genes carried on the chromosome encoded for these

toxins, these *E.coli* strains cause spectrum of human illness ranging from uncomplicated diarrhea to hemorrhagic colitis and hemolytic-uremic syndrome (Herbert *et al*., 1999; Johnson *et al*., 2000).

E. Capsules

Capsules represent extra protective barrier which make the host more susceptible and provide the protection of bacteria against desiccations and act as antiphagocytosis, anticomplement and serum resistance (Mark *et al*., 2004).

1.2.6.2 Genus *Proteus*

Enterobacteriaceae is considered as one of the biggest family from the five groups of Bergy's key of classification 1994, *Proteus* is one of important medical genera which return to this family. Bacteria in this genus is Gram negative rod, measuring (1.5-3) μm in length and 0.5 μm in diameter, motile by peritrichous flagella, facutatively anaerobic, non-sporforming, uncapsulated, most isolates having fimbriae, it's oder is very strong (Cruickshank *et al*., 1975).

Proteus applied its name by Hauser in 1885 for their different shapes, from short vegetative swimmer cell to elongated highly flagellated forms referred as swarmer cell. The most important feature which differentiated *Proteus* from other genera in the *Enterobacteriaceae* family is the swarming phenomena (Mobley and Belas, 1995).

The genus *Proteus* has four species which are *P.mirabilis, P.vulgaris, P.penneria* and *P.myxofaciens* (Jawetz *et al*.,1998).

It could be isolated from clinical specimen such as urine,

wound, and blood and it also found in normal flora in the intestine of healthy human, but it is considered as apportunistic pathogens that causing many infection when moves from their normal site (Ananthanarayan and Pariker,1988; Davis *et al*., 1990).

Proteus play a particularly important role in UTIs, which can be subdivided into two categories: haematogenous infections (also known as systemic infections) and ascending infections, in which bacteria colonize, step by step, the introitus, urethra, bladder, ureter, and, in the end, the kidneys, (Rubin *et al*.,1992).

The second type of UTI is more common to *Proteus* strains. *P. mirabilis* most frequently causes UTI in patients with urinary catheters in place or with structural abnormalities, as well as after surgical inter vention in the urinary tract (Penner,1992; Warren, 1996; Rozalski *et al*.,1997).

1.2.6.2.1 Virulence factors of *Proteus mirabilis*

Proteus mirabilis. have many Virulence Factors which help in causing the infections these are:

A. Fimbriae and adherence ability of *Proteus mirabilis*

The ability to adhere to uroepithelium is considered as important virulence factor in *Proteus sp.* (Mobley and Chippendle,1990).

Strain of *Proteus sp.* attached only to sequamous and not to transitional epithelial cell (Eden *et al*.,1980).

B. Swarming of *Proteus mirabilis*

Swarming was also considered as an important phenomenon during life cycle of *Proteus*, it is a cyclical differentiation process in which typical vegetative rods , aseptate filaments that posses up to 50 fold more flagella per unit cell surface area (Liaw *et al*.,2000).

Proteus spp. posses particular problems in the care of patients undergoing long-ter bladder catheterization. Infections with this organism result in the formation of extensive crystalline biofilms on the catheters that can block the flow of urine from the bladder. The crystalline material, composed of magnesium and calcium phosphates, precipitates out of solution under the alkaline conditions generated by the *P. mirabilis* urease enzyme (Hedelin *et al*.,1984; Stickler *et al*., 1993; Morris *et al*.,1997).

The obstruction of the flow of urine through the catheter can induce serious complications. Urine either leaks around the outside of the catheter causing patients to become retained in the bladder resulting in painful distension of the bladder and reflux of urine to the kidneys, which can initiate episodes of pyelonephritis, septicemia, and shock. It has been suggested that the well-known ability of *P. mirabilis* to swarm rapidly over surfaces may play a role in the pathogencity of this species in the catheterized urinary tract. On contact with a solid surface, *P. mirabilis* swimmer cells (1-2) µm in length differentiate into elongated, hyper flagellated swarmer cells (up to 80 µm long), which exhibit increased expression of virulence factors (Allison *et al*.,1994; Belas,1996; Kunin, 1997).

C. Urease production by *Proteus mirabilis*

Urease is an important agent responsible for the pathogenesis of *Proteus* in the kidney, it is a cytoplasmic multimeric, nicked metallo enzyme. This enzyme hydrolyzed urea, which is present in urine generating ammonia and carbon dioxide. Ammonia raises the pH and normally soluble ions precipitated to form stones, usually composed of magnesium ammonium phosphate and calcium phosphate (Mobley and Chippendle, 1990) Catheters are the most commonly deployed prosthetic medical devices. Unfortunately, the care of many patients undergoing long-term catheterization is frequently complicated by infection with *Proteus spp.* these organisms colonize the catheter, forming surface biofilm communities, and their urease activity generates ammonia from urea, elevating the pH of the urine and the biofilm. Under these alkaline conditions, crystals of magnesium ammonium phosphate and calcium phosphate are formed and become trapped in the biofilm. As the biofilm spreads and develops it obstructs the flow of urine through the catheter, causing either incontinence due to leakage of urine around the catheter or retention of urine in the bladder. In the latter case, painful distension of the bladder and reflux of infected urine to the kidneys can cause pyelonephritis and septicaemia (Stickler and Zimakoff, 1994; Kunin, 1997; Morris *et al.*, 1999; Darouiche, 2001).

D. Hemolysin production by *Proteus mirabilis*

Two distinct hemolysins have been found among *proteus* isolate which are HpmA and HlyA (Pablo *et al*., 2003).

The HpmA which is calcium independent hemolytic activity produced by all strains of *P. mirabilis* and most strain of *P. vulgeris*, while HLYA which is calcium dependent hemolysin activity is not found in *P. mirabilis* but it is found in some strains of *P.* vulagaris. In addition, hemolytic strains of *P. mirabilis* are more virulence than non-hemolytic strains, when injected intravenously into mice (Swihart and Welch, 1996; Liaw *et al*., 2000).

E. Production of Protease by *Proteus mirabilis*

Strains belong to *P. mirabilis*, associated with human urinary tract infections have previously been shown to secret an extracellular metaloproteinase which cleave both sub classes of immunoglobulin proteins which are IgA and IgG and also non immunoglobulin proteins such as secretory component casein and bovine serum albumin (Loomes *et al*.,1992; Allison *et al*., 1992).

1.2.6.3 Genus *Klebsiella spp.*

It was first described by the German microbiologist Edwin Klebs in 1885 and it was named on his name.

Klebsiella are belonged to the enterobacteriaceae family, and they are Gram negative rods, non-sporing, non-motile bacilli which tend to be

short and thick, about (0.3-1) μm in diameter and (0.6-6) μm in length. They form a well-defined polysaccharide capsule (Holt *et al*., 1994).

Temperature range for growth is (12-43)^{o}C, optimum 37^{o}C. Most strain ferment lactose and their colonies on MacConkey's medium are pink. They ferment other sugars and producing acid and gas, oxidase negative, catalase positive, not producing H_2S in TSI agar or liquefy gelatin (Holt *et al*.,1994; Collee *et al*.,1996; Abbott,1999).

Members of the *Klebsiella* genus typically express 2 types of antigens on their cell surface. The first is a lipopolysaccharide (O-antigen); the other is a capsular polysaccharide (K- antigen) **(**Ørskov, 1984).The genus was originally divided into 3 main species based on biochemical reactions. 7 species with demonstrated similarities in DNA homology are known. These are (1) *Klebsiella pneumoniae,* (2) *Klebsiella ozaenae,* (3) *Klebsiella rhinoscleromatis,* (4) *Klebsiella oxytoca,* (5) *Klebsiella planticola,* (6) *Klebsiella terrigena,* and (7) *Klebsiella ornithinolytica*. *K pneumoniae* is the most medically important species of the group. *K oxytoca* and *K rhinoscleromatis* have also been demonstrated in human clinical specimens. In recent years, klebsiellae have become important pathogens in nosocomial infections (Sakazaki *et al*.,1989).

1.2.6.3.1 Virulence Factors of *Klebsiella pneumoniae*

A number of bacterial factors that contribute to the pathogenesis of these bacteria. Both *in vitro* and *in vivo* models have been established to

investigate the interaction of bacterial cells and the host. *Klebsiella* has several factors which are:

A.Capsular Antigens

Klebsiellae usually develop prominent capsules composed of complex acidic polysaccharides. Capsules are essential to the virulence of *Klebsiella*. The capsular material forms thick bundles of fibrillous structures covering the bacterial surface in massive layers. This protects the bacterium from phagocytes by polymorphonuclear granulocytes and prevents killing of the bacteria by bactericidal serum factors. Apart from their antiphagocytic function, *Klebsiella* capsule polysaccharides have been reported to inhibit the differentiation and functional capacity of macrophages in vitro (Yokochi *et al.*,1979; Highsmith and Jarvis,1985; Amako *et al.*,1988; Podschun and Ullmann,1992; Podschun *et al.*,1992).

B. Pili (Fimbriae)

As a critical first step in the infectious process, microorganisms must come as close as possible to host mucosal surfaces and maintain this proximity by attaching to the host cell (adherence). The adhesive properties in the *Enterobacteriaceae* are generally mediated by different types of pili. Pili (otherwise known as fimbriae) are filamentous projections on the bacterial surface. These structures are up to 10 µm long and have a diameter of 1 to 11 nm; they consist of polymeric globular protein subunits (pilin) with a molecular mass of 15 to 26 kDa (Ofek and Doyle, 1994). Pili are demonstrated mainly on the basis of their ability to agglutinate erythrocytes of different animal species. Depending on whether the reaction is inhibited by D-mannose, these adhesins are

designated as mannose-sensitive or mannose-resistant hemagglutinins (MSHA and MRHA), respectively (Ottow, 1975).

C. Siderophores

Many bacteria attempt to secure their supply of iron in the host by secreting high-affinity, low-molecular-weight iron chelators, called siderophores that are capable of competitively taking up iron bound to host proteins (Griffiths *et al.*, 1988).

Klebsiella are synthesis two different type of siderophores. This siderophore appears to comprise the main iron uptake system of enterobacteria and is synthesized by almost all clinical isolates of *E. coli* and *Salmonella* spp. (Griffiths, 1987).

1.2.6.4 Genus *Pseudomonas spp*.

Gram negative rods, motile by one polar flagella, aerobic while some can grow unaerobically in the presence of nitrates and using it as an electron acceptor. This bacteria do not ferment sugars but utilize it oxdatively as other organic compounds. Member of this genus are variable in their metabolic activity and widely distributed in water, waste water, crude milk, fishes and poultry. The optimal temperature for the growth of the member of this genus is 37C but it can grow at 42C, and the optimal pH for its growth is between (7.6-7.4) (Collee *et al.*, 1996; Jawetz *et al.*, 1998)

These bacteria are clinically important because they are resistant to most antibiotics and they are capable of surviving in conditions that few other organisms can tolerate. They also produce a slime layer that is resistant to phagocytosis. *Pseudomonas* is often encountered in hospital and clinical work because it is a major cause of hospital acquired

(nosocomal) infections. Its main targets are immunocompromised individuals, burn victims, and individuals on respirators or with indwelling catheters. *P. aeruginosa* is more frequently found in hospital-acquired urinary tract infection due to antibiotics which fervor it's selection in hospital patients. (stolp and Gudkari, 1984; Glauser, 1986).

P. aeruginosa has many virulence factors such as exotoxin which has toxic effect on corneal tissue. Also this bacteria has the ability to produce proteolytic enzymes and hemolysin that destroy cells and tissue (Ijiri *et al*., 1993).

1.2.6.5 *Candida spp.*

The genus *Candida* belongs to the kingdom Fungi, division Eumycota (true fungi), which relates to class Deuteromycetes (fungi imperfectial) and the family Saccharomycetaceae (budding yeast) containing different genera of yeast, the genus of *Candida* which is one of the most common yeast (Hannula, 2000).

Candida is a simple diploid eukaryote organism lacking the sexual cycle. It's cells take different shapes (cocci, ovoid, cylindrical or elongate), which can be staining by Gram's and also by lacto phenol blue staining. Ovoid yeast cell ranging from (3-5) μm in size. *Candida*, which are commonly part of the normal flora of mouth, skin, intestinal tract, and vagina, it is a necessary yeast as part of the normal flora for human health (Lodder, 1974; Barefoot and Klaenhammer; 1983).

Growth requirement of *Candida* are (20-38) C^o within the range of pH (2.5-7.5). The colonies of *Candida* spp. are cream colored to yellowish, which may be pasty, smooth, glistening or dry, wrinkled and dull, depending on the species (Odds, 1988; Larone,1995).

There are many species of the genus *Candida* which cause the disease known as candidiasis, the clinical spectrum of candidiasis is extremely diverse. Almost any organ or system in the body can be affected. Candidiasis may be superficial and local or deep-seated and disseminated infections which arise from haematogenous spread from the primarily infected locus. *Candida albicans* is the most pathogenic and most commonly encountered species among all. Its ability to adhere to host tissues, produce secretory aspartyl proteases and phospholipase enzymes, and transform from yeast to hyphal phase are the major determinants of its pathogencity. *Candida* is a commensal organism found in (40-80) % of normal humans, and is present in the mouth, gut, and vagina. Problems start when a person experiences some alteration in:

- Cellular immunity: e.g. immuno suppression or AIDS
- Normal body flora: e.g. loss of normal bacterial flora due to antibiotic or steroid therapy
- Normal physiology: e.g. cardiac surgery or indwelling catheters (Abi-Said *et al.*,1997).

The genus *Candida* includes around 154 species. Among these, six are most frequently isolated in human infections. While *Candida albicans* is the most abundant and significant species, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, and *Candida lusitaniae* are also isolated as causative agents of *Candida* infections. Importantly, there has been a recent increase in infections due to non-albicans *Candida* spp., such as *Candida glabrata* and *Candida krusei* (Arif *et al.*, 1996).

There are two groups of methods to distinguish *Candida* strains. The first are phenotyping methods, which can be divided into two methods:

a. Biotyping: - based on differences in metabolic properties of yeast isolates. Recently, widely use of commercial test (API 20c) system for assimilation of 19 different carbohydrates.

b. Serotyping:- based on detecting reactions between the antigen and the antibody raised against it. A commercial slide agglutination test uses monospecific antisera raised with the polysaccharide antigens extracted from *Candida albicans.*

The second are genotyping methods, which include different molecular methods for detection of *Candida albicans* like karyotyping, restriction endonuclease analysis (REA), and restricted fragment length polymorphism (RFLP). While, Arbitrarily Primed Polymers Chain Reaction (AP – PCR) is the more recent methods for detection of *Candida albicans* strains (Hannula, 2000).

1.2.6.5.1Virulence factor of *Candida albicans*

Multiple factors have been implicated in the enhancement of *Candida albicans* pathogenicity; these include

A. Phospholipase production

Phospholipase D (PLD) activity is the first enzyme described in *Candida albicans* (CaPLD1). This enzyme was stimulated by inducers of dimorphic transition and an important regulatory to its. Activity of the enzyme is stimulated upon receptor ligation by agonists, resulting in of modification various lipid constituents of the membrane, degradation or phosphorylation (membrane – damaging enzyme), and generation of one or more products that are able to recruit or modulate specific target

proteins. These enzymes were responsible or essential for sporulation of *Candida albicans* (Nealoo and Joseph,1997; Mordechai *et al*.,2000; Bernhard *et al*., 2001).

