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Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Sciences
Biotechnology Department

Bacteriological and Genetic Study on Virulence Factors of *Klebsiella pneumonia* Isolated from Urinary Tract Infections

**Submitted to the College of Science of AL-Nahrain University
As partial fulfillment of the requirements for the degree of Master of Science in Biotechnology**

By

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December **2005**

الاهداء

الى جميع الباحثين عن نور المعرفة

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Summary

One hundred and fifty isolates were isolated and identified from patients with urinary tract infection, and they were belonged to five genera: *E. coli* 70 isolates (46.7%), *Klebsiella* 20 isolates (13.3%) [in which 18 isolates were *Klebsiella pneumonia* and 2 isolates were *Klebsiella oxytoca*], *Proteus* 18 isolates (12%), *Enterobacter* 13 isolates (8.7%), *Pseudomonas* 12 isolates (8.0%) and *Staphylococcus* 17 (11.3%) isolates [in which *Staphylococcus epidermides* 9 isolates and *Staphylococcus aureus* 8 isolates]. Ninety isolates (60%) were obtained from female while sixty isolates (40%) from male.

The antibiotic sensitivity pattern was examined for all (20) *Klebsiella* isolates and results showed that all the isolates (100%) were resistance to penicillin, 16 isolates (80%) were resistance to ampicillin and streptomycin 15, isolate (75%) were resistance to gentamicin and, 14 isolates (70%) were resistance to chloramphenicol, while all the isolates (100%) were sensitive to imipenem, and 14 isolates (70%) were sensitive to norfloxacin, 13 isolates (65%) were sensitive to cephalexin, 12 isolates (60%) were sensitive to tetracycline and cefotaxime and 11 isolates (55%) were sensitive to aztreonam.

A number of virulence factors of *Klebsiella* were detected, results showed that all the isolates (100%) were encapsulated, having colonization factor antigen type I (CFA / I), while 16 isolates (80%) were have colonization factor antigen type III (CFA / III). All the isolate (100%) were able to produce siderophore, while none of them were able to produce hemolysin.

Results showed that 75% of *Klebsiella* isolates were resistance to the serum bactericidal effect, while 25% of the isolates were sensitive. Also, all the isolates (100%) showed the ability to adhering to epithelial cells. However, MR1, MR2, MR10 and MR16 isolates had the highest level of adherence with an average of 20 bacteria per epithelial cell. All the isolates showed high resistance to the phagocytosis in which the phagocytosis percentage was

decreased for all isolates and it was ranged between 7.5-15.0%.

To study the relationship between plasmids of *Klebsiella pneumoniae* MR1 and its pathogenicity, bacteria was treated with ethidium bromide. Results showed that there was a number of cured isolates which lost resistance for many antibiotics. Chloramphenicol and aztreonam resistance markers in this bacterium were carried on two different plasmids, while ampicillin and tetracycline resistance markers were carried on one plasmid. And the streptomycin and gentamicin resistance markers were carried either on chromosome or on mega plasmid which can not be cured.

The cured isolates MR1C1 and MR1C2 (which lost resistance to ampicillin, tetracycline, chloramphenicol and aztreonam) were choused to be tested for other virulence factors. Results showed that both isolates were capsulated but the capsule size was reduced, also both isolates were still have the ability to produce colonization factor antigen type I and III (CFA / I and III) and the ability to produce siderophores. However, resistance to serum bactericidal effect was decreased compared to MR1 isolate, and adherence ability of these two cured isolates was decreased in which the average of adherence cells became 2-7 bacterial cells / epithelial cell, also phagocytosis resistance was decreased in which phagocytosis percentage reached to 53% and 60% for the MR1C1 and MR1C2 respectively.
From these results it was concluded that genes encoding for the colonization factor antigen (CFA I / III) and siderophore production of *Klebsiella pneumoniae* MR1 are carried on chromosome, while gene encoding for capsule synthesis was carried on chromosome and regulated by plasmid gene (s). The changes in other properties (resistance to serum bactericidal effect, adhesion ability and phagocytosis resistance) may be due to the capsule reduction.

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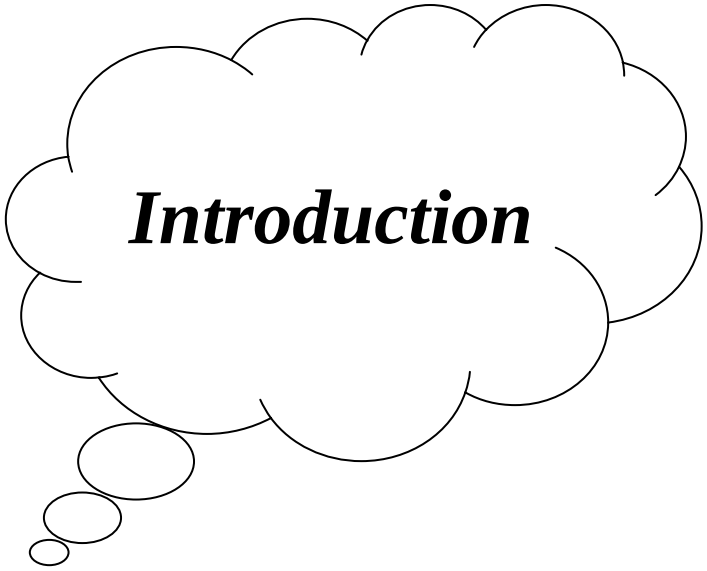
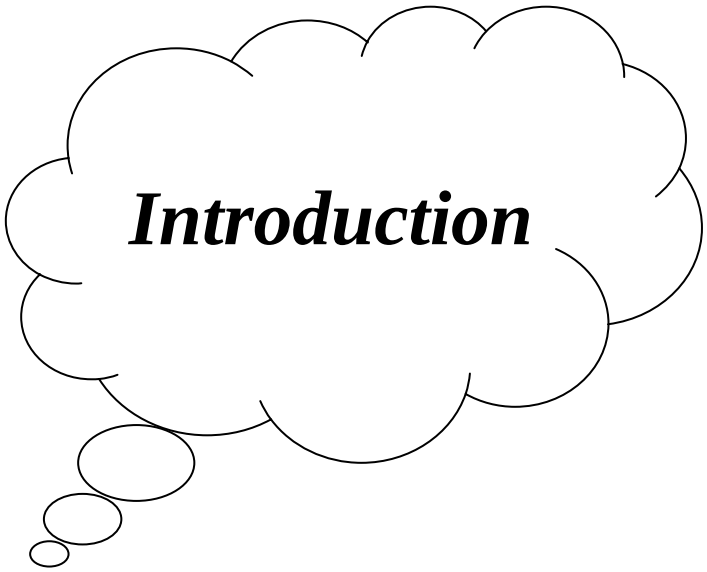
List of Abbreviations