B. Expression of drug resistance genes

Antifungal drug resistance is quickly becoming a major problem in the expanding population of immunocompromised persons. It has resulted in a drastic increase in the incidence of opportunistic and systemic fungal infections. Clinical resistance is defined as persistence or progression of an infection despite appropriate antimicrobial therapy. Resistance is considered primary when an organism is resistant to the drug before exposure, whereas secondary resistance is that which develops in response to exposure to the drug. Major genes that contribute to drug resistance are those coding for multidrug efflux pumps, the up – regulation of which can result in a multidrug-resistant phenotype. *Candida albicans* possesses different types of efflux pumps: adenosine triphos-phate–binding cassette (ABC) transporters encoded by the CDR genes (CDR1 and CDR2) and major facilitators encoded by the MDR genes (Mary *et al*., 2004).

C. Production of an extracellularly Secreted aspartyl proteinase (Sap)

Secreted aspartyl proteinases (Sap) appear to be a virulence-associated attribute of *Candida* species. These enzymes can cleave several proteins, which are important in host defenses, such as antibodies of both immunoglobulin G and A isotypes. Also, (Sap) may promotes colonization, penetration, and invasion by *Candida albicans*. The

function of (Sap) is through catalyzing the hydrolysis of peptide bonds (CO—NH) in proteins due to degradation of human proteins and structural analysis in determining (Sap) substrate specificity. Less pathogenic or nonpathogenic *Candida* species do not appear to produce significant amounts of these enzymes (Flavia *et al.*, 1995; Julian *et al.*, 2003).

D. Adhesion

Capacity of *Candida albicans* to adhere to many different host tissues is broadly considered a virulence trait to initiate invasive activity. Adhesion, either directly or by inference with the stated growth conditions, that hydrophobic *Candida albicans* cells adhere better and with greater site diversity than hydrophilic cells to endothelial cells, epithelial cells, and other host tissues (Pati *et al.*, 2001).

E. Hyphal formation

Hyphae growth, initiation by germ tube formation which increases adherence properties of yeast. Switching between growth forms is influenced by many factors including temperature, pH, carbon source, nitrogen source, and cell concentration. The ability of the pathogenic fungus *Candida albicans* to switch from yeast to a hyphal morphology in response to external signals is implicated in its pathogenicity. Hyphal cell walls are more adherent than yeast cell walls to human endothelial and epithelial cells and contain slightly more chitin. In addition, true hyphae express a specific constellation of genes. Hyphae are thought to play an important role in pathogenesis because their filamentous growth pattern

can facilitate invasive growth. In addition to their unique morphology, hyphae are further distinguished from budding cells and pseudohyphae by the production of hypha-specific virulence factors. Formation of germ tubes can be triggered by a variety of inducers, including temperature, pH, and serum. Serum has been described as the 'magic potion' for induction of germ – tube formation by *Candida albicans* (Cutler, 1991; Cheryl *et al.*,2001; Andre *et al.*,2002; Eric *et al.*,2002; Stephen and James, 2004; Debbie *et al.*,2004).

1.2.7 Adhesion and Biofilm formation

Bacterial adhesion play very important role in bacterial colonization in most environments and cause infection, because it enable them to colonize environment under condition where they otherwise would be washed away (Mills and Powelson, 1996).

There are two types of bacterial adhesion:-

1.2.7.1 Non- specific adhesion

Non specific adhesion was defined as interaction due to over all macroscopic surface properties as charge or surface free energy. Non specific adhesion is the most common form of adhesion in nature it involves non covalent bonds and hydrophobic interaction, the non covalent bonds which involved electrostatic forces, hydrogen bonds and finally vandervaals forces (Razato *et al.*,1998; Briandet *et al.*,1999).

1.2.7.2 Specific adhesion

Specific adhesion involve a lock and key bond between complementary receptor and adhesin molecule. An adhesin is a protein produced by microorganism and it has complementary structure on the surface of the host cell. Adhesin molecule can easily interact with receptor and not be affected by interfering molecule structure and negatively charged molecules present on the bacterial surface (Ofek and Doyle, 1994; Kuehen *et al.*, 1994; Salyers and Whitt, 1994).

Many member of *Enterobacteriaceae* specially *E.coli* express different types of adhesin like intimin which represent attaching and effacing agent and fimbriae, these fimbriae which involved in the adhesive ability of certain strains and play an active role in the specific adhesion (Graafde and Gastra, 1994; Martindale *et al.*, 2000).

Fimbriae were shown to be fiber like structures (hence their name), made of polymer of polypeptide arranged around a central canal, each bacterial cell posses several hundreds of fimbriae which are spread over the cell and are anchored to the outer membrane, fimbriae have specific receptors on the surface of target cell that attach with it (Reisner *et al.*, 2003)

Although most of time bacteria are free but prefer to make accumulation of it's cells to resist the bad environmental condition, this accumulation is named the adhesion layer or biofilm. The term biofilm use to describe the matrix–enclosed bacterial populations adhere to each other and \or to surface (Davey and O' Toole, 2000; Stoodley *et al.*,2002).

Biofilm formation is favoured under most nutrient –sufficient environments, biofilm structure show extremely complex consisting of microcolonies composed of cells of single or multiple species. Biofilm is

extracellular polymer substances mainly consist of glycoprotein, which contain glucose, galactose, mannose, fructose , raminose and N-acetyle glucose amine (Dolan and costerson,2002; Prigent *et al*.,2001; Reisener *et al*.,2003).

Microcolonies of biofilm are separated by water –filled channels which hypothesized to promote the influx of nutrient, biofilm may form on a wide variety of surfaces including solid moisture surfaces, soft living tissue and medical devices like catheter. Biofilm become hundreds of microns in depth thus are difficult to treat (Danese *et al*.,2002; Nicola and Lazazzera, 2004; Romanova *et al*., 2006).

Flagella, motility and chemotaxis play an important role on the biofilm formation, flagella required for attachment to a biotic surface thus facilitating the initiation of biofilm formation. Motility necessary to enable a bacteria to reach the surface there by facilitating growth and spread of the biofilm, finally that chemotaxis is required for bacteria to swim towards nutrients associated with a surface (Leslie and Kolter,1998; Mcbride,2001).

Biofilm is have an active role in bacterial pathogenesity because the bacteria embedded in a matrix of host proteins and microbial slime, which provide a home for organism and promote increased drug resistance (O'Toole *et al*., 2000 ; Hassett *et al*., 2003; Soto *et al*., 2007).

In the case of catheterized patients , biofilm may contain organisms have the ability to hydrolyze urea in the urine to form free ammonia which rise the pH of the biofilm liquid led to precipitations of minerals such as calcium phosphate, these minerals can become entrapped in the biofilm and cause the encrustation of catheter which become completely blocked. Specific gene that encode and control for biofilm formation in

E.coli and the mutant strain lack the ability for biofilm formation (Stamm *et al*., 1991; Rodney and Donan, 2002).

1.2.8 Antibiotics resistance

Antibiotics are biochemical compounds naturally produced by certain types of microorganism (bacteria and fungi) that inhibit the growth or kill other microorganism (Doi *et al*., 2002).

Different types of antibiotics are discovered, some of them classified as broad spectrum antibiotic which effect on a wide range of bacteria (Gram positive and Gram negative), while others classified as narrow spectrum antibiotics effected on a limited type of microorganism (Hvidberg *et al*., 2000).

Certain types of bacteria are inherently resistant to the effect of particular antibiotic, this is called innate or intrinsic resistance, while resistance of other bacteria to antibiotic types considered as acquired resistance which may result through spontaneous mutation or the acquisition of new genetic information (Greenwood *et al*.,1997; Nester *et al*., 2001).

The resistance of bacteria to particular antibiotic may result from mutation which change the components of bacterial cell or the bacteria may have the plasmid carrying genes encoded for these resistance or by transposons that encoded for resistance and have the ability to transfer to another plasmid which lack to the resistance property, the acquired of resistance between the bacterial cells may result from conjugation , or transformation or transduction (Schlessinger and Eisenstein,1999 ;Hryniewicz *et al*.,2001).

Antibiotic division depends on their chemical structure or mode of action ,some antibiotics effect on the cell wall synthesis like β- lactams and cephlosporins, polymyxins effect on cell membrane integrity(Todar,2002).

Aminoglycosides, tetracycline and macrolides act as protein synthesis inhibitor while quinolones inhibit nucleic acid synthesis (Hooper,2000; Todar,2002; Brooks *et al.*,2004).

The evaluation of bacterial antibiotic resistance depend on many factors such as the type of chemical structure of antibiotic, drug mode of action, period of use, type of bacteria and it's ability to develop it's resistance mechanism against particular antibiotic(Pitout *et al* .,1997).

1.2.9 Role Of Plasmids In Antibiotics Resistance

A plasmid is a DNA molecule separate from the chromosomal DNA and capable of autonomous replication. It is typically circular and double-stranded. It usually occurs in bacteria, sometimes in eukaryotic organisms (e.g in *Saccharomyces cerevisiae*), size of plasmids varies from 1 to over 400 kilobase pairs (kbp) and the molecular weights of plasmids range from about 10^6 dalton for the smallest plasmids to slightly more than 10^8 dalton for the largest one. There may be one copy, for large plasmids, to hundreds of copies of the same plasmid in a single cell, or even thousands of copies, for certain artificial plasmids selected for high copy number. A plasmid contains genes normally not essential for cell growth or survival, some plasmids can integrate into the host genome, be artificially constructed in the laboratory and serve as vectors (carriers) in cloning (Hardy, 1986; Chakrabarty, 1996; Lewin,2000).

Some plasmids are conjucative (transmissible) and others are non conjucative (non transmissible) plasmids.In addition to conjugation, plasmid can be transferred by transformation. Such transfer will increase the chance of spreading the antibiotic resistance between bacterial species, and thus the incidence of bacterial infection will increase also (Satta *et al*., 1987; Davies,1994).

Normally, plasmid are dispensable to its host cell, though many-plasmids contain genes that may be essential in certain environment. Resistance members of enterobacteriacea frequently contain multiple plasmids which carry genes that confer resistance to numerous antibiotics, the larger of which can carry for resistance to 10 or more antimicrobial agents. Environmentally, a cell containing such plasmids had a better chance to survive. For example resistance genes to the drugs tetracycline, penicillin, ampicillin, chloramphenicol, streptomycin, kanamycin and sulfonamide are located on R-plasmids. Plasmid may also encode resistance to wide variety of agents that are toxic to bacteria such as bacteriocins, heavy metals, detergents, serum components and other environmental poisons. These virulence factors have been found in plasmids that also contain drug resistance determinants (Gowal *et al*., 1985; Molnar, 1988; Jacoby and Han, 1996; Jawetz *et al*., 2001).

1. 2.10 Plasmid Curing

In nature plasmid can be lost spontaneously from a very few cells, but the probability of this loss is extremely low, ranging from 10^{-5} - 10^{-7} (Molnar, 1988). However, the majorities of plasmids are extremely

stable, and require the use of curing agents or other procedures that might increase the plasmid loss, and these form the basis of artificial plasmid elimination (Trevores, 1986; Molnar,1988).

Elimination of antibiotic resistance at high frequency is of interest to assert extrachromosomal location of genetic determinants, and obtaining a plasmid-cured derivative will allow a direct comparison to be made between the plasmid-containing and plasmid-cured cells. As a result of earlier studies it is already known that acridine orange, ethidium bromide and sodium dodecyle sulphate (SDS) affect plasmid replication (Tomoeda *et al*.,1968).

Elevated temperature and thymine starvation also affect on plasmid replication (Trevores,1986).

Some antibiotics like rifampicin, chloramphenicol and mitomycin C also have a moderate effect on plasmid replication (Fenwick and Curtiss, 1973). Some tricyclic compounds like promethiazine and impramine were shown to have antiplasmid activity (Molnar *et al*., 1978).

Chapter Two

Materials & Methods

2.1Materials

2.1.1 Apparatus and Equipments

Apparatus	Company/Country
Autoclave	KarlKolb/Germany
Automatic pipettes	Brand/Germany
Auto vortex	Stuart scientific/UK
Balance	Ohaus/France
Compound light microscope	Olympus/Japan
Cooling centrifuge	MLW/Germany
Deep freezer	Sanyo/Japan
Electrophoresis	LKB/Sweden
Hot plate magnetic stirrer	Gallenkamp/England
Incubator	Gallenkamp/England
Micro centrifuge	Eppendorf/Germany
Millipore Filters	Millipore and Whattman/England
Oven	Gallenkamp/England
pH-meter	Gallenkamp/England
portable centrifuge	Gallenkamp/England
Power supply	LKB/Sweden
Sensitive balance	Mettler/Swizerland
Spectrophotometer	Shimadzu/Japan
UV Transillaminator	Biorad
Water bath	Gallenkamp/England

2.1.2 Chemicals

Materials	Company /Country
Absolute ethanol (99%)	Iraq
Acetic acid	BDH/England
Agar –Agar	Himedia /India
Agarose	Sigma /USA
Boric acid	Analar/England
Bromo phenol blue	BDH/England
Chloroform	BDH/England
Corn Meal Infusion	Oxoid/England
Cornmeal agar	Oxoid/England
Crystal violet	BDH/England
Dextrose	BDH/England
Dimethyl- α-naphthylamine	BDH/England
Disodium ethylene diamine tetra-acetic acid	BDH/England
Ethidium bromide	BDH/England
Ethyle alcohol	BDH/England
Glucose	BDH/England
Glycerol	BDH/England

Materials	Company /Country
Hydrogen Peroxide (H_2O_2)	BDH/England
Iodine ,safranine	BDH/England
Isopropanol	BDH/England
Lactose	BDH/England
Phenol red	BDH/England
Sodium chloride (NaCl)	Fluka/Switzerland
Sodium dodecyl sulphate (SDS)	Fluka/Switzerland
Sucrose	Analar/England
Sulfanilic acid	BDH/England
Tween 80	Oxoid/England
Tris-Cl	Fluka/Switzerland
Urea	Himedia/India
Yeast extract	Himedia/India
α – Nephthol	Fluka/Switzerland

2.1.3 Media

2.1.3.1 Ready prepared media

Medium	Company/Origin
Brain heart infusion broth	Difco / U.S.A
Cornmeal agar	Oxoid / England
Eosine methlyine blue	Himedia/ India
MacConkey agar	Himedia/ India
MR-VP Media (methyle red-Voges Proskauer)	Himedia/ India
Muller – Hinton agar	Himedia/ India
Nutrient agar	Himedia/ India
Nutrient broth	Himedia / India
Sabouraud dextrose agar	Oxoid / England
Simmon - citrate agar	Difco / U.S.A
Triple sugar Iron agar (TSI)	Difco / U.S.A
Tryipticase soya broth	Himedia / India
Urea agar base	Difco / U.S.A

They were prepared as recommended by the manufacturing company and sterilized by autoclaving.

2.1.3.2 Laboratory prepared media

2.1.3.2.1 Blood Base Agar (Collee *et al.*,1996).

Ready prepared according to the information of company, pH was adjusted to7 and then steriled by autoclaving at [121^{O}C,15 Ib/in^{2}], previously cooling to 50 ^{O}C then 7% blood was added and mixed well, poured in sterile pettry dishes and saved at 4 ^{O}C.

2.1.3.2.2 Chocolate Agar Medium (Atlas *et al.*, 1995).

It was used for cultivated of fastidious bacteria, and prepared as in item (3.1. 3.2.1) then heating it until it turned to characteristic brown color.

2.1.3.2.3 Peptone Water or Indol Medium (Atlas *et al.*, 1995).

This medium composed of the following (g/L):

Peptone	20gm
Sodium chloride	5gm

pH was adjusted to 7, mixed thoroughly and distributed into tubes and sterilized by autoclaving.

2.1.3.2.4 Urea agar base medium (Collee *et al.*,1996).

Prepared by adding 24 gm of urea agar base to 950 ml of distilled water, adjust pH (6.8- 7.0) and sterile by autoclaving for 10 minutes, then let to cool for 50 °C and added 50 of 20% urea solution which sterile by filtration by (0.22 millipore filter unit), after that put it in sterile tubes to solidify as slants.

2.1.3.2.5 Semi solid agar medium (Collee *et al*., 1996).

It was prepared by dissolving 0.5 % of agar in nutreint broth, the final medium should be quite and transparent, 10 ml were dispensed in test tubes and let to set in the vertical position, it was used for motility test.

2.1.3.2.6 Nitrate medium (Atlas *et al.*, 1995).

This medium composed of peptone 5gm supplémented with 0.2gm KNO_3 in 1L of D.W. distributed into tubes and sterilized by autoclaving.

2.1.3.2.7 Luria – bertani broth medium (Sambrook *et al.*,1989).

This medium composed of :

Trypton	10gm
Yeast extract	5gm
NaCl	5gm
Glucose	1gm

Components were dissolved in 950 ml of D.W., pH was adjusted to 7 then sterilized by autoclaving.

2.1.3.2.8 Sabouraud Dextrose Agar with Antibiotic (SDA) (Jong,1981).

(SDA) contain:

Peptone	10gm
Dextrose	20gm
Agar	15gm
D.W.	100ml

It was prepared by dissolving this compound in D.W. and the pH was adjusted to 7, after autoclaving the medium was supplemented with chloromphenicol antibiotics at concentration of 0.05 mg/ml. This medium was used for primary isolation of dermatophytes and it was used as selective media for dimorphic fungi and dermatophytes.

2.1.3.2.9 Sabouraud Dextrose Broth medium (SDB)

(Buffo *et al.*,1984).

(SDB) contain:

Peptone	10gm
Dextrose	20gm
D.W.	100ml

It was prepared by dissolving this compound in D.W. and the pH was adjusted to 7, then sterilized by autoclaving. This medium was used for growth and identification of fungi.

2.1.3.2.10 Corn –Meal Agar With 80% Tween Medium

(Forbes *et al.*, 1998).

Contain:

Corn-Meal Agar	17gm
Tween	10gm
D.W.	1L

After adjusting the pH to 7, the medium was sterilized by autoclaving. This medium was used for morphological identification of yeast.

2.1.3.2.11 Fermentation Basal Medium (Hassan *et al.*, 2004).

This medium composed of :

Yeast extract	5g
Peptone	15g
Phenol red indicator	10g
D.W.	1L

2% of sugars (glucose, lactose, maltose, and sucrose) were added to fermentation basal media. pH was adjusted to 7 and autoclaved for 10 min. This media for identification of *Candida* spp.

2.1.4 Normal human plasma (Oliver *et al.*,1982).

Human plasma obtained from single donor was taken from blood bank center of Alyarmouk hospital in Baghdad, sterilized by filtration and kept frozen at -20 °C.

2.1.5 Reagents

2.1.5.1 Ready to use reagents

Reagents	Company/Origin
Kovacs reagent	Bio Merieux /France
Oxidase reagent	Bio Merieux /France
Catalase reagent 3% Hydrogen Peroxide(H_2O_2)	Iraq

2.1.5.2 Laboratory prepared indicators

2.1.5.2.1 Methyl red reagent (Collee *et al.*,1996).

Used with methyl red test (production of acid) which prepared by dissolving 0.1 gm of methyl red pigment in 300 ml of ethanol and then added 200 ml of distilled water.

2.1.5.2.2 Vogas- Proskour reagents (Collee *et al.*,1996).

Used with acetone formation test which consist of:-

A- First solution: 40% KOH, prepared by dissolving 40 gm of KOH in 100 ml of Distilled water.

B-Second solution: prepared by dissolving 5 gm of α –Nephthol in 100 ml of Absolute ethanol.

2.1.5.2.3 Nitrate test reagent (Atlas *et al.*,1995).

Used with nitrate test which consist of :-

Solution A: It was prepared by adding 0.8 gm of sulfanilic acid to 100 ml of 5*N* acetic acid.

Solution B: It was prepared by adding 0.5 gm of Dimethyl- α-naphthylamine to100 ml of 5*N* acetic acid.

Equal volumes of solution A and B were immediately mixed before using.

2.1.5.2.4 Phenol red reagent

It was prepared by dissolving 0.1 gm of phenol red in 10 ml of D.W.

2.1.6 Bacterial Strain

Strain	Source
E.coli MM294	Department of biotechnology of AL-Nahrain university.

2.1.7 Antibiotics

A- Antibiotic disks:

The following antibiotic discs were used in this study:

Antibiotics	Abbreviations	Concentration (μg)	Company(origin)
Ampicillin	AM	10	AL-Razzi (Iraq)
Cefotaxime	CTX	30	AL-Razzi (Iraq)
Cephalexin	CL	30	AL-Razzi (Iraq
Chloramphenicol	C	30	AL-Razzi (Iraq)
Ciprofloxacin	CIP	5	AL-Razzi (Iraq)
Gentamycin	CN	10	AL-Razzi (Iraq)
Nalidixic acid	NA	30	AL-Razzi (Iraq)
Penicillin	P	10	AL-Razzi (Iraq)
Rifampicin	RA	5	AL-Razzi (Iraq)
Streptomycin	S	10	AL-Razzi (Iraq)
Tetracycline	TE	30	AL-Razzi (Iraq)
Trimethoprim + Sulfmethaxazol	SXT	1.25 + 3.75	AL-Razzi (Iraq)

B-Antibiotic Powder

Antibiotic	Company
Chloramphenicol	Troge-Germany
Ketazole	Medipharm-Syria

2.1.8 Api -20 E Kit

Api system kit obtained commercially from (Bio Merieux / France)

Which included:-

A- Galleries: The gallery is a plastic stripe with 20 microtubes containing dehydrated reactive ingredients.

B- Api 20 E Reagent:-

1- Oxidase reagent .

2- Kovac's reagent.

3- Voges – proskure reagent.

a- Vp 1 (40 % Pottasium hydroxide).

b- Vp 2 (6 % alpha nephthol).

4- ferric chloride 3.4 %.

2.1.9 Solutions and Buffers

2.1.9.1 Sugar stock solutions

Sugar stock solutions were prepared by dissolving 2 gm of each sugar used (glucose, lactose, maltose and sucrose) in 100 ml of distilled water, then they were sterilized by autoclaving at 121 ^{O}C for 10 min.

2.1.9.2 Physiological saline solution (NaCl) (Atlas *et al.*,1995).

It was prepared by dissolving 8.5gm of NaCl in 950 ml of D.W. After pH was adjusting to 7, it was sterilized by the autoclaving.

3.1.9.3 phosphate buffer saline (PBS) pH=7

(Cruckshank *et al.*,1975).

It was prepared by dissolving 8 gm NaCl, 0.2 gm KCl, 0.2 gm KH_2PO_4 , and 1.15 gm K_2HPO_4 in 950 ml of D.W. pH was adjusted to 7 and sterilized by autoclaving at 121 ºC 15 Ib/in^2 for 15 minutes .

2.1.9.4 Antibiotic Solutions

They were prepared as follows:

1. Ampicillin,gentamicin,cephalexin,chloramphenicol, cefotaxime,pencillin and trimethoprim- sulfmethaxazol were prepared as stock solutions by dissolving 10 mg /ml of antibiotic powders in D.W., sterilized by filtration and stored at -20 ºC**.**

2. Tetracycline was prepared as stock solution of 10 mg /ml of tetracycline hydrochloride in ethanol /water (50% v/v), sterilized by filtration and stored at -20 ºC.

3. Nalidixic acid was prepared by dissolving 0.2g of the antibiotic in 50 ml of D.W., few drops of absolute alcohol and NaOH (0.1M) was added till the antibiotic was completely dissolved, then volume was completed to 100 ml with D.W., and sterilized by filtration.

4. Chloramphenicol stock solution (Al-Jeboury, 2001).

   It was prepared by dissolving 100 mg of chloramphenicol in 10 ml distilled water.

5. Ketazole stock solution

   It was prepared by dissolving 200 mg of Ketazole in 10 ml of D.W. Then the solution was filter-sterilized through 0.22 mm millipore filter papers.

2.1.9.5 DNA Extraction buffers & solutions

2.1.9.5.1 SET buffer (Maniatis *et al*., 1982).

Composed of Sucrose 75 mM, EDTA 25 mM, Tris–Hcl 20 mM, pH was adjusted to 8 and sterilize by autoclave at 121 °C for 15 minutes .

2.1.9.5.2 lysozyme solution. (50 mg / ml) (Maniatis *et al*., 1982).

It was freshly prepared by dissolving (50 mg) of lysozyme in 1ml of sterile distilled water.

2.1.9.5.3 Proteinase K solution (20 mg/ml) (Maniatis *et al.*,1982).

It was prepared by adding (20mg)of proteinase K in 1 ml of sterile D.W.

2.1.9.5.4 Sodium Dodecyl Sulphate Solution (SDS) (100 mg/ml). (Maniatis *et al.*,1982).

Prepared by dissolving 10 gm of SDS in 100 ml of distilled water.

2.1.9.5.5 NaCl Solution. (Maniatis *et al.*, 1982).

It was prepared by dissolving 29.2 gm of NaCl in 800 ml of D.W. complete the volume to 1 liter by D.W., then sterilized by autoclaving.

2.1.9.5.6 TE Buffer (Maniatis *et al.*,1982).

Comprised of Tris –Cl 10 mM and EDTA 1mM, pH was adjusted to 8 and sterilize by autoclave.

2.1.9.5.7 Solution I (Maniatis *et al.*,1982).

Comprised of Glucose 50 mM, Tris.Cl 25 mM (pH = 8), EDTA 10mM, autoclaved for 15 min. at 10 lb/in^2 and stored at 4 °C.

2.1.9.5.8 Solution II (Maniatis *et al.*, 1982).

Comprised of NaOH 0.2 N, SDS 1%, solution II should be made up from stock solution of 10 N NaOH and 20 % SDS.

2.1.9.5.9 Potassium acetate solution (Maniatis *et al.*,1982).

Comprised of Potassium acetate 60 ml (5M), Glacial acetic acid 11.8 ml, H2O 28.5 ml, pH was adjusted to 4.8.

2.1.9.6 Gel Electrophoresis Buffers and Solutions

2.1.9.6.1 TBE Buffer (pH=8) (Maniatis *et al.*, 1982).

Comprised of Tris base 54 gm, Boric acid 27.5 gm, 0.5 M EDTA 20 ml, dissolve in 1000 ml of D.W.

2.1.9.6.2 Gel Loading Buffer (Maniatis *et al.*, 1982).

Comprised of Bromo phenol blue 0.25 % (w/v), Sucrose solution40% (w/v)

2.1.9.6.3 Ethidium Bromide solution (10 mg/ ml) (Maniatis *et al.*, 1982).

It was prepared by dissolving 1gm of ethidium bromide in 100 ml of D.W. and stirred on magnetic stirrer for few hours to ensure that the ethidium bromide has been dissolved then was filtered, and stored in dark bottle, ethidium bromide is powerful mutagen, gloves and masks were worn during weighting and through all steps of handling.

2.1.9.7 DNA Marker (1kb ladder/ Biron).

One kb DNA ladder is a convenient marker for determining the size of double –stranded DNA .The ladder consist of 13 fragment that range in size from 250 to 10000 base pair.

2.2 Methods

2.2.1Sterilization methods

2.2.1.1 Moist heat sterilization

Autoclave was used to sterilize media, buffers and solutions at 121°C for (15 Ib/ in^2**)** for 15 minutes**.**

2.2.1.2 Dry heat sterilization

Electric oven was used to sterilize glass wares and others by heating at 180 °C for 2 hours.

2.2.1.3 Filteration (membrane sterilization)

Millipore filter unit was used to sterilize the sugars, urea, antifungal drug and crystal violet after dissolving in D.W.

2.2.2 Collection of samples

Urine samples from catheter were collected in sterile swabs containing 5 ml of normal saline from patients of (Al- Yarmouq hospital , The centeral child hospital , Al-Kathmya hospital and Al- Alwya hospital) during the period from Oct.-1-2005 to Jun.-1 -2006.

A total of 350 samples were collected and transported to the laboratory during 1 hour by using a cool box because (low temperature serves to inhibit bacterial and yeast replication in the urine sample until processed in laboratory). This important because the number of bacteria in the urine sample is important in determining if there is clinically significant bacteriuria, if the sample is not properly stored, small number contaminating bacteria may multiply to large numbers and create a false impression of significant bacteriuria.

2.2.3 Isolation of bacteria

Swab was streaked on nutrient agar, blood agar, macConkey agar plates and SDA. Plates then were incubated over night at 37°C.

2.2.4 Identification of Isolates

2.2.4.1 Identification of bacteria

2.2.4.1.1 Culture Characteristics

Bacterial isolates were studied depending on the colony size, shape, edge, color, and odor (Atlas *et al.*, 1995).

2.2.4.1.2Morphological Characteristics

The second step in the identification of bacteria was done by Gram stain. A loopfull of bacteria suspension was fixed on a slide, and then stained by Gram stain to examine Gram reaction, shape, spore forming and capsule of isolated bacteria (Atlas *et al.*,1995).

2.2.4.1.3Biochemical Tests

Biochemical tests which are more specific in identification of bacteria that include many tests:

A. Catalase test (Atlas *et al.*,1995).

A single colony was placed onto a clean glass microscope slide with a sterile toothpick, then a drop of hydrogen peroxide (3%) was placed onto the colony. The production of gaseous bubbles indicates the presence of catalase.

B. Oxidase test (Atlas *et al.*, 1995).

This test was done by using filter paper with a few drops of oxidase reagent (tetramethyl-p-phenylene diamine dihydro-chloride). A clump of cells was picked up from the slant growth with sterile wooden stick and smeared on filter paper. The development of a violet purple color within 5 -10 seconds indicate a positive result.

C. Indole test (Collee *et al.*, 1996).

The peptone water was inoculated by isolate and incubated at 37 °C for 24 hr, then added 0.05 ml of Kovac's reagent and mixed gently . Positive result was recorded by the appearance of pink ring on the surface of broth. This test demonstrates the ability of bacteria to decompose the amino acid tryptophan to indol which accumulate in the medium.