Abbreviation	Mean
Amp	Ampicillin
Atm	Aztreonam
BA	Blood Agar
BHI	Brain Heart Infusion
C	Chloramphenicol
CFA / I	Colonization Factor Antigen type I
CFA / III	Colonization Factor Antigen type III
Cn	Gentamicin
CPS	Capsular Polysaccharide
D.W.	Distilled Water
ESBL	Extended Spectrum Beta Lactamase
LF / NLF	Lactose Fermenter / Non-Lactose Fermenter
LPS	Lipopolysaccharides
Mac	MacConkey Agar
MIC	Minimum Inhibitory Concentration
MR/K-HA	Mannose Resistance / *Klebsiella*-Like hemagglutinins
MRHA	Mannose Resistance Hemagglutinins
MR-VP	Methyl Red – Voges Proskauer
MSHA	Mannose Sensitive Hemagglutinins
NA	Nutrient Agar
NCCL	National Committee for Clinical Laboratory Standard
PAD	Phenylalanin Deaminase

PAIs	Pathogenicity Islands
PBS	Phosphate Buffer Saline
PNHS	Polled Normal Human Serum
S	Streptomycin
Tc	Tetracycline
TSI	Triple Sugar Iron
UTI	Urinary Tract Infection



Chapter One



Chapter One

1-1 Introduction

Urinary tract infection (UTI) is the most common problem affecting human of all ages. It is a disease of world wide importance as a cause of both morbidity and mortality. The vast majority of infections are caused by enterobacteriaceae originating from the gut (Hannan *et al*., 1993).

Due to the development of antimicrobial agents, the ratios of UTIs were decreased. Distribution of bacteria causing UTIs and their antibiotics sensitivities vary from place to place and from time to time depending on the environment and the choice of treatment which require a continuous assessment to establish the resistance pattern of the microorganisms (Gruneberg, 1984).

Resistance to commonly antimicrobial agents is an increasing problem in many developing countries due to the overuse and misuse of antibiotics which result in their ineffectiveness against multidrug resistant bacteria responsible for many life-threatening diseases (Burke and Levy, 1985).

The wide spread of bacterial strains resisting to several antibiotics becomes one of the major problems in treating the UTI. The antibiotic resistance can be coded by chromosomal or plasmids genes. Many of plasmid carrying antibiotic resistance genes can be transferred from one bacterial cell into another by conjugation and transformation, thus spreading the resistance to antibiotics (Satta *et al.,* 1987). The associations of transmissible plasmids with the multiresistance to several antibiotics were established in several bacterial species causing UTIs (Livrelli *et al.,* 1996).

Klebsiella spp. is well known to most clinicians as a cause of community-acquired bacterial pneumonia, occurring particularly in chronic alcoholics and showing characteristic radiographic abnormalities due to a severe pyogenic infection which has a high fatality rate if untreated. The vast majority of *Klebsiella* infections, however, are associated with hospitalization. As opportunistic pathogens, *Klebsiella* spp. primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such

as diabetes mellitus or chronic pulmonary obstruction. Nosocomial *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae*, the medically most important species of the genus (Podschun and Ullmann, 1998).

Klebsiella spp. have different virulence factors which give the bacteria the ability to invade the host, such as **capsule** which increases its resistance to phagocytosis and serum killing factors, adhesion **pili** which help cells to adhere to the epithelial cells, and their ability to chelating ferric compound from the environment by the production of **siderophore**. And some other factors such as the production of urea, enterotoxin and lipopolysaccharide production (Podschun and Ullmann, 1998). However, antibiotic resistance properties are the major factor in its pathogenicity that it resists for wide spectrum of antibiotics and specially β-lactam antibiotics. This is due to the presence of R-plasmids (Vernet *et al*., 1995).

To study the microbial pathogenicity at the molecular level, *Klebsiella* were used to study its virulence factors for the following reasons:

1. It's considered one of the five most infectious agents of the urinary tract infection.
2. Few studies were performed about *Klebsiella* and its virulence factors compared with other members of enterobacteriaceae family.
3. *Klebsiella* is considered responsible of outbreaks, nasocomial infections and represent serious problem in the medical and surgical kits and instrument.

Aims of the Study

Isolation and identification of some bacterial causative agents of UTI and select *Klebsiella* spp. isolates for:

1. Studying the resistance pattern of *Klebsiella* isolates to antimicrobial agents.
2. Study some of the virulence factors of these bacteria.
3. Investigate the correlation of plasmids contents and the pathogenicity of these bacteria.

Chapter Two

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2-1 Urinary Tract Infection

Urinary tract infection (UTI) is among the commonest cause of illness in the community and major cause of illness in hospitalized patients (Hannan *et al*., 1993). It is particularly common in females; 10-20% of women have UTI at some time in their life and a significant number have recurrent infection (Mims *et al*., 1987). Its important lies in the fact that a considerable proportion of the population may acquire asymptotic infection (Santoro and Kaye, 1978) and it is the most common bacterial infection of human of all ages and both sexes (Tanagho and McAninch, 1995)., ~~that i~~It may occur in about 1% of girls, 2% of pregnant women and up to 20% of elderly men and women (Gabriela, 2000; Jawetz *et al*., 2001).

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). When present, the clinical presentation of UTI may be that either of lower or of upper urinary tract symptoms (Glauser, 1986). Lower urinary tract symptoms most commonly result from vaginitis, cystitis, uretheritis or prostitis while upper urinary tract symptoms means acute bacterial infection of the kidney (Truck, 1981; Pingle, 1984).

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2-2 Pathogenesis of Urinary Tract Infection

Urinary tract infections (UTIs) occur as a result of the interaction of bacterial virulence factors (such as: increased adherence to vaginal and uroepithelial cells, resistance to serum bactericidal activity, a higher quantity of K antigen, the presence of aerobactin, cytotoxic necrotizing factor type 1, and hemolysin production) and host biological and behavioral factors as opposed to highly efficient host defense mechanisms. There are three possible routes by which bacteria can invade and spread within the urinary tract and these routs

include: **the ascending**, **hematogenous**, and **lymphatic pathways** (Mandell *et al*., 2000).:

A: The Ascending Route

The urethra is usually colonized with bacteria. Studies using suprapubic puncture techniques have revealed the occasional presence of small numbers of microorganisms in the urine of uninfected persons. 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.

B: 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 bacteremia or endocarditis, 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 the intravenous route with gram-negative enteric bacilli, the common pathogens in urinary tract infection, is difficult. Additional manipulations such as the creation of ureteral obstruction are often necessary. It would appear that in humans, infection of the kidney with gram-negative bacilli rarely occurs by the hematogenous route.

C: 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)

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2-3 Epidemiology of Urinary Tract Infection

More than 95% of urinary tract infections are caused by a single bacterial species. There is a great difference between the bacterial flora of the urine in patients with an initial episode of urinary tract infection compared with the flora from those with frequent recurrences of infection (Jellheden *et al*., 1996).

The main causes of UTI are gram-negative bacteria and especially the ~~Enterobacteriaceae~~ ~~enterobacteriaceae~~enterobacteriaceae family (Stamey ~~*et al.*,~~ ~~1971~~1973). *Escherichia coli* is by far the most frequent infecting organism in acute infection (Mobley *et al*., ~~1986~~1988). In recurrent urinary tract infections, especially in the presence of structural abnormalities of the urinary tract (such as obstructive uropathy, congenital anomalies, neurogenic bladder malfunction, and fistulous communication involving the urinary tract), the relative frequency of infection caused by *Proteus*, *Pseudomonas*, *Klebsiella*, and *Enterobacter* spp. and by *Enterococci* and *Staphylococci* increases greatly. In the presence of structural abnormalities, it is also relatively common to isolate multiple organisms from the urine. Since instrumentation and repeated courses of antimicrobial therapy are common in these patients, antibiotic-resistant isolates might be expected (Bronsema *et al*., 1993).

The hospital environment is an important determinant of the nature of the bacterial flora in urinary tract infection. *Proteus, Klebsiella, Enterobacter*, and *Pseudomonas* spp. and *Staphylococci* and *Enterococci* are more often isolated from inpatients, compared with a greater preponderance of *E. coli* in an outpatients population (Williams, 1974; Takeuchi *et al*., 1984). Cross-infections

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are important in the pathogenesis of hospital-related urinary tract infections, especially with indwelling catheters (McLean *et al*., 1985).

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2-4 The Correlation between UTI with Sex and Age

The problem of urinary tract infection spans all age-groups, beginning with neonates (Nordqvist *et al*., 1984; Kunin, 1997). UTI are much more common in women than in men. Many of these patients previously had urinary tract infections as children and continue to have infections as adults ~~(Febore *et al*., 1999)~~. Once a woman develops infection, she is more likely to develop subsequent infections than a patient who has had no previous infections (Febore *et al*., 1999).

The frequency of urinary tract infection in infants is about 1 to 2%. It is much more common in boys during the first 3 months and thereafter occurs more often in girls (Auckenthaler, 2000).~~.~~

During the preschool years, urinary tract infection is more common in girls than in ~~boys,~~boys; its frequency is about 4.5% for girls and about 0.5% for boys (Zimakoff *et al*., 1995).

The presence of bacteriurea in childhood defines a population at higher risk for the development of bacteriurea in adulthood (Ouslander *et al*., 1987).

Once adulthood is reached, the prevalence of bacteriurea increases in the female population. The prevalence of bacteriurea in young nonpregnant women is about 1 to 3%. Each year, about 25% of bacteriuric women clear their bacteriurea, and an equal number become infected (often women who have had urinary infection previously). At least 10 to 20% of the female population experience a symptomatic urinary tract infection at some time during their life (Nicolle *et al*., 1988). Other conditions may increase the frequency in female such as diabetic women but not men have been found to have a higher prevalence of bacteriurea than nondiabetic patients and black women with sickle

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cell trait have a higher prevalence of bacteriurea during pregnancy than black women without the sickle trait (Mohler *et al.,* 1987; Pearman *et al*., 1991).