D. Methyle red test (Collee *et al.*,1996).

Test tubes containing MR-VP broth were inoculated with single colony, and incubated at 37 °C for 48h. Then 5 drops of methyl red indicator was added to each tube and mixed. Positive results are bright red and negative are yellow. This test is employed to detect the production of sufficient acid during the fermentation of glucose.

E. Voges-Proskauer test (Collee *et al.*,1996).

Test tubes MR-VP broth was inoculcated with single colony of each isolate, and incubated at 37 °C for 48h. Then 1 ml of VP1 and 3 ml of VP2 was added to 5 ml of cultured broth and shacked for 30 seconds. The formation of pink to red color indicates a positive result. This test is employed to detect the production of acetoin.

F. Citrate utilization test (Atlas *et al*., 1995).

Simmon citrate agar was inoculated by bacteria and incubated at 37 ºC for 24 hr , changing the color of media from green to blue indicate a positive result while the green color indicates a negative result. This test to detect the ability of bacteria to utilize citrate as the sole of carbon and energy source for growth and an ammonium salts as the source of nitrogen.

G. Urease test (Atlas *et al*., 1995).

This test to examine the presence of urease enzyme. Urease production detected by inoculating the surface of Christensen urea agar slants with bacteria and incubate at 37 ºC for 24 hr, after incubation the appearance of pink color mean a positive result while yellow color mean a negative result.

H. Triple Sugar Iron Test (TSI test) (Atlas *et al.*,1995).

Test tubes containing triple sugar iron (TSI) agar slant were inoculated by stabbing with single colonies, and incubated at 37˚C. Results are as following:

Color	pH :Slant/Bottom	Result Utilization
Red / Yellow	Alkaline / Acid	Glucose only fermented Peptones utilization
Yellow / Yellow	Acid / Acid	Glucose fermented; lactose and /or sucrose fermented
Red /Red	Alkaline / Alkaline	No fermentation of glucose, lactose or

		sucrose /Peptones utilization

While the formation of black precipitated indicates H_2S production and pushing agar to the top indicates CO_2 formation.

I. Motility test (Collee *et al.*,1996)

Test tubes containing semisolid agar media, were inoculated with single colony with a straight wire, making a single stab down the center of the tube to about half depth of the medium incubated at 37 ˚C for 24- 28 hrs, motile bacteria was swarmed, it was easily recognized by the naked eye. Motile bacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque. 0.5 % agar dissolved in nutrient broth. It is important that the final medium should be quite clear and transparent.

J. Nitrate reduction test (Atlas *et al.*,1995).

Test tubes with nitrate media 5ml were inoculated with bacterial culture. All tubes were incubated at 37 ^{O}C for 24 hr. After incubation, 0.1ml of the test reagent was added to each tube. The immediate formation of red color indicates positive results; that nitrate reduced to nitrite.

2.2.4.1.4 Identification of bacteria by Api-20E Kit

Identification of isolates was carried out by sub-culturing representive colonies from MacConkey agar plates on api-20E microtubes systems. This system is designed for the performance of more than 20 standard biochemical tests from a single colony on plate medium. Each test in this system is performed within a sterile plastic microtube

which contain the appropriate substrate and affixed to an impermeable plastic strip (gallery). Each gallery contains 20 microtubes (each of which consists of a tube and a couple selection).

2.2.4.2 Isolation and Identification of Yeast (*Candida spp.*)

2.2.4.2.1**Morphology of colony** (Savage and Balish, 1971).

After cultivation of suspected isolates on SDA and incubated at 37 °C for 48 hr, morphology of colonies was examined and described.

2.2.4.2.2**Microscopic examination** (Kreger-van, 1984).

Part of the colony was fixed on a glass slide, stained by Gram's stain, then cell were examined under oil-immersion lens of a compound light microscope.

2.2.4.2.3 **Surface growth** (Forbes *et al.*, 1998).

This test was used for detection the ability of *Candida* spp. to grow on surface of SDB by transferring part of a colony to tubes contained this medium. After incubation at 37°C for (24–72) hrs. Presence of gas bubbles (Co_2) was indicated a positive result.

2.2.4.2.4 **Germ tube formation test** (Evans and Richardson,1989).

This test was used for detection the ability of *Candida* spp. to form germ tube. Formation of germ tube was examined by adding 1 ml of human serum in a sterile tube and re–suspended with part of colony then incubated at 37 °C for (2 – 4) hrs. After incubation, a drop from suspension was put by a pasture pipette on a slide covered with cover

slipe. Result was recorded under light microscope by observed formation of germ tube.

2.2.4.2.5 Sugar fermentation test (Forbes *et al.*,1998).

This test was used for detection the ability of *Candida* spp. to ferment of certain sugar (glucose, sucrose, lactose and maltose) and production of CO_2. *Candida* isolates were incubated in fermentation basal media contained 2% of each sugar in test tubes at 37^{o}C for (48-72) hrs. The positive result was recorded by changing red color to yellow and production of CO_2 gas bubbles, in comparison with control.

2.2.4.2.6 Chlamydospore formation test (Barnett *et al.*, 2000).

This test was used for detection and identification of *Candida albicans*. The isolates *Candida* were cultured on Corn – meal agar with tween 80 and incubated at 37 ^{0}C for (5 – 7) days. Round blastoconidia bunched together with pseudohyphae or true hyphea were observed under light microscope.

2.2.5 Maintenance of isolates

2.2.5.1 Maintenance of bacterial isolates

Maintenance of bacterial strains was performed according to (Maniatis *et al*., 1982) as follow:

A- Short- Term storage (few weeks)

Bacterial isolates were maintained for few weeks on nutrient agar plates; the plates were wrapped tightly with parafilm, and then stored at 4 ^{o}C.

B-Medium-Term storage (1-3months)

Bacterial isolates were maintained for few months by stabbing nutrient agar slants in screw-capped tubes containing 5-8 ml of nutrient agar medium and incubated at 37 ºC for 24hr. then stored at 4 ºC.

C- Long Term storage

Single colony were cultured in nutrient broth and incubated at 37 ºC for 24 hr, and then 8.5 ml of cell suspension was mixed with 1.5 ml (15%) of glycerol, and stored for long time at -20 ºC.

2.2.5.2 Maintenance of *Candida* Isolates

The isolates were inoculated on SDA slants contained 0.5mg/ml chloromphenicol. After incubation at 37^0C for 48 hrs, the slants placed in 4^0C as stock culture. These slants were recultured every three months (Oliver *et al.*, 1982).

2.2.6 Detection the ability of bacteria and *Candida* for biofilm formation (Test tube Method) (Christensen *et al.*, 1982).

By using Christensen method for detection the ability of bacterial isolates and Candida isolates for biofilm formation, this method included inoculation 5 ml of (Trypton soya broth) with particular isolates and incubated for 48 hours at 37 ºC, after that, the contents of the tubes were removed carefully and added the crystal violet stain (1%) to each tube for 15 minutes then rinsed the tubes and let tubes to dry at room temperature (20-25)ºC. The result was read by notice the formation of biofilm as a layer at the internal wall of tubes by naked eye and comprise with the

negative control (tube contains Tsb medium without inoculation), thickness and color of layer consider a parameter of isolates (bacteria and *Candida*) ability for biofilm formation.

2.2.7 Antibiotic sensitivity test of bacteria isolates (Atlas *et al*., 1995).

Sensitivity of bacterial isolates (*E. coli, Proteus mirabilis, Klebsiella pneumonia* and *Pseudomonas aeruginosa*) were tested toward antibiotics (Ampicillin (AM), Cefotaxime (CTX), Cephalexin (CL), Chloramphenicol(C), Ciprofloxacin (CIP), Gentamicin (CN), Nalidixic acid (NA), Pencillin (P), Rifampicin (RA), Strteptomycin (S), Tetracycline (TE), Trimethoprim- Sulfmethaxazol (SXT)) by using the modified disc diffusion method. Ten ml of nutrient broth medium was inoculated with bacterial isolate, and incubated at 37 ºC for 18 hours, transfer 0.1 ml (1.5×10^{8}cell/ ml) of freshly broth(Growth of bacteria was monitored by McFarland tube No. 5 turbidity standard, which as equivalent to bacterial concentration for inoculum 1.5×10^{8} organism / ml) to Mullar–Hinton agar plate and streaked by sterile cotton swab three times by rotating the plate approximately 60 mm between streaking to ensure even distribution of the inoculum, the inoculated plates were placed at room temperature for 10 minutes to allow absorption of excess moisture, then antibiotic disks were applied by sterile forceps on the surface of plates and incubated at 37 ºC for 18 hours in an inverted position .

After incubation, measured the diameter of inhibition zone (clear area around disks) by ruler which indicate the sensitivity of bacteria to that antibiotic and the result were compared with NCCLs (1994).

2.2.8 Sensitivity of *Candida albicans* isolates to antifungal agent and to determination of minimum inhibition concentration (MIC) (Santos and Hamdon, 2004).

Ten ml of SDB were inoculated by 33 isolates of *Candida albicans* and incubated at 37 °C for (24 – 48) hrs. After incubation period, their stationary phase was determined by spectrophotometer 20 through estimating the optical density (O.D. 490 nm) in range from (1.0 to 1.2). Then serial dilutions were made to obtain 10^5 cell/ml. In other hand, Ketazole was diluted to prepared different concentration (fold dilution) which were (4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048) µg/ml**.** All these dilution were made in tubes containing SDB at final volume 5ml in each tube. 0.05ml SDB of 10^5 cell/ml were added to each tube, then incubated at 37°C for 48 hrs. The result was recorded by determined the activity of antifungal drugs on *Candida* isolates. The suitable concentration was cultured on SDA. After incubation period 48 hrs at 37°C, the result was recorded and compared with control (SDB with *Candida* isolates) to determine the minimum inhibition concentration of antifungal.

2.2.9 Extraction of DNA

DNA extraction was done for(5isolates which gave the highest resistance for antibiotic) by salting out method which describe by Pospiech and Neumann(1995).The isolates named *E.coli* (RS1), *P. mirabilis* (RS2), *K. pneumonia* (RS3) *and P. aeruginosa* (RS4) and *C. albicans* (C13).

- culture of bacteria and yeast in nutrient broth(for bacteria) and SDB(for yeast) at 37 ºC for 18 hours , which was pelleted from 10 ml by centrifugation at 6000 rpm for 15 minutes.

- The pellet was washed with 3 ml of SET buffer and resuspended the cells with 5 ml SET buffer, then added 0.1 ml freshly prepared lysozym (final concentration 1 mg/ ml) was added and incubated at 37 ºC for 30 minutes
- Added 0.14 ml of proteinase K solution, mix 0.6 ml of 10 % SDS mix by inversion and then incubated 2 hours at 55 ºC invert occasionally.
- Added 2 ml of Nacl 5M, mix thoroughly by inversion, let cool to 37 ºC .
- Added chloroform, mix by inversion for 30 minutes, centrifugation (6000 rpm) for 15 minutes.
- The aqueous phase (upper) was transferred to another sterile test tube and 0.6 ml of DNA volume of isopropanol was added , mixed by inversion and kept at room temperature (20-25)ºCfor 5 minutes.
- Isopropanol was discarded and rinse DNA in 0.5 ml of 70 % ethanol, dry and then dissolve in 2 ml of TE buffer.

2.2.10 Plasmid DNA isolation

Plasmid DNA isolated according to method described by (Maniatis *et al*., 1982) and including the following steps:

- Resuspend the bacterial pellet (4 isolates) from a 50 ml culture in 1 ml of solution I containing 5 mg/ml lysozyme (powdered lysozyme should be dissolved in the solution just before use). Let stand at room temperature for 5 min.

- Then add 2 ml of freshly made solution II. Cover the top of the tube with parafilm and mix the contents by gently inverting the tube several times. let stand on ice for 10 min.

- Then add 1.5 ml of an ice–cold solution of a 5 M potassium acetate (pH 4.8), cover the top of the tube with parafilm and mix the contents by inverting the tube sharply several times. leave the tube to stand on ice for 10 min.

- Then centrifuge at 20000 rpm for 20 min at 4 Cº. The cell DNA and bacterial debris should form a tight pellet on the bottom of the tube .

- Then transfer the supernatants to another tube and add 0.6 volume of isopropanol to the tube. mix well and let stand at room temperature for15 min.

- Then recover the DNA by centrifugation at 12000 rpm for 30 min. at room temperature. (salt may precipitate if centrifugation is carried out at 4 ºC).

- Then discard the supernatant. Wash the pellet with 70% ethanol at room temperature discard as much ethanol as possible, then let it to dry.

- Then dissolve the pellet in a total volume of 0.8 ml of TE (pH = 8).

2.2.11 Spectrophotometer Determination of DNA

Ten microliters of each DNA sample were added to 490 micro liter of distilled water and mixed well. A spectrophotometer was used to measure the optical density (O. D.) at wave length of 260 nm and 280 nm. An O.D. of 1 corresponds to approximately 50 mg/ml for double stranded DNA. The concentration of DNA was calculated according to formula described below (Sambrook *et al*., 1989).

DNA concentration (mg/ml) = O.D 260 nm X 50X Dilution factor

2.2.12 Agarose Gel Electrophoreses (Sambrook *et al*., 1989)

Agarose gels was prepared by dissolving 0.7 gm of agarose in 100 ml of TBE buffer 1X, and heated on hot plate till all agarose crystals were dissolved. After cooling to 50 ºC it was poured gently in the apparatus tray and cooled to 25 ºC. Then the tray containing agarose gel was transferred and immersed in apparatus tank containing TBE buffers solution.

Samples of DNA were mixed with 1/10 volume of the loading buffer and added to the wells in gel. Generally, gels were run for 6 hrs. at 5 volt/cm and the gel buffer added up to the level of horizontal gel surface. Agarose gel was then stained by immersing in 0.5 µg/ml ethidium bromide for (30-45) minutes.

DNA bands were visualized by UV-illumination at 302 nm on an UV-transilluminator. Gels were distained in distilled water for (30-60) minutes to gel ride of background before photographs were taken.

2.2.13 Curing of Plasmid DNA

Curing experiment was performed by using Ethidium bromid according to Trevors, (1986) and Salzono *et al*., (1992) and as fallows:

A- Solutions

10 mg/ml of Ethidium bromid stock solution was prepared by dissolving 0.2g of Ethidium bromid in (20) ml of D.W. stock solution will sterilized by filtration .

B-Procedure

- Single colony of the locally isolated bacteria (RS1, RS2, RS3and RS4) was used to inoculate 5 ml of BHI broth and incubated at 37 ºC for 3.5 hours. Then 0.1 ml a liquates was taken from the growth culture and used to inoculate universal tubes containing 5 ml of BHI broth and specific concentration of ethidium bromide solution in each tube (0,20,50,100,200,250,300,400,800,1000 and 1600 µg/ml).All the tubes were incubated with shaking (150 rpm) at 37 ºC for 24 hours.

- The growth density of different universals was measured visually and compared with the control to determine effect of each curing agent on bacterial growth.