The prevalence of bacteriurea in adult men is low (0.1% or less) until the later years, when it rises. The increase in bacteriurea in older men is probably mainly related to prostatic disease and the resultant instrumentation. In young men, a lack of circumcision increases the risk of urinary tract infection caused by uropathogenic strains of *E. coli,* including the development of symptomatic urethritis (Melekos and Asbach, 1986).

In elderly people, at least 10% of men and 20% of women older than 65 years have bacteriurea. In contrast to young adults, in whom bacteriurea is 30 times more frequent in women than in ~~men,~~men; in those older than 65 years the ratio alters dramatically, with a progressive decrease in the female/male ratio (Steinhardt and McRoberts, 1980; Bakke, 1993~~Steinhardt and McRoberts, 1980; Bakke, 1993~~).

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2-5 Diagnosis of Urinary Tract Infection

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2-5-1 Collection of the Specimen

Urine in the bladder is normally sterile. Since the urethra and periurethral areas are very difficult to sterilize, even the most carefully collected specimens are frequently contaminated. By quantitating bacteria in midstream, clean-voided urine, it is possible statistically to separate contamination from urinary tract infection. Patients with infection usually have at least 10^5 bacteria / ml urine in the bladder, and therefore voided urine usually contains at least 10^5 bacteria / ml. Patients without infection have sterile bladder urine, and with proper collection, voided urine usually contains less than 10^4 bacteria / ml. (Schaeffer *et al*., 1988; Jawets *et al*., ~~1995~~2001).

Acceptable methods for urine collection include (1) midstream clean catch, (2) catheterization, and (3) suprapubic aspiration. The clean-catch method is preferred for the routine collection of urine for culture.

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Disinfectants should not be used for cleaning because they may lower the bacterial count if they get into the urine sample (Glauser, 1986). And ideally, samples should be collected before antimicrobial therapy, or has done within the last 24 hr, this should be stated clearly on the request form (Mims *et al*., 1987).

The urine should be processed immediately, or if refrigerated at 4°C, it can be cultured within 24 hours.

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2-5-2 Laboratory Investigations

Microscopic examination of the urine is the first step in the laboratory diagnosis of urinary tract.

A clean-catch midstream urine specimen is centrifuged for 5 minutes at 2000 rpm, and then the sediment is examined under high power. Each leukocyte seen represents about 5 to 10 cells / mm^3 of urine; 10 to 50 white cells / mm^3 are considered the upper limit of normal (Hammarsten and Lindqvist, 1992). With this criterion, 5 to 10 leukocytes per high-power field in the urine specimen is the upper limit of normal, as they represent 50 to 100 cells / mm^3. In fact, most have hundreds of leukocytes per cubic millimeter. Red blood cells may appears, this may be indicative of other disorders such as calculi, tumor, vasculitis, glomerulonephritis, and renal tuberculosis However, pyuria without infection remains common (O'Kelly *et al*., 1995).

~~However, red blood cells may be indicative of other disorders such as calculi, tumor, vasculitis, glomerulonephritis, and renal tuberculosis.~~

Calibrated loops serve as a simple inexpensive way to examine quantitatively the bacteriologic characteristics of urine specimens (Ouslander *et al*., 1987). Platinum loops that deliver 0.01 ml and 0.001 ml are used to streak urine onto agar plates. After incubation at 37°C for 24 hours, the number of colony-forming units is counted, and the total number of organisms originally

present in the specimen is estimated by multiplying the colony count by 10^2 or 10^3, respectively. **Bacteriurea** is defined as "**Significant**" when the numbers of bacteria in voided urine that exceed the numbers usually due to contamination from the anterior urethra (i.e., $\geq 10^5$ bacteria/ml).

A further refinement of the technique involves the use of differential agars to allow isolation from mixed cultures and more rapid identification (DeGroot *et al.*, 1988).

2-6 Management of Urinary Tract Infection

The selection of an appropriate antimicrobial agent has become complex because of the increasing number of compounds available, each with its characteristic spectrum and toxic properties. However, in most cases, any of many available agents are perfectly satisfactory. Given two or more drugs with equivalent activity against the infecting microorganism, the agent with the least toxicity should be chosen (Sobel and Kaye, 2000).

Antimicrobial agents should only be administered when there is reasonable evidence of infection in the urinary tract. Symptoms are not a reliable indication of infection. The diagnosis of infection in the asymptomatic patient should be made on no fewer than two cultures of clean-voided, midstream urine in which the same microorganism is present in significant titers. If the patient is symptomatic, one specimen suffices, and therapy should be started (Sobel and Kaye, 2000).

A rational approach to the treatment of urinary tract infection depends on an appreciation of the prognosis of the untreated infection and the long-term results to be expected from therapy. The side effects, cost, and inconvenience of different therapeutic regimens must also be considered (Sobel and Kaye, 2000).

2-7 *Klebsiella*

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~~ ~~enterobacteriaceae~~enterobacteriaceae family, and they are gram negative rods, non-sporing, non-motile bacilli which tend to be short and thick, about 0.3-1 ~~-0.3~~ µm in diameter and 0.6-~~0.~~6 µm in length. They form a well-defined polysaccharide capsule (~~Pergy~~Holt *et al.*,~~,~~ 1994).

They grow well on ordinary nutrient media and on glucose-ammonium salt agar unsupplemented with growth factors. Temperature range for growth is 12-43ºC, optimum 37ºC. Colonies are large, raised, moist and mucoid and the degree of mucoidness depends on the amount of carbohydrate in the culture medium as well as varying from strain to strain. 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 produce H_2S in TSI agar or liquefy gelatin and (G + C) ratio is about (53-59 %) (Holt *et al.*, 1994; Collee *et al.*, 1996; Abbott, 1999).

2-8 Taxonomy of the Genus *Klebsiella*

The taxonomy of *Klebsiella* is characterized by a nomenclature reflecting its colorful taxonomic history. Originally, the medical importance of the genus *Klebsiella* ~~(family *Enterobacteriaceae*)~~ led to its being subdivided into three species corresponding to the diseases they caused: *K.* ~~*pneumonia*~~*pneumoniae*~~*e*~~, *K. ozaenae*, and *K. rhinoscleromatis*. As the taxonomy became increasingly refined due to the development of new methods such as numerical taxonomy, the species classification in this genus was continually revised. In time, three main classifications emerged, those of Cowan, Bascomb, and Ørskov (Table 2-1).

Table 2-1 Species classification of the genus *Klebsiella* by different taxonomic systems (Podschun and Ullmann, 1998).

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Classification by:		
Cowan	**Bascomb**	**Ørskov**
K. aerogenes K. edwardsii	*K. aerogenes/oxytoca/ edwardsii*	*K. ~~pneumonia~~pneumoniae~~e~~* subsp. *~~pneumonia~~pneumoniae~~e~~*
subsp. *edwardsii*	*K. ~~pneumonia~~pneumoniae~~e~~*	subsp. *ozaenae*
subsp. *atlantae*	sensu stricto	subsp. *rhinoscleromatis*
K. ~~pneumonia~~pneumoniae~~e~~	sensu lato	*K. oxytoca*
K. ozaenae	*K. ozaenae*	*K. terrigena*
K. rhinoscleromatis	*K. rhinoscleromatis*	*K. planticola* (syn. *K. trevisanii)*
	K. "unnamed group	
	Enterobacter aerogenes	*K. ornithinolytica*

In the early 1980s, *Klebsiella* isolates from the environment, which had previously been classified as "*Klebsiella*-like organisms" (groups J, K, L, and M), were increasingly being classified into provisional taxa (Gavini *et al*., 1977). These groups gave rise to four new species: *K. terrigena*, *K. ornithinolytica*, *K. planticola* and *K. trevisanii* (Sakazaki *et al*., 1989). In 1986, the last two species were combined into one species, *K. planticola*, because of their extensive DNA sequence homology (Gavini *et al*., 1986). While originally considered to be without clinical significance and restricted to aquatic, botanical, and soil environments, *K. terrigena* and *K. planticola* have been reported as occurring in human clinical specimens (Podschun and Ullmann, 1994~~; Podschun and Ullmann, 1994~~). According to these findings, particularly *K. planticola* has been isolated from human infections with a surprisingly high frequency of 3.5 to 18.5% among clinical isolates of *Klebsiella* species. More than half of these isolates were recovered from respiratory tract secretions; wound and urine isolates were the next most common ~~(Posdchun and Ullmann, 1994)~~. Thus, at present it seems possible that in addition to *K. ~~pneumonia~~pneumoniae~~e~~* and *K. oxytoca*, a third *Klebsiella* species exists that is able to cause human infections~~.~~ (Posdchun and Ullmann, 1994).

The adoption of a consistent nomenclature has been further complicated by the fact that Great Britain and the former Commonwealth countries adhere to the

classification of Cowan while the USA prefers Ørskov's classification. Consequently, the same bacterium may be called *K. ~~pneumonia~~pneumoniae~~e~~* in one country and *K. aerogenes* in another. Most European countries follow the American example and recognize the worldwide predominant classification of Ørskov (Podschun and Ullmann, 1998).

2-9 Differentiation of *Klebsiella* Species

Klebsiella species are usually identified and differentiated according to their biochemical reactions (Table 2-2). The genus is defined as containing gram-negative, nonmotile, usually encapsulated rod-shaped bacteria of the family ~~Enterobacteriaceae~~enterobacteriaceae ~~(Appendix A)~~, which produce lysine decarboxylase but not ornithine decarboxylase and are generally positive in the Voges-Proskauer test (Edwards and Ewing, 1986). ~~Within the genus *Klebsiella*, the individual species can be differentiated on the basis of the features listed in (Appendix B).~~ Whereas most *Klebsiella* species can be identified by standard microbiological laboratory tests, the species *K. terrigena* and *K. planticola* require special, nonconventional reactions (such as utilization of *m*-hydroxybenzoate or hydroxy-L-proline, pectate degradation, acid from melezitose, or growth at 10°C) (Podschun and Ullmann, 1998).

Table 2-2 Biochemical reactions of *Klebsiella* species (Podschun and Ullmann, 1998).