- The lowest concentration of each curing agent that inhibited bacterial growth was considered as the minimum inhibitory concentration (MIC).

- Samples were taken from universals containing the highest concentration of each curing agent that still allows bacterial growth, which is known as (sub lethal concentration).

- Serial dilutions from the tubes containing growth and sub-lethal concentration of ethidium bromide was achieved, then 0.1 ml of 10^{-8} dilution was spread on nutrient agar plates, and incubated at 37 ºC for 24 hours.

2.2.14 Selective of Cured Cell (Trevors, 1986).

After treatment of bacterial isolates (RS1, RS2, RS3 and RS4) with curing agent, 100 colony that still able to grow on nutrient agar were selected randomly and were replica plated (using tooth pick) on nutrient agar plates containing the antibiotic to which the wild isolate was resist. Plates then incubated at 37 ºC for 24 hours, the resultant colonies may by arise

from cured cells. If a colony was able to grow on the master plate but not on the selective agar containing the appropriate antibiotic, it means that the cells of this colony were may be cured cells that lost the resistance to this antibiotic. The percentage of cured cells was determined.

Chapter Three

Results & Discussion

3.1 Isolation and identification of microbial isolates

Three hundred and fifty urine samples were collected from catheterized patients (the swab obtained from connection region of this device) in hospitals which suffered from complicated UTI in Al-kadhumia hospital, Al- Yarmook hospital , Al- Alwya hospital and Centeral child hospital during the period (from 1st- Oct. -2005 to1st- Jun. -2006).

Urine samples (swabs) were cultivated on common and selective media (for each suspected bacterial and yeast genus or species). Then all isolates were identified using cultural ,morphological and biochemocal tests.

Cultural examination showed that colonies of the bacterial species, after incubation on different media at 37 °C for 24 hrs, were (lactose fermenter, flat, dry, pink and non viscous colonies) for *E.coli*, (non lactose fermenter, small, pale colonies swarming motility after 42 hr of incubation) for *Proteus spp.*, (lactose fermenter, mucoid, large, pink and viscous colonies) for *Klebsiella spp.*, (non lactose fermenter, pale colonies small colonies ,greenish pigment) for *Pseudomonas spp.*,(lactose fermenter, raised, pink colonies) for *Enterobacter spp.*, (medium to large, smooth, entire slightly, raised translucent; they are yellow to creamy colonies and beta hemolytic) for *Staphylococcus spp.*, and each of *Citrobacter spp.*, *Kluvyra spp* and *Pantoea spp.* are lactose fermenter and simellar to *E. coli* in morphological characteristic.

Moreover, under the oil immersion objective of the compound light microscope, cells of the suspected bacterial isolates were all gram negative and bacilli except *Staphylococcus spp.* isolates were gram positive and cocci.

Cultural characteristic of the yeast isolates showed that their colonies appeared on SDA as white to creamy, glossy, smooth, soft and circular. Such characteristics come in accordance with those belonged to genus *Candida*.

After Gram staining of smears obtained from colonies of isolates (36) grown on SDA, cells appeared under the oil-immersion objective as violet, oval having short extension shape, budding and spore former.

Results showed, 339 (96.857%) isolates [303 (89.380%) bacteria and 36 (10.619%) yeast] obtained from catheterized patients, while 11(3.142%) samples were showed no growth of microorganisms from total samples (350) figure (3-1).

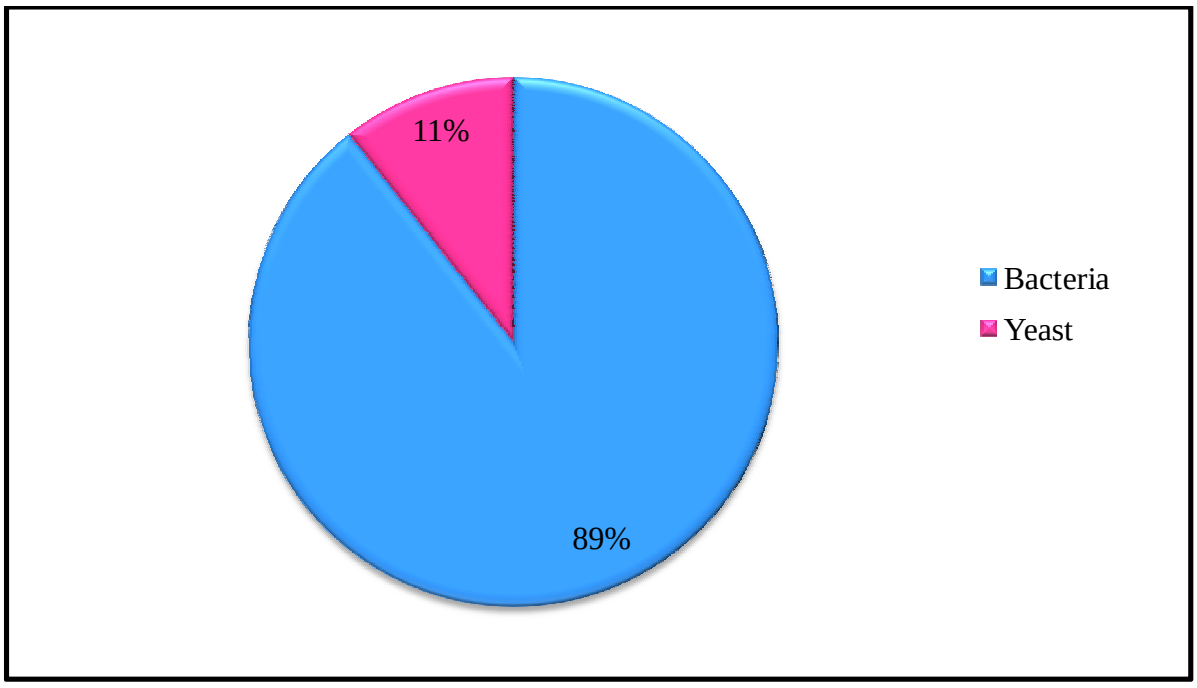


Fig (3-1) percentage of bacteria and yeast isolated from catheterized patients.

Further biochemical tests illustrate in table (3-1) and api 20-E system identification showed that 9(2.57%) isolates were identified as *E.coli,* 109(31.14%) isolates as *Proteus mirabilis*, 23(6.57%) isolates as *Proteus vulgaris* , 74(21.14%) isolates as *Klebsiella pneumonia*, 39(11.14%) isolates as *Pseudomonas aeruginosa*, 16(4.57%) isolates as *Enterobacter spp*., 16(4.57%) isolates as *Staphylococcus spp*., 4(1.14%) isolates as *Citrobacter spp*., 7(2.0%) isolates as *Kluvyra spp*., 6(1.71%) isolates as *Pantoea spp*. and 36(10.28%) samples showed yeast isolates belonged to *Candida spp.*,which 33 (9.42%) isolates belonged to *Candida albicans* .

Results of biochemical tests and morphological tests of *Candida* isolates illustrated in table (3-3) which included (surface growth on SDB, formation of germ tube when cultured in human serum for 2-4 hrs., chlamydospore formation and sugar fermentation). Depending on such characteristics, and in accordance with Ping (2002), such results are usually related to the yeast *Candida albicans* that represented 33 (9.42%) out of 36 isolates.

Table (3-1) The percentage of different microorganisms isolated from urine of catheterized patients.

Isolates		Urine sample from catheterized patients	
		No.of Isolates	Percentage %
1-	*E.coli*	9	2.57
2-	*Proteus mirabilis*	109	31.14
3-	*Proteus vulgaris*	23	6.57
4-	*Klebsiella pneumonia*	74	21.14
5-	*Pseudomonas aeruginosa*	39	11.14
6-	*Enterobacter spp.*	16	4.57
7-	*Staphylococcus aureus* (G+ve)	16	4.57
8-	*Citrobacter spp.*	4	1.14
9-	*Kluvyra spp.*	7	2.00
10-	*Pantoea spp.*	6	1.71
11-	*Candida spp.*(*Candida glabrata and Candida tropicalis*)	3	0.86
12-	*Candida albicans*	33	9.42
13-	No growth	11	3.14
14-	Total	350	100

Table (3-2) Biochemical characteristics of bacterial isolates.

Test		*E coli*	*Klebsiella pnemoniae*	*Enterobacter spp.*	*Pseudomonas aeruginosa*	*Proteus mirabilis*	*Proteus vulgaris*	*Citrobacter*	*Kluvyra*	*Pantoea*	*Staphylococcus aureus*
Indol production		+	_	_	_	_	+	+	+	_	_
Methyl red		+	_	_	_	+	+	+	+	ND	ND
Vogas–proskuar (VP)		_	+	+	_	ND	_	_	_	+	+
Citrate(Simmon)		_	+	+	ND	ND	_	+	+	+	ND
Catalase		+	+	+	+	+	+	+	+	+	+
Oxidase		_	_	_	+	_	_	_	_	_	_
Urease		-	+	-	ND	+	+	ND	_	_	ND
Motility		+	_	+	+	+	+	+	+	+	_
Nitrate reduction		+	+	+	ND	+	+	+	+	+	ND
TSI	H2S	_	_	_	+	+	+	_	_	_	ND
	CO2	+	+	ND	+	+	+	ND	ND	ND	ND
	Acid	A/A	A/A	ND	Alk/A	Alk/A	Alk/ A	ND	ND	ND	ND

(+): positive result. (−): negative result. (ND): not determined. (Alk): alkaline reaction. (A): acid reaction.

Table (3-3) Morphological characteristics and biochemical tests of *Candida* isolates.

Test	*C. albicans*	*C. glabrata*	*C. tropicalis*
Morphology	White to creamy, glossy, smooth, soft and circular	small, pasty, white to cream in color and glistening	cream-colored with a slightly mycelial border
Surface growth	–	–	+
Germ tube	+	–	–
Clamydospore	+	–	–
Glugose fermentation	+	+	+
Lactose fermentation	–	–	–
Maltose fermentation	+	–	+
sucrose fermentation	–	–	+

Complicated UTIs which caused by bacteria but they occur as a result of some anatomical or structural abnormality. Often associated with catheter use in the hospital setting like bladder and kidney dysfunction, or kidney transplant prostates enlargement . The common features in most complicated UTIs is the inability of the urinary tract to clear out bacteria because of a physical condition that causes obstruction to the flow of urine or problems that hinder treatment success.

Proteus and *Klebsiella* represent the main causative agent of complicated UTIs in collected samples. This is probably because *Proteus* is swarming and *Klebsiella* forming capsule and each of them were able to produce a potent urease which acts on urea to produce ammonia, rendering the urine alkaline.

These result was near that of Warren (1996) who found that *proteus* and *Klebsiella* isolates were representing (54.5%) and (20%) of UTIs in catheterized patients respectively.

Although many reports were pointed that *E.coli* represent important uropathogene for complicated UTIs of catheterized patients but result of this study showed low frequency because these samples obtained from patients posses catheter (3-5 days) and the *E.coli* bacteria prefers long term catheterized urinary tract or the patients may obtained a high dose of antibiotic inhibit *E.coli* before and after using of catheter. This result were near with recently Iraqi study by Al-Chalabi (2007).

Enterobacter and *pseudomonas* represent the second causative agents of UTIs of catheterized patients because each of them are more frequently found in hospital acquired UTI due to their resistance to antibiotics favors their selection in hospital patients (Pitout *et al.*, 2005).

Other species of bacteria may contributed in contamination of catheter and led to infection but with low frequency like *Citrobacter spp.*, *Kluvyra spp.*, *Pantoea spp.* (Mims *et al.*, 1987; Anderson *et al.*, 2004).

Gram positive bacteria are known to be involved in UTI of catheterized patients like *Staphylococcus spp.* (Oni *et al.*, 2003).

Other microorganism like fungi can cause UTIs in catheterized patients was *Candida albicans* because due to their ability to adhere to host tissues, produce secretory aspartyl proteases and phospholipase enzymes, and transform from yeast to hyphal phase which initiation by germ tube formation, these are the major determinants of its pathogenicity (Kauffman *et al.*, 2000).

It has been observed that nosocomial urinary tract infections have increased in the two last decades, probably due to many predisposing factors associated with occurrence of candiduria. Several risk factors such as use of indwelling urinary devices, diabetes mellitus, antibiotic use, immunosuppressive therapy, extended hospitalization, extremes of age and female sex have been identified as associated with increase of *Candida* growth in urine .Although, non-*albicans* species as *C. tropicalis* and *C. glabrata* have been frequently found in these catheterized patients, *C. albicans* is still the most frequent yeast recovered from urine cultures. Many risk factors may favor or contribute to patient colonization. Some investigators have showed that use of antibiotics and prolonged hospital permanence represent important risk factors for the development of microorganism (Kauffman *et al.*, 2000; Nucci, 2000; Blumberg *et al.*, 2001; Sobel, 2002; Simpson *et al.*, 2004).

Prolonged antibiotic or steroid therapy destroys the balance of normal flora in the intestine allowing the endogenous Candida to

overcome the host. Invasive procedures, such as cardiac surgery and indwelling catheters, produce alterations in host physiology and some of these patients develop Candida infections (candidiasis). These drugs play a critical role in the pathogenesis of candiduria which emerges during antibiotic face fungal colonization, with ready access to the urinary tract especially in the presence of indwelling catheter (Fisher *et al.,* 1982, Sobel & Vazquez 1999; Álvarez-Lerma *et al.* 2005) showed that in most cases of candiduria the patient had a urinary catheter (92.6%). Similar results have been obtained by (Kobayashi *et al.*;2004) who verified that 84.4% of the patients with candiduria had a catheter. According to Klotz and Smith (1990), yeasts are able to adhere to the catheter allowing colonization in this device. Although infection by yeasts in patients with indwelling urinary catheter has not been well defined till now, use of invasive procedures has been reported as an important factor for development of *Candida spp*. infection (Álvarez-Lerma *et al.*, 2005).

3.2 Prevalence of UTIs among gender of catheterized patients

Out of the 339 positive cases, 221(63.138%) were from female patients while 118 (33.711%) of the cases were from the male (figure 3-2). It is known that incidence of UTIs is generally higher in females than in males worldwide for several reasons like the shorter female urethra is less effective deterrent to infection than the male urethra, sexual intercourse facilitates the movement of microorganisms up the urethra particularly in female, so that the incidence of UTIs is higher among sexually active than celibate women. However the antibacterial properties of prostatic fluid may also account for increased resistance to UTI observed in men. In male infants UTIs are more

common in the circumcised and this is associated with colonization of the inside of prepuce and urethra with fecal organisms (Glauser, 1986; Mims *et al*., 1987).