Characteristic	*Klebsiella pneumoniae*			*K. oxytoca*	*K. terrigena*	*K. planticola*	*K. ornithinolytica*
	subsp. *pneumoniae*	subsp. *ozaenae*	subsp. *rhinoscleromatis*				
ndole	−/−	−/−	−/−	+	−/−	v	+
Ornithine	−/−	−/−	−/−	−/−	−/−	−/−	+

decarboxylase							
ysine decarboxylase	+	v	−	+	+	+	+
ectate degradation	−	−	−	+	−	−	−
as from lactose at 44.5°C	+	−	−	−	−	−	−
rowth at 10°C	−	−	−	+	+	+	+
cid from:							
D-Melezitose	−	−	−	v	+	−	−
L-Sorbose	v			+	+	+	
tilization of:							
m-ydroxybenzoate	−	−	−	+	+	−	−
Hydroxy-L-roline	v			v	v	+	
Malonate	+	−	+	+	+	+	+
Methyl red test	−	+	+	−	+	v	+
oges-roskauer eaction	+	−	−	+	+	+	+

v, variable reaction

2-10 Typing of *Klebsiella* Isolates

Varieties of methods have been used with various degrees of success in *Klebsiella* typing and are discussed below

Biotyping

Biotyping based on an extended panel of biochemical and culture tests. Biotyping can be carried out by using macrotube tests alone (Haverkorn and Michel, 1979) or by combining a commercially available miniaturized system such as the API 20E system with additional macrotube tests (Podschun *et al.*, 1992). However, because of the large number of reactions to be tested and the

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often long cultivation times up to 90 days for demonstration of gelatinase ~~(Stenzel *et al*., 1972)~~ biotyping of *Klebsiella* spp. is not very suitable as an epidemiological tool (Stenzel *et al*., 1972).

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Serotyping

Serotyping is currently the most widely used technique for typing *Klebsiella* spp. It is based mainly on a division according to the capsule antigens. *Klebsiella*~~e~~ usually have well-developed polysaccharide capsules, which give their colonies their characteristic mucoid appearance. Of 82 capsule antigens described, 77 types form the basis for an internationally recognized capsule antigen scheme. Although 12 different O-antigen types of *Klebsiella* have also been described, they are difficult to classify because their determination is hampered by the heat-stable capsules (Ørskove and Ørskove, 1984). Capsule typing, by contrast, shows good reproducibility and is capable of differentiating most clinical isolates (Ayling-Smith and Pitt, 1990). The drawback of this method is the large number of serological cross-reactions that occur among the 77 capsule types. Thus, individual sera have to be absorbed with the cross-reacting K-antigens. Moreover, the typing procedure is cumbersome because of the time needed to perform the test and is susceptible to subjective interpretations because of weak reactions that are not always easy to interpret. Since anti-capsule antisera are not commercially available, this technique is practiced mostly in specialized laboratories. However, in contrast to capsule typing, neither biochemical typing, bacteriocin typing, nor phage typing alone is sufficiently discriminative and reproducible for epidemiological purposes except under certain conditions (Ørskove and Ørskove, 1984). The combined use of biotyping and capsule typing enables the differentiation of a large number of bioserotypes (Renine and Duncan, 1974).

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Phage Typing

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Phage typing of *Klebsiella* was first developed in the 1960s (Przondo-Hessek, 1966). Although the phage reaction is easily read and the reproducibility of the method is acceptable, this technique shows a relatively poor typing rate of 19 to 67% (Rubin, 1985). Since it is not an alternative to capsule typing, this procedure has never become widespread and is useful mainly as a secondary method in combination with serologic testing. It should be stressed, however, that it is possible to develop capsule- and O-antigen-specific phage typing if appropriate efforts are made, as a number of reports have demonstrated (McCallum *et al*., 1989; Pieroni *et al*., 1994).

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Bacteriocin Typing

Bacteriocins are bactericidal substances, usually proteins, produced by bacteria to inhibit the growth of other bacteria, usually members of the same species. An isolate can be characterized either by its ability to inhibit specific indicator strains or by its sensitivity to bacteriocins synthesized by a set of producer strains. Since the synthesis of bacteriocins is not frequent enough in *Klebsiella*, the latter technique has become the method of choice for bacteriocin typing of organisms belonging to this genus (Hall, 1971). ~~A modification~~Modifications of the "scrape-and-point" procedure, in which the bacteriocins are synthesized on an agar medium immediately before the strains to be typed are inoculated by a multipoint inoculator. This method has proven superior for bacteriocin typing of clinical and environmental *Klebsiella* strains as well as of nosocomial outbreaks of *Klebsiella* (Bauernfeind *et al*., 1993; Podschun and Ullmann, 1993; Podschun and Ullmann, 1996).

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Molecular Typing Methods

~~Molecular typing methods, as applied to the genus *Klebsiella*, are still in their infancy.~~ Preliminary descriptions have been presented on plasmid profiles, ribotypes, multilocus enzyme analyses, and applications of pulsed-field gel electrophoresis. The procedures vary from laboratory to laboratory and lack

standardization, making it difficult to compare them (Bauernfeind *et al*., 1993; Arlet *et al.,* 1994; Nouvellon *et al*., 1994).

2-11 Epidemiology

Klebsiella spp. are ubiquitous in nature. *Klebsiellae* probably have two common habitats, one being the environment, where they are found in surface water, sewage, and soil and on plants, and the other being the mucosal surfaces of mammals such as humans, horses, or swine, which they colonize. In this respect, the genus *Klebsiella* is like *Enterobacter* and *Citrobacter* but unlike *Shigella* spp. or *E. coli*, which are common in humans but not in the environment (Seidler *et al*., 1975).

In humans, *K. pneumoniapneumoniae* is present as a saprophyte in the nasopharynx and in the intestinal tract. Carrier rates differ considerably from study to study. The detection rate in stool samples ranges from 5 to 38%, while rates in the nasopharynx range from 1 to 6 % (Rosenthal and Tager, 1975).