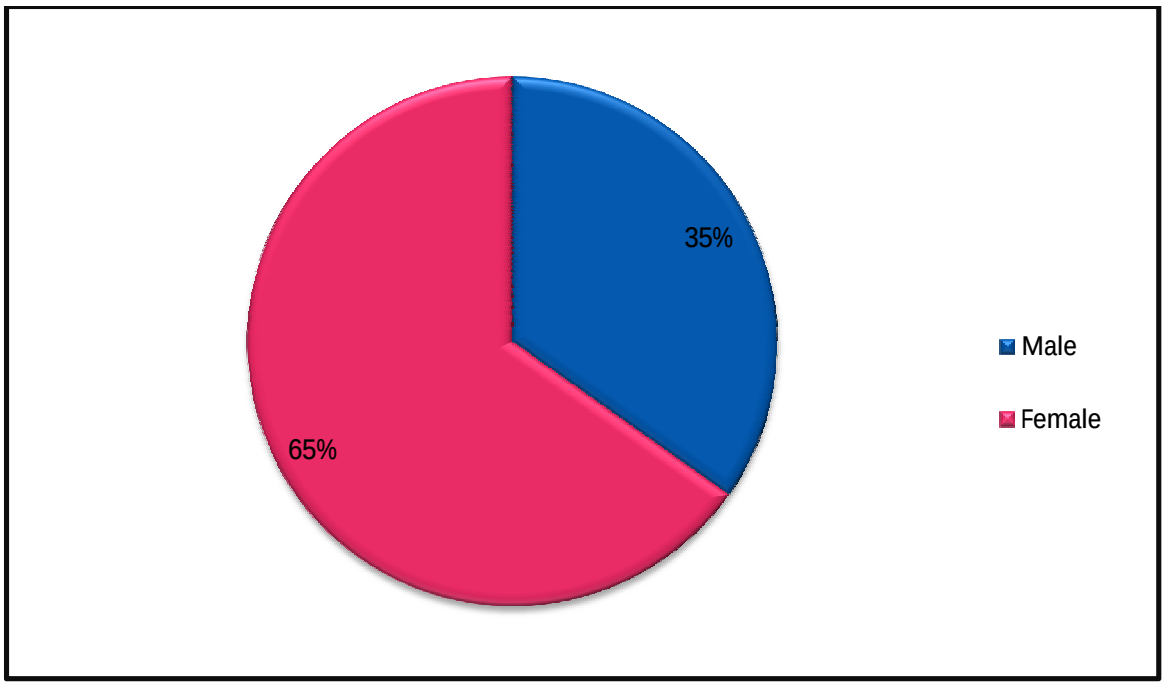


Fig (3-2) Prevalence of UTIs among gender of catheterized patients.

3.3 biofilm formation

Biofilms formation play an important role in the pathogenesis, persistence, and eventual treatment of UTIs (Mireles *et al.*, 2001).

Test tube method used to detect ability of pathogenic bacteria and yeast isolates (*E. coli, P. mirabilis, P. vulgaris, K. pneumonia, P. aeruginosa. and C. albicans*) which isolated from catheterized patients for biofilm formation fig (3-3).

Fig (3-3) Biofilm formation by isolates in test tubes method.

[(1)Control (nutrient broth only). (2)*P. aeruginosa* (3) *E coli*(4) *C. albicans* (5) *P. mirabilis* (6) *K. pneumonia* (7) *P. vulgaris*]

Results showed that 306 isolates(90.265%) form biofilm with difference in thickness of formed layer which *P. aeruginosa* isolates were able to produce high quantity for biofilm and followed by *E.coli*, *C. albicans*, *P. mirabilis*, *K. pneumonia* and *P. vulgaris* were decreased in biofilm formation.

Biofilm production by bacteria and other microorganisms were genetically controlled property, which represent one of the most

important virulence factors in productive organism (Mireles *et al.*, 2001; Santo *et al.*, 2006).

Biofilm can increase bacterial resistance to antimicrobial agents by some mechanisms which included impairment of antimicrobial agents diffusion, locally alteration of microenvironment that impair the activity of antimicrobial agent, reduced bacterial growth rate and finally bacterial within biofilm may facilitate plasmid exchange and enhance the spread of antimicrobial resistance (Watnick and Kolter 2000; Donlan, 2001).

Sauer and Camper (2001) notice that microorganisms differ in their ability to produce biofilm, thickness of biofilm differ according to the genus and species of producing bacteria, conditions like temperature, pH and type of UTIs.

Biofilm producing bacteria show much greater resistance to antibiotics than their free-living counterparts. One potential reason for this increased resistance is the penetration barrier that biofilms may present to antimicrobials (Lynch *et al.*, 2007).

Bacterial biofilm described bacterial populations in natural and pathogenic ecological systems in terms of a floating or planktonic population of bacteria interacting with a more important matrix-enclosed sessile population of bacteria associated with or adherent to a surface. Although the urinary tract is a hostile environment for bacteria, infections occur when bacteria go up the urethra from the opening. Infection results when the bacterial virulence factors overcome the numerous host defenses. As adherent cells grow, they form encapsulated microcolonies, which are small clumps of morphologically identical cells (often 2-10 cells) immediately adjacent to each other, growth of adjacent microcolonies toward each other will lead to the development of a mature biofilm. Sessile bacteria as the permanently attached bacteria within these films. They behave quite

differently from free-floating or planktonic bacteria. The attached bacteria are more resistant to both antibiotics and the body's own immune response, These bacteria also create their own environment such as urinary calculi or stones. Nevertheless, these bacteria can spread to other organs such as the kidney (Stewart and Costerton, 2001; Parsek and Singh, 2003).

Antibiotic resistance is the most problematic and costly characteristic of biofilms, planktonic, or floating, cells in all these systems are completely eradicated at the antibiotic levels but more than 100 times as much antibiotic is needed to eradicate bacteria within a biofilm. Antibiotics can't penetrate within the film or that bacteria within a biofilm go into a reduced-level of metabolic activity, and don't absorb antibiotic toxins, essentially protecting themselves by going dormant (Curtis and Nickel, 2002).

The role of biofilms in infections associated with catheters that a variety of micro-organisms, including fungi, can be involved, which are identified morphologically by scanning or transmission electron microscopy, are recovered by routine culture method which the formation of Candida biofilms carries important clinical repercussions because of their increased resistance to antifungal therapy and the ability of cells within biofilms to withstand host immune defenses (Douglas, 2002).

Biofilm formation on medical devices can negatively impact the host by causing the failure of the device and by serving as a reservoir or source for future continuing infections (Kojic and Darouiche, 2004).

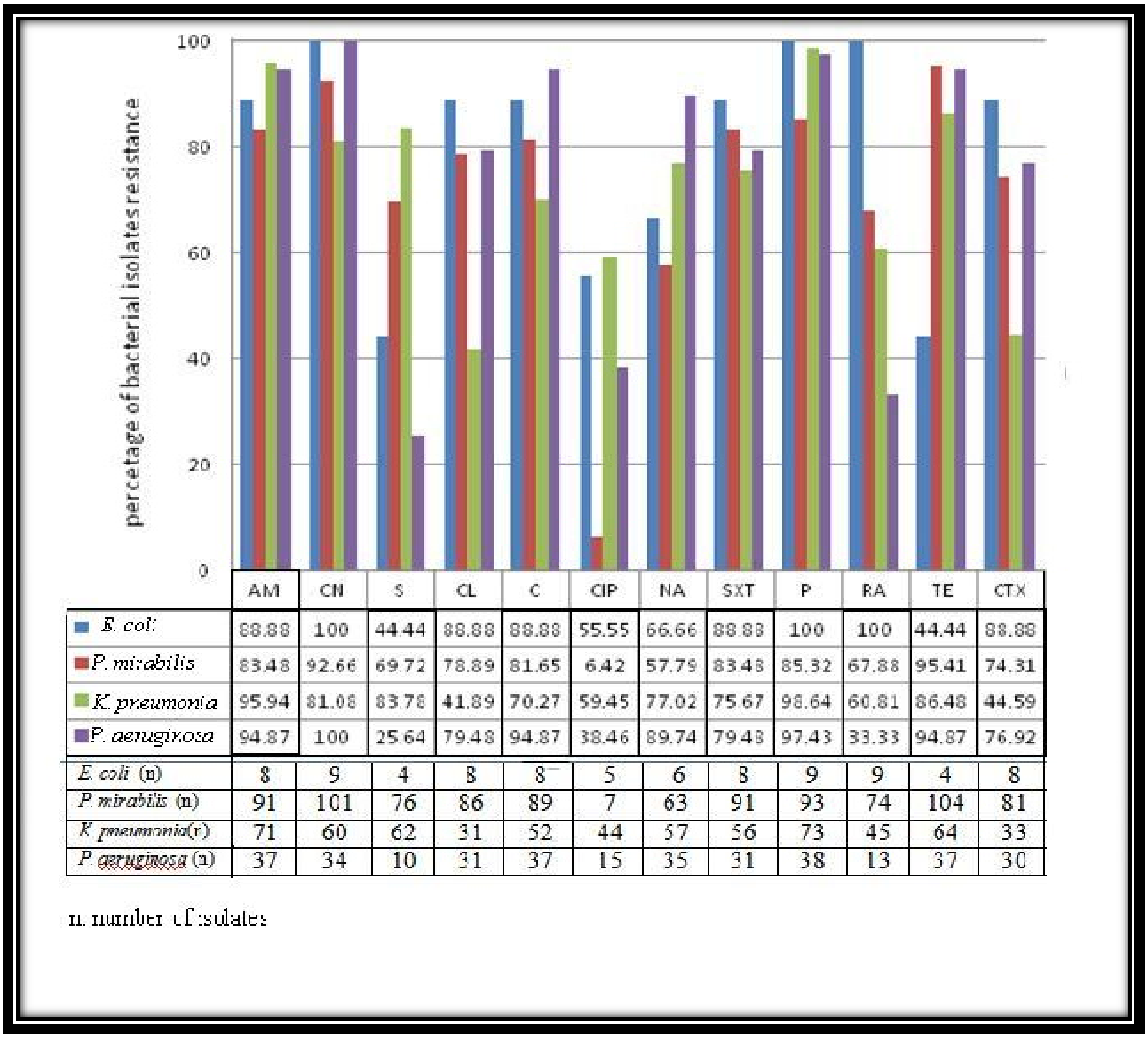
3.4 Antibiotic sensitivity of bacterial isolates

The emergence of prevalence of antibiotic resistance strains is consider as a major therapeutic problem that can be explained by several hypothesis such as the influence of excessive and / or inappropriate antibiotic use (Sotto *et al.*, 2001).

The disk diffusion method was used to determine the sensitivity of (*E.coli, P. mirabilis, K. pneumonia and P. aeruginosa*) to different antibiotics. Result showed all isolates were resistance to antibiotic figure (3-4). The antibiotic resistance among isolates varied according to the genus and species of isolates and to the nature of antibiotics. In general, the isolates were highly resistance to Pencillin (P), Gentamicin (CN), Tetracycline (TE), Chloramphenicol (C) and Trimethoprim-Sulfmethaxazol (SXT). They were moderate in their resistance to Ampicillin (AM), Nalidixic acid (NA), Cephalexin (CL), Strteptomycin (S), Cefotaxime (CTX) and Rifampicin (RA). they were sensitive to the antibiotic, especially to the Ciprofloxacin .

However, Pencillin was the most effective antibiotic which represent 92.20% of all isolates, this is due to the lack of pencillin binding proteins (PBPs) or the microorganism could change their permeability to the drug (Malkawi and Youssef, 1996).

All isolates were resistance to gentamicin and tetracycline which represent 90.47%, on other hand chloramphenicol and trimethoprim-Sulfmethaxazol which represent 80.51%, these results were in agreement with Godfrey and Evens(2000) who found that their isolates were resistance to the same antibiotics.



	AM	CN	S	CL	C	CIP	NA	SXT	P	RA	TE	CTX
E. coli	88.88	100	44.44	88.88	88.88	55.55	66.66	88.88	100	100	44.44	88.88
P. mirabilis	83.48	92.66	69.72	78.89	81.65	6.42	57.79	83.48	85.32	67.88	95.41	74.31
K. pneumonia	95.94	81.08	83.78	41.89	70.27	59.45	77.02	75.67	98.64	60.81	86.48	44.59
P. aerugiosa	94.87	100	25.64	79.48	94.87	38.46	89.74	79.48	97.43	33.33	94.87	76.92
E. coli (n)	8	9	4	8	8	5	6	8	9	9	4	8
P. mirabilis (n)	91	101	76	86	89	7	63	91	93	74	104	81
K. pneumonia(n)	71	60	62	31	52	44	57	56	73	45	64	33
P. aeruginosa (n)	37	34	10	31	37	15	35	31	38	13	37	30

n: number of isolates

Fig (3-4) The percentage of antibiotic resistance of bacterial isolates.

Resistant to antibiotics may be come from the antibiotic resistance genes; some of these genes may be located on chromosomal DNA, while the others may be located on plasmid DNA (Jawetz *et al*., 1998).

From the results, it may be concluded that fluoroquinolones and aminoglycosides groups of antibiotics had remarkable effect on bacteria isolates of patients suffering from UTIs of catheterized patients. This came almost in agreement with that of Power *et al.*, (1993) who found that there were no significant differences between effects of ciprofloxacin and chloramphenicol against 57 positive-cultures of catheterized patients suffering from UTIs. Nalidixic acid was introduced as the first type of quinolone used for treatment of UTIs, this antibiotic was active against *E.coli, Klebseilla spp.* and *Proteus spp.* which represent the main uropathogenes. Different studies showed that nalidixic acid play important role in treatment of UTIs specially caused by *E.coli* (Brooks *et al*., 2004).

The ability of bacterial cell to resist chloramphenicol is either due to their ability to produce chloramphenicol acetyl transferase which modifies the antibiotic or due to mutation that happened to the 23S rRNA which results in decrease the sensitivity of cells to the chloramphenicol while resistance to tetracycline is generally due to decrease accumulation, either through decrease uptake or increase efflux (Prescott *et al*., 1990; Nester *et al*., 2001).

Initially most of antibiotic resistance genes are found on chromosomal DNA, such as genes responsible for penicillins and cephalosporins resistance (Eggman *et al*., 1997), but these genes were carried also on self transmissible or mobilizable plasmid, and the transfer of such plasmid from one strain to another via conjugation or transformation was one of the major reasons for spreading the antibiotics resistance between bacterial population specially those that belong to the family *Enterobacteriaceae* which represent the major causative agent of UTIs (Livrelli *et al*., 1996; Rice *et al*., 1996).

Incorrect and long used of many antibiotics led to appear genes that not found previously, causes increase in resistance of the bacterial strain (Chen *et al.*, 1999).

The multidrug resistance of bacteria to several antibiotics could be due to the permeability of the outer membrane, which might prevent the entry of antibiotic into cell, or due to certain mutations that occur as a result of over use and misuse of antibiotics, but arise spontaneously are generally resistant to only one antibiotic (Malkawi and Yousef, 1996; AL- Shallchi, 1999; and Rasool *et al*., 2003).

In addition to mutations, R plasmids offer resistance to antibiotics and are transmissible from one cell to another by direct cell contact. Conjugation (direct *in vivo* gene transfer) is a convenient method of transferring drug resistant genetic determinants among intra- and intergreneric bacterial populations (Rasool *et al*., 2003; and Amyes *et al*., 1989).

Biofilm play important role in pathogenesity, so in the abiotic surface (catheters) which lack the intrinsic defenses present in host tissues, antibiotic frequently are not able to clear resistant bacterial biofilms from catheter surfaces(Donlan, 2001).