These carrier rates change drastically in the hospital environment, where colonization rates increase in direct proportion to the length of stay. Reported carrier rates in hospitalized patients are 77% in the stool, 19% in the pharynx, and 42% on the hands of patients (Cooke *et al*., 1979). The high rate of nosocomial *Klebsiella* colonization appears to be associated with the use of antibiotics rather than with factors connected with delivery of care in the hospital (Pollack *et al*., 1972). Previous antibiotic therapy is significantly associated with acquisition of *Klebsiella* by the patient. Furthermore, widespread use of antimicrobial therapy has often been held responsible for the occurrence of multiply multiple resistant *Klebsiella* strains in hospitals (Tullus *et al*., 1988).

Apart from medical equipment (contaminated due to faulty hygienic procedures) and blood products, the principal reservoirs for transmission of *Klebsiella* in the hospital setting are the gastrointestinal tract of patients and the hands of hospital personnel (Montgomerie, 1979).

2-12 Pathogenicity Factors of *Klebsiella*

The terms "pathogenicity factor" and "virulence factor" are used synonymously by some authors (Schaechter and Eisenstein, 1993), while others lay emphasis on a clear-cut distinction between them. The term "pathogenicity" defines the ability of a bacterium to cause disease while "virulence" is the measurement or degree of pathogenicity of any bacterial species (Podschun and Ullmann, 1998**).**

Nosocomial *Klebsiella* infections most commonly involve the urinary and respiratory tracts. Since these two body sites differ considerably with respect to the host defense mechanisms, it should be expected that the pattern of virulence factors found in UTI-causing strains of *Klebsiella* will differ from that observed in strains isolated from pulmonary sources of patients with pneumonia (Podschun and Ullmann, 1998**).**

A number of bacterial factors are contributed to the pathogenesis of *Klebsiella*. Both *in vitro* and *in vivo* models have been established to investigate the interaction of bacterial cells and the host.

Klebsiella has several factors (Fig 2-1) which may involve in its pathogenicity.

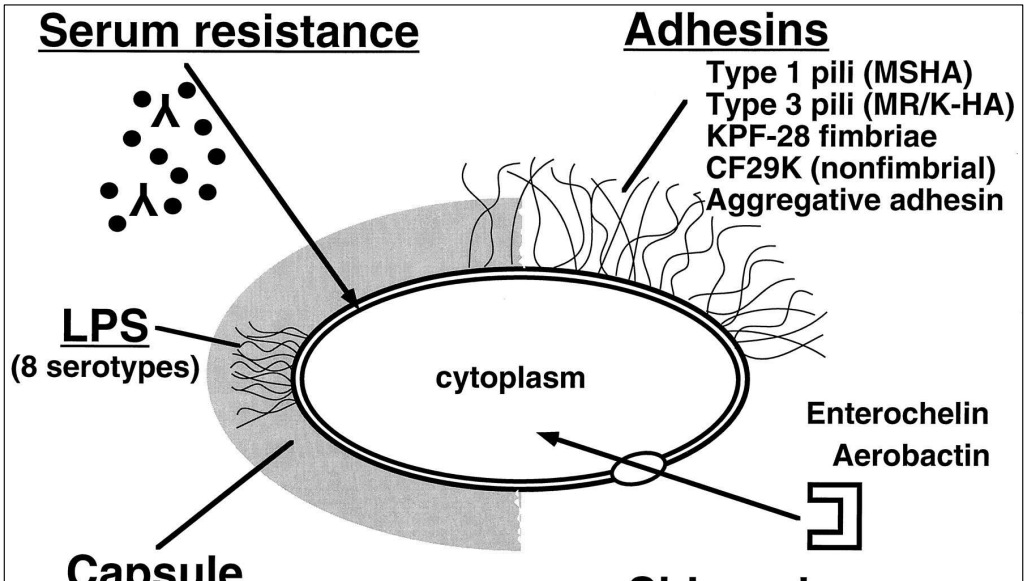

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Figure (2-1) Schematic representation of *Klebsiella* pathogenicity factors (Podschun and Ullmann, 1998)

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2-12-1 Capsular Antigens

Klebsiella usually develop prominent capsules composed of complex acidic polysaccharides. The capsular repeating subunits, consisting of four to six sugars and, very often, uronic acids (as negatively charged components), can be classified into 77 serological types ~~(Ørskov and Ørskov, 1984)~~. Capsules are essential to the virulence of *Klebsiella* (Highsmith and Jarvis, 1985). The capsular material forms thick bundles of fibrillous structures covering the bacterial surface in massive layers (Amako *et al.*, 1988). This protects the bacterium from phagocytosis by polymorphonuclear granulocytes, on the one hand, and prevents killing of the bacteria by bactericidal serum factors, on the other (Podschun and Ullmann, 1992; Podschun *et al.*, 1992). 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).

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While *Klebsiella* capsular polysaccharide (CPS) were generally considered to mediate virulence properties, this consideration has recently been abandoned because of the great differences in virulence observed among different capsular types: strains expressing the capsule antigens K1 and K2 were found to be

especially virulent in a mouse peritonitis model, whereas isolates of other serotypes showed little or no virulence (Mizuta *et al*., 1983).

The degree of virulence conferred by a particular K antigen might be connected to the mannose content of the CPS. Capsular types with low virulence, such as the K7 or K21a antigen, contain repetitive sequences of mannose-α-2/3-mannose or L-rhamnose-α-2/3-L-rhamnose. These sequences are recognized by a surface lectin of macrophages, which mediates opsonin-independent (i.e., complement- and antibody-independent) phagocytosis, known as lectinophagocytosis ~~(Athamna *et al*., 1991)~~. Thus, *Klebsiella* strains bearing capsule types devoid of these mannose or rhamnose sequences should be more closely associated with infectious diseases~~.~~ (Athamna *et al*., 1991).