This multidrug resistance led to antibiotic ineffectiveness against bacteria responsible for UTIs and other life threatening diseases (White *et al*., 2002).

Chomarat (2000) mention that taking the antibiotics even for short time and in small dose lead to increase the development of resistant isolates.

3.5 Antifungal Sensitivity of yeast

3.5.1 Determination of minimum inhibition Concentration (MIC)

In this study, ketazole was used as an antifungal agent due to its remarkable inhibitory effect on *Candida albicans* isolate (C13), which highly producing to the germ tubes. Figure (3-5) shows that among the 33 isolates of *Candida albicans,* (C13) isolate was the highest producer of germ tubes from the budding cell with a number of (85×10^{-4}). For this reason, it was chosen for further studies.

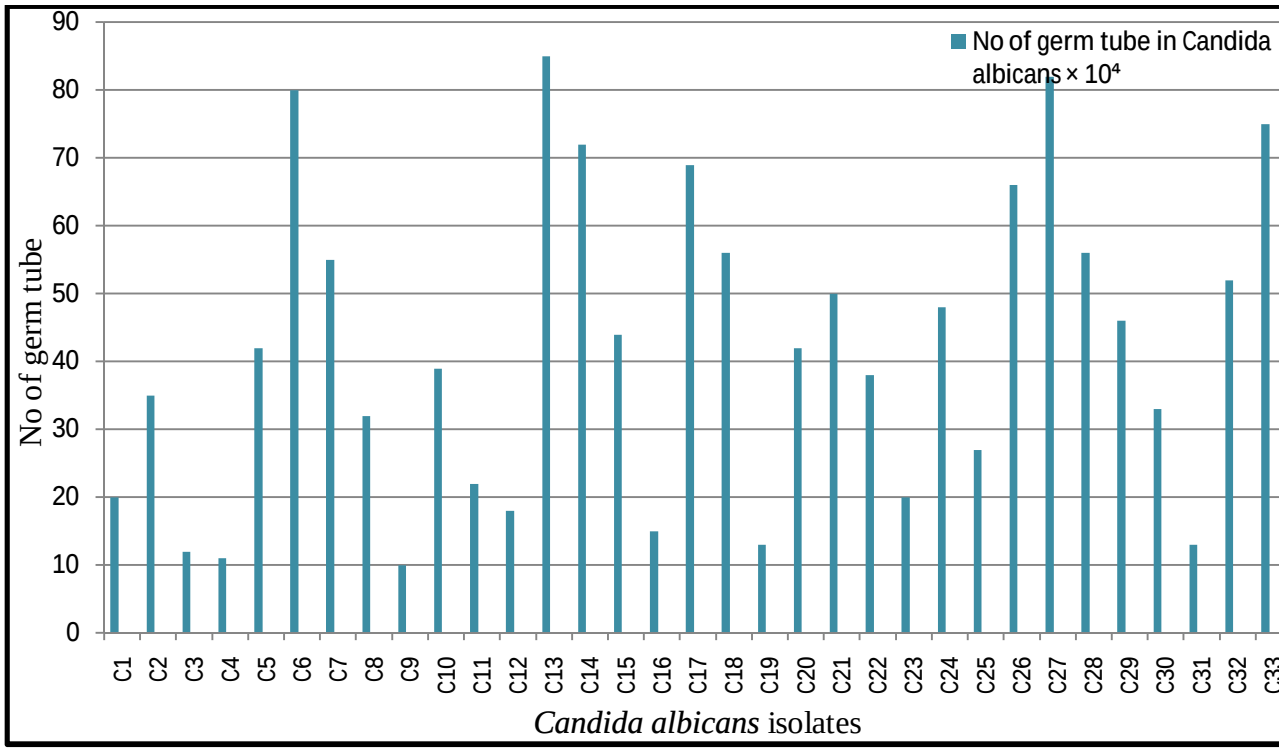


Fig (3-5) Ability of *Candida albicans* isolates to form germ tube in human serum.

The minimum inhibitory concentrations (MICs) of ketazole were 128 µg/ml in SDB against 28 isolates of *C. albicans*, on the other hand the rest of *C. albicans* which represent 5 isolates, the MICs of ketazole were 64 µg/ml. Ketazole was able to inhibit growth of fungi including *Candida albicans*. Daniel *et al* (1999) stated that there is a general agreement that it prevents the synthesis of ergosterol (a major component of fungal plasma membrane) by inhibiting the cytochrome P-450-dependent enzyme lanosterol demethylase. Upon fungal exposure to ketazole, depletion of ergosterol and accumulation of 14α-methylated sterols will take place. This interferes with the functions of ergosterol in fungal membranes and disrupts both the structure of the membrane and several of its functions, such as nutrient transport and chitin synthesis, leading to inhibit fungal growth (Daniel *et al.*, 1999). Same authors added that ketazole inhibit fungal growth by preventing 14-demethylation of lanosterol and effectively block synthesis of ergosterol. Also, some of these agents, at higher concentrations, destroy membranes directly, causing rapid killing. Depending on the former results, it can be concluded that *Candida albicans* yeast was more sensitive to the ketaconazole than other fungus. Inder and Davids (1985) obtained similar finding when they noticed that the antifungal activity of ketazole was better against yeast than fungi.

3.6 Extraction of DNA

Salting out method which described by Pospiech and Neumann (1995) was used for total genomic DNA extraction of different locally(*E. coli* , *P. mirabilis*, *K. pneumonia* , *P. aeruginosa* and *C. albicans*) isolates, the protocol involve main procedures of preparation of pellet from bacterial cultures, total genomic DNA extraction and measurement the purity of extracted DNA by spectrophotometer then separated the product on 0.8% agarose gel by electrophoresis (2.5hours,60 volt) as shown in Fig (3-6).

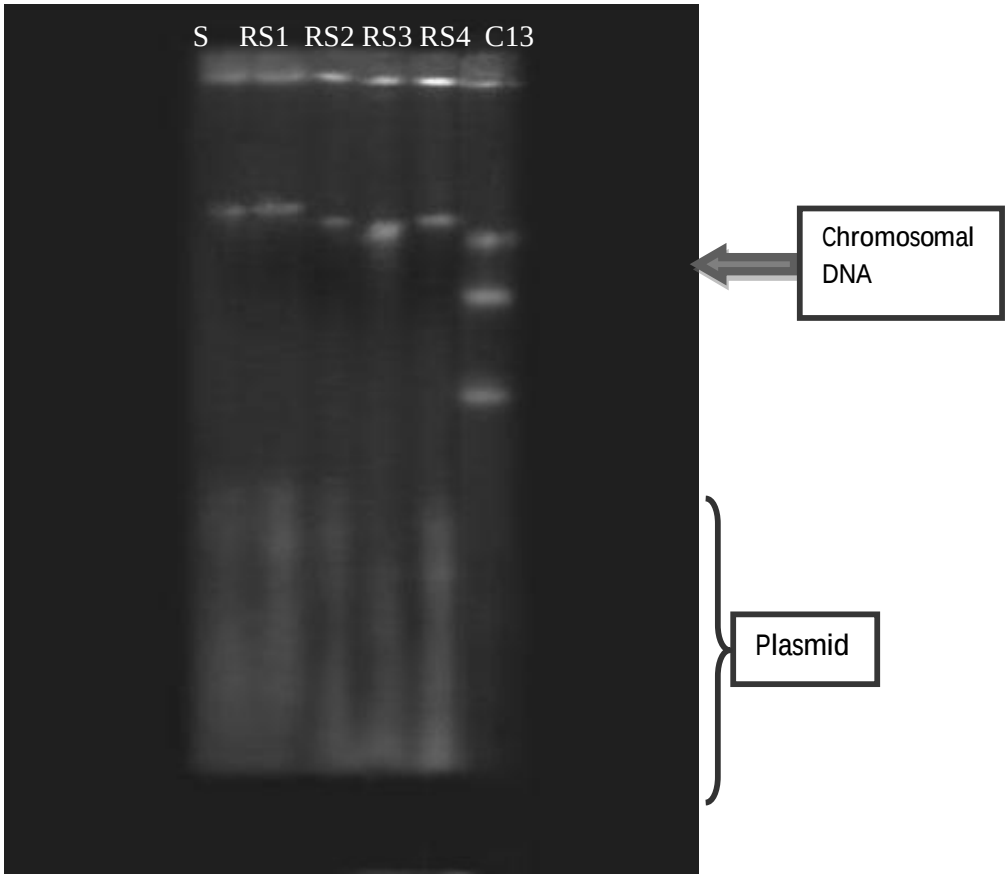


Fig (3-6) Agarose gel electrophoresis of DNA samples extracted from isolates (RS1, RS2, RS3, RS4, C13)

Lane S: represent DNA of *E. coli* MM 294 which considered as standard strain.

RS1: *Ecoli* isolates.

RS2: *P. mirabilis* isolates.

RS3: *K. pneumonia* isolates.

RS4: *P. aeruginosa* isolates.

C13: *C. albicans* isolates.

3.7 Plasmid Profile

Plasmid profile of different locally isolates (*E. coli*, *P. mirabilis*, *K. pneumonia* and *P. aeruginosa*) were studied in order to know the role of plasmid DNA in the resistance of these isolates to different antibiotics. Plasmid DNA was extracted according to the modified alkaline lysis method described by Birnboim and Doly described by Larvery *et al.*, (1979).

To determine the plasmid profile of the efficient isolates, (RS1,RS2,RS3 and RS4), antibiotic resistance of these isolates to different antibiotics was studied. Results mentioned in table (3-4) showed that RS1, RS2, RS3 and RS4 were resistant to nine, seven, seven and eight antibiotics respectively.

Table (3-4) Antibiotic resistance of selected bacterial isolates for plasmid isolation

Bacterial isolates	Antibiotic resistance
RS1	AM,CN,CL,C,NA,SXT,P,TE,CTX
RS2	AM,CN,S,C,SXT,P,TE
RS3	AM,CN,C,SXT,P,TE,CTX
RS4	AM,CN,CL,C,SXT,P,TE,CTX

Plasmid profile of these isolates mentioned in figure (3-7) showed that RS1 isolate harboring four plasmid which were named as follows (pRS11, pRS12, pRS13 and pRS14).

The size of pRS11 and pRS12 ranging from 8 kbp-10kbp, while pRS13 size ranging from 5-6kbp and pRS14 about 3kbp.

RS2 isolate represented three plasmid which were named as follows (pRS21, pRS22 and pRS23). pRS21 and pRS22 sizes ranging from 8-10kbp, while pRS23 size ranging from 3-4kbp.

RS3 isolate represented also three different plasmid bands which were named as follows (pRS31, pRS32 and pRS33). It was found that the mwt. of pRS31 about 8-10kbp, pRS32 about 4-5kbp and pRS33 about 2.5-3kbp.

RS4 isolate harboring seven bands which were named as (pRS41, pRS42 pRS43, pRS44, pRS45, pRS46 and pRS47). Size of pRS41and pRS42 ranging from 8-10kbp, pRS43 size about 4kbp, while pRS44 size ranging from 2.5-3 kbp. pRS45 size about 1.5-2kbp, finally pRS46 and pRS47 had a molecular weight range from 0.75-1kbp.

The bacterial isolates tested in this study may be containing another plasmids does not detected (may be because of its large size).

Alkaline lysis method is a suitable method for extraction of plasmid DNA. Lysozyme treatment is an important step in cell lysis, while proteinase k has been useful in the preparation of lysates and probably aids in breaking up DNA-protein-membrane complexes after cell lysis (Clewell *et al.*, 1974).

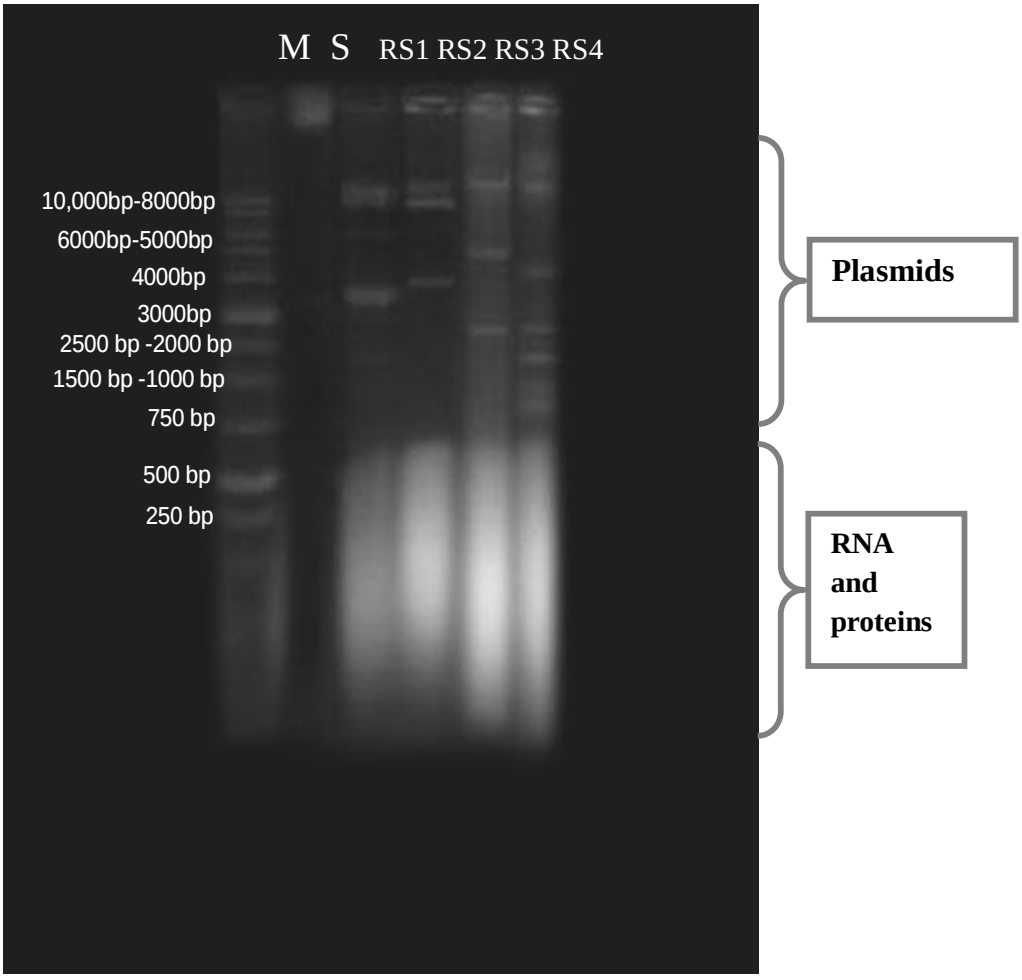


Fig (3-7) Plasmid Profile of RS1, RS2, RS3 and RS4 on agarose gel (0.8%) and voltage 60 V for 2.5hrs

Lane S: represent DNA of *E.coli* MM 294 Which considered as standard strain .

From top to bottom RS1 isolate: pRS11, pRS12, pRS13 and pRS14 plasmid band.

RS2 isolate: pRS21, pRS22 and pRS2 plasmid band.

RS3 isolate: pRS31, pRS32 and pRS33 plasmid band.

RS4 isolate: pRS41, pRS42 pRS43, pRS44, pRS45, pRS46 and pRS47 plasmid band.

3.8 Plasmid Curing

Plasmid curing of bacterial isolate was used to know whether the gene(s) responsible for antibiotic resistance is located on the plasmid or chromosomally encoded, for this purpose, many attempts were done in order to cure plasmid DNA of locally isolates *E.coli*, *P. mirabilis*, *K. pneumonia* and *P. aeruginosa* by using ethidium bromide as a curing agent according to procedure described by Trevors, (1986). Results in table (3-5), showed that the sublethal concentration of ethidium bromide that allows the growth of RS1, RS2, RS3 and RS4 were 800, 1000, 800 and 1000 µg/ml respectively

Table (3-5) Effect of Ethidium Bromide on the growth of RS1, RS2, RS3 and RS4

Ethidium bromid	Growth			
	RS1	RS2	RS3	RS4
0	+++	+++	+++	+++
20	++	++	++	++
50	++	++	++	++
100	++	++	++	++
200	+	+	+	+
250	+	+	+	+
300	+	+	+	+
400	+	+	+	+
800	±	+	±	+
1000	-	±	-	±
1600	-	-	-	-

Where: (–): no growth (0%), (±):slight growth (25% -20%), (+):moderate growth (50% - 30%), (++):good growth (90% - 70%), (+++):very good growth(100%)

The mode of action of ethedium bromide in curing of plasmid DNA is the inactivation of plasmid DNA replication during cell division, which leads to the presence of plasmid-less cell in the next generations. Furthermore ethidium bromide was a good agent in curing of plasmid DNA, if it compared with physical and other chemical agents (Hohn and Korn, 1969).

To prepare for this experiment, 100 ml of cultures incubated with sublethal concentration were taken and incubated for 24 hrs at 37˚C to determine the sub-lethal concentration of ethidium bromide .

From these treatment, appropriate dilutions were made and spreaded on nutrient agar plates .One hundred colonies were selected and each one of these colonies was grown in the defined medium containing different antibiotics with AM,CN,CL,C,NA,SXT,P,TE,CTX for RS1, AM,CN,S,C,SXT,P,TE for RS2, AM,CN,C,SXT,P,TE,CTX for RS3 while AM,CN,CL,C,SXT,P,TE,CTX for RS4 in order to determine the cured colonies, which lost its ability to grow in this defined medium, the obtained cured colonies were retested in this medium to ensure loosing their ability to grow.

Result showed that most of RS1 colonies were still resist to these antibiotics, while few colonies became sensitive to AM, CN, TE, P and CL, while RS2 colonies became sensitive to AM, CN, TE and P, RS3 colonies fail to grow on plates containing AM, TE, P and CTX, finally RS4 colonies fail to grow on plates containing AM, CN, TE, P and C. This may due to plasmid curing caused by the effect of ethidium bromide as a curing agent and as it was mentioned by Bouanchaud *et al.*, (1969).

From these results it can be concluded that resistance to ampicillin, tetracycline, gentamycin and pencillin for (RS1, RS2, RS3, RS4), cephalexin for RS1, cefotaxime for RS3 and chloramphenicol for RS4 were located on plasmid DNA (plasmid encoded phenotypes), while other antibiotics phenotypes were chromosomally located.

To confirm this result, cells that suspected to be cured were taken and grown in trypton soya broth for 24 hrs at 37˚C with shaking (150 rpm), and then plasmid profile was examined by extraction of plasmid DNA using alkaline lysis method. Results in figure (3-8) showed that after the curing, RS1isolate represented only one band which was named as pRS14. Antibiotic sensitivity to this isolate indicated that was the reason for losting three plasmid.

As for the two isolates RS2, RS3 the loosing of all plasmids after the curing causing losing a number of resistance properties (AM, CN, P,TE) for *P. mirabilis* and *K. pneumonia* that they were carried by chromosome not by plasmid.

While for RS4 isolate, five plasmid bands were lost after curing, and only two band shown in fig (3-8) which were named as pRS43 and pRS45. Lost of five plasmid band might indicated the retain of the isolate to be sensitive to following antibiotic which were (AM, CN, P, TE, C), but resistance to (STX, CL, CTX) might be carried on the other two plasmid bands.

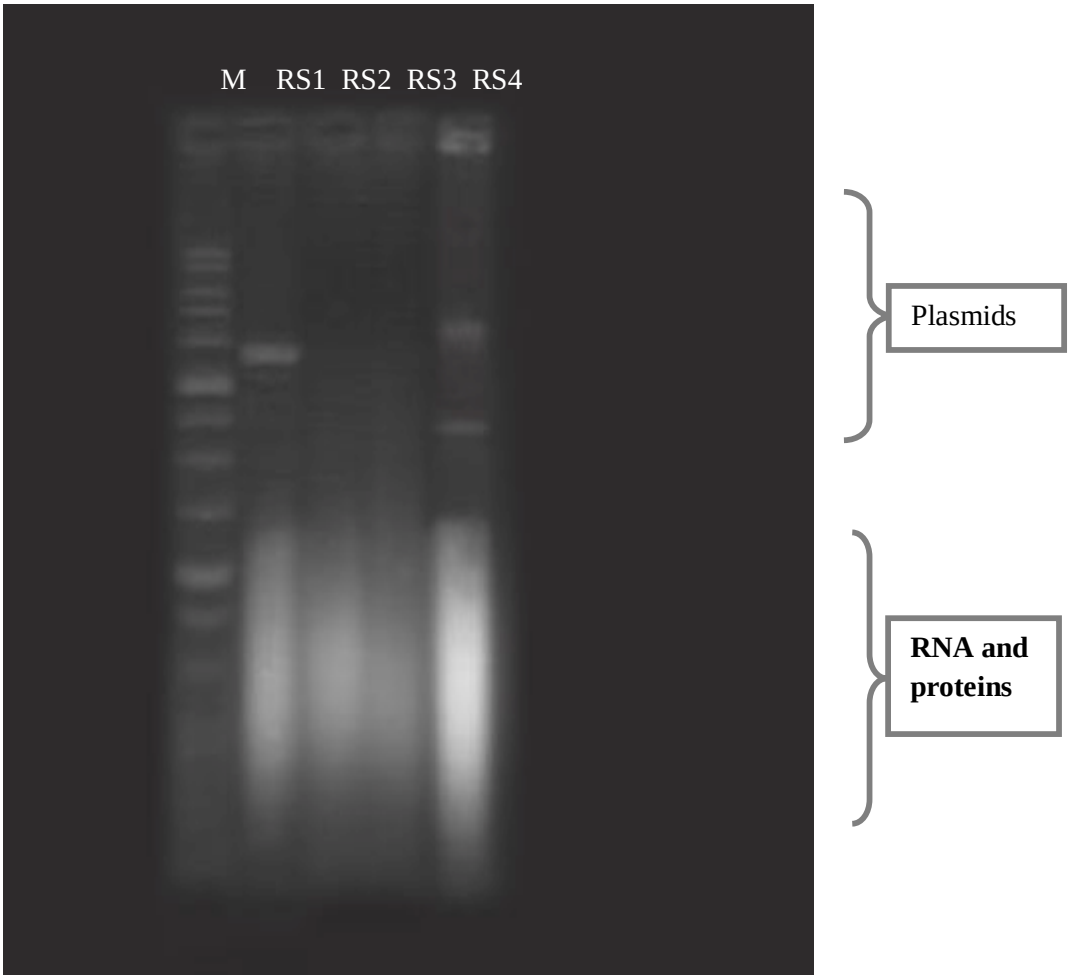


Figure (3-8) Gel electrophoresis of total DNA extracted from isolates(RS1, RS4) after curing by ethidium bromide.

Agarose concentration 0.8 % (W/V) and voltages 60 V for 2.5 hours.

The above results indicated that ethidium bromide is a powerful agent in eliminating antibiotic resistance plasmids and it was mentioned by (Bouanchaud *et al.*, 1969).

Uchechi, *et al* (2007) founded that ampicillin, tetracycline and cephalexin resistance determinants were encoded by plasmid.

Khan and Musharraf (2004) founded that gentamycin and pencillin resistance markers of *Proteus mirabilis* were identified as plasmid mediated.

It was known that *Klebsiella* have a number of drug resistance plasmid (Jacoby and Han, 1996; Al- Saeed, 1997). Al-Saeed(1997) found that ampicillin and tetracycline resistance genes were carried on one plasmid *klebsiella pneumonia*. Also Sirot *et al* (1991) found that *K. pneumonia* CF1314 have a 150 kb plasmid which carried genes encoded for tetracycline, amikacin, and sulfonamide.

In general it is documented that very few of the conventional antibiotics are active against *P. aeruginosa,* because many studies demonstrated a cluster of genes, which either plasmid or chromosomally encoded, responsible for antibiotic resistance in *P. aeruginosa* (Holloway *et al*., 1979; Paddila and Vasquez, 1993).

Chapter Four

Conclusions & Recommendations

Conclusion

1. Many species of uropathogenes(bacteria and yeasts) responsible of complicated UTIs with different pathogenesity rate according to their virulence factors . *Proteus sp., Klebsiella sp. and Candida sp.* represent the main causative agents of UTIs of catheterized patients suffering from complicated UTIs .
2. Most uropathogenic bacteria and yeast reflect high ability to produce biofilm specially with catheterized patients . catheter has been considered good environment which provide bacteria and yeast with optimal conditions of biofilm formation.
3. Biofilm formation represents the main factor cause initiation of infection and responsible for antibiotic resistance that increase according to thickness of biofilm layer .
4. Biofilm impairs the activity of antibiotics against uropathogenes thus increase the complexity of UTIs .
5. Most resistant genes of uropathogenic bacteria carried on plasmids .

Recommendations

1. Performing futural studies about different virulence factors closely associated with complicated UTIs.

2. Accurate use of catheter to avoid side effects like contamination led to cause infection.

3. Correct choose of antibiotics for catheterized patients treatments to minimize the chance of reinfection.

4. Use other development technique about plasmids resistance genes location.

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الخلاصة

تضمنت هذه الدراسة عزل وتشخيص انواع مختلفة من الممرضات(بكتريا-خمائر) لمرضى انابيب القثطرة الذين يعانون من التهابات مجاري بولية معقدة وتحديد قابلية هذه الممرضات على انتاج طبقة الغشاء الحيوي كعامل ضراوة اساسي في احداث الاصابة المرضيةمع تحديد علاقة المقاومة للمضادات الحياتية بالبلازميدات وقد كانت خطوات الدراسة كالاتي:

- **المرحلة الاولى:** تشخيص العزلات البكتيرية والخمائر انابيب القثطرة للمرضى و من نموذج من الادرارالماخوذ من قد وجد انه 339عينة بنسبة(96.85%)بالزرع مثلت نتيجة موجبة بينما 11 عينة بنسبة (3.142%) مثلت نتيجة سالبة وقدكانت 221 عينة بنسبة (63.138%) ماخوذة من النساء و 118عينة بنسبة (33.711%) من الرجال وقد تضمنت العينات الموجبة للزرع 303 عزلة بكتيرية بنسبة(89.380%) و 36 عزلة خميرة بنسبة (10.619%) وقد اوضحت لل 339 عزلة ان عزلات Api 20نتائج الاختبارات الكيموحيوية والتشخيص باستعمال نظام البكتيرية والخمائرتضمنت على: *E.coli Proteus spp.; Pseudomonas spp.;* هي تمثل المسببات الرئيسية لالتهابات المجاري و *Klebsiella spp.; Candida spp. and* البولية المعقدة وبنسب مختلفة حسب الانواع وضراوتها.

- **المرحلة الثانية:** تحديد قابلية الممرضات الرئيسية المشخصة على انتاج طبقة الغشاء الحيوي %) هي 90.265عزلة بنسبة (306 باستعمال طريقة انابيب الاختبار وقد اظهرت النتائج انه *Pseudomonas*منتجة لطبقة الغشاء الحيوي مع الاختلاف في سمك الغشاء المتكون وقد كانت *Proteus* ومن ثم *Candida spp. E.coli* مكونة لاسمك طبقة غشاء حيوي تليها *spp.* على التوالي. *spp. Klebsiella spp.*

- **المرحلة الثالثة :** دراسة مقاومة العزلات البكتيرية المشخصة للمضادات الحياتية المستعملة في علاج هؤلاء المرضى وقد اعطت النتائج مقاومة عالية تجاه:Penicillin, gentamycin, tetracyclin, chlormphenicol and trimethoprim- sulfmethaxazol ومقاومة متوسطة تجاهAmpicillin, nalidixic acid , cephalexin , cefotaxime

الذي يمثل المضاد ciprofloxacin واقل مقاومة تجاه streptomycin and rifampicin, الاكثر فعالية تجاه الممرضات البكتيرية للجهاز البولي.

يمثل المضاد المستخدم للتخلص من الخمائر والفطريات وقدكان اقل تركيز مثبط من Ketazol هو 128اما بقيه العزلات فقد كان اقل تركيز *Candida albicans* الكيتازول ضد 83%من مثبط هو 64.

للممرضات الرئيسية (البكتريا –الخمائر) حيث اعطت نتائج **DNAالمرحلة الرابعة** : عزل DNAانه العزلات البكتيريه *E.coli* (RS1), *P. mirabilis* (RS2), *K. pneumonia* (RS3) *aeruginosa* (RS4) and. *P* تمتلك كروموسوم وبلازميدات مختلفه من حيث العدد تمتلك فقط كروموسوم وغير ممتلكه للبلازميدات ومن ثم تم C13 والنوع بينما العزله الخميره مقاومة المضادات فيما اذا كانت محمولة على الكروموسوم او على تحديد لتحديد صفة اما نتائج التحييد اظهرت مايأتي:,البلازميدات

Ampicillin, penicillin , gentamycin and tetracycline-ان صفة المقاومة لمضادات للعزلات محمولة على البلازميد.

محموله على البلازميد.RS1 من قبل Cephalexin-مقاومه

محمولة على البلازميد. RS3 من قبل عزله Cefotaxim- مقاومة

محمولة على البلازميد . RS4 من قبل عزله Chlromphnicol- مقاومة

وصفة المقاومة لبقية المضادات تكون محمولة على الكروموسوم.

بسم الله الرحمن الرحيم

فَبدأ بِأوعيتهِم قَبلَ وِعاءِ أخِيهِ ثمَ استَخرجَهَا مِن
وِعَاءِ أخِيهِ كَذَلكَ كِدنَا لِيُوسُفَ مَا كَانَ لِيَأخُذَ
أخَاهُ فِي دِينِ المَلكِ إلَا أن يَشَاءَ اللّهُ نَرفعُ
دَرَجَاتٍ مَّن نَّشَاءَ وَفَوقَ كُلِ ذِي عِلمٍ عَلِيمٌ

صدق الله ألعلي العظيم

سورة يوسف

الآية 76

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم/ قسم التقانة الأحيائية

انتاج الغشاء الحيوي كعامل ضراوه في البكتريا والخمائر الممرضه للجهاز البولي

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم

في التقانة الاحيائية

مِن قِبَل

رغده سعد مكيه

بكلوريوس تقانة احيائية / كلية العلوم

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