2-12-2 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~~enterobacteriaceae are generally mediated by different types of pili. ~~Pili (otherwise known as fimbriae) are nonflagellar, 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). Of the different types of pili described in enterobacteria, there are two predominant types in *Klebsiella* spp.

Type 1 (common) pili. They are MSHA which agglutinate guinea pig erythrocytes. The adhesion protein in this pilus type is located on the fimbrial

shaft and is capable of binding to mannose-containing trisaccharides of the host glycoproteins (Babu *et al*., 1986). The relevance of these pili to bacterial virulence is thought to arise mainly from binding of the bacteria to mucus or to epithelial cells of the urogenital, respiratory, and intestinal tracts (Venegas *et al*., 1995). Their role in the pathogenesis of UTI was clarified mostly in studies on *E. coli* but has also been described for *K. ~~pneumonia~~pneumoniae~~e~~* in animal models (Maayan *et al*., 1985; Fader and Davis, 1982). These structures have been shown to bind effectively to proximal tubulus cells, soluble, mannosyl-containing glycoproteins in urine, such as the Tamm-Horsfall protein, or in saliva (Virkola *et al*., 1988; Reinhardt *et al*., 1990). These findings provide an explanation for the fact that type 1 pili mediate bacterial colonization of the urinogenital and respiratory tracts (Clegg and Gerlach, 1987).

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Type 1 pili are important for host colonization, but their contribution to subsequent steps of pathogenesis is less clear.

~~Gerlach *et al*., (1989) referred that the gene which responsible of type 1 pili are carried either on chromosome or on conjugative plasmid.~~

Type 3 pili. Unlike other fimbriae, type 3 pili agglutinate only erythrocytes that have been treated with tannin. Although its name, mannose-resistant~~,~~ / *Klebsiella*-like hemagglutination (MR/K-HA), implies that this fimbrial type is synthesized only by *Klebsiella*, ~~later~~ studies demonstrated that type 3 pili occur in many enteric genera, but they are not identical in all genera of enterobacteria, (Old and Adegbola, 1985). Originally described as the adhesion organelles of *Klebsiella* inhabiting plant roots (Korhonen *et al*., 1983), these pili were later found to be capable of binding to various human cells. Strains of *K. ~~pneumonia~~pneumoniae~~e~~* expressing type 3 pili adhere to endothelial cells, epithelia of the respiratory tract, and uroepithelial cells (Tarkkanen *et al*., 1990; Hornick *et al*., 1992~~; Würker *et al*., 1990; Tarkkanen *et al*., 1997~~). In the kidneys, these pili mediate bacterial adhesion to tubular basement membranes,

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Bowman's capsules, and renal vessels (Tarkkanen *et al*., 1990). Binding to tannic acid-treated erythrocytes is inhibited by spermidine, a polyamine that is also secreted in urine (Gerlach *et al*., 1989). Since spermidine is exposed on the cell surface of damaged erythrocytes, it has been suggested that MR/K hemagglutination is mediated by spermidine (Gerlach *et al*., 1989). This might explain why type 3 pili bind to tannic acid- or heat-treated erythrocytes but not to untreated erythrocytes.

The role of this fimbrial type in the pathogenetic process is largely unknown.

Three new types of *Klebsiella* adhesins have been recently reported. The R-plasmid-encoded CF29K adhesin of *K. pneumoniae* has been demonstrated to mediate adherence to the human intestinal cell lines Intestine 407 and CaCo-2 (Darfeuille-Michaud *et al*., 1992). This adhesin type seems to be identical to the CS31-A adhesive protein of human diarrheal *E. coli* strains (Di Martino *et al*., 1995) and belongs to the K88 adhesin family. The available data suggest that CF29K probably is a product of the transfer of CS31A genetic determinants from *E. coli* to *K. pneumoniae* strains in the human intestine. A particular adherence pattern characterized by aggregative adhesion to intestinal cell lines is mediated by another new *Klebsiella* adhesin that seems to be composed of capsule-like extracellular material (Favre-Bonte *et al*., 1995). While the two adhesins mentioned above are nonfimbrial, a third putative colonization factor of the human gut is a new fimbria that has been termed KPF-28 (Di Martino *et al*., 1996). Interestingly, this fimbrial type has been found in the majority of *K. pneumoniae* strains producing CAZ-5/SHV-4 type ESBL (Di Martino *et al*., 1996).

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2-12-3 Serum Resistance and Lipopolysaccharide

The first line of defense by the host against invading microorganisms includes, in addition to phagocytosis, the bactericidal effect of serum. The serum

bactericidal activity is mediated primarily by complement proteins. After their cascade-like activation, these proteins accumulate as membrane attack complex on the surface of the microorganism (Taylor, 1983). This complex consists of the terminal complement proteins C5b-C9, which produce a transmembranous pore in the outer membrane of gram-negative bacteria, leading to an influx of Na^+ and subsequent osmotic lysis of the bacteria (Ramm *et al*., 1983; Taylor and Kroll, 1985).

Most commensal gram-negative bacteria are sensitive to the bactericidal effect of human serum, whereas pathogenic strains often exhibit serum resistance properties (Ollings, 1977). Thus, clinical isolates of enterobacteria often show resistance to serum, and the feature "serum resistance" has been correlated with the onset of infection and severity of symptoms (Roantree and Rantz, 1960; Gower *et al*., 1972; Virkola *et al*., 1988). Since the main role of the serum bactericidal system is thought to prevent microorganisms from invading and persisting in the blood, even differences in the degree of bacterial serum susceptibility may determine whether a strain is able to infect as well as the length of time it takes the organisms to establish the infection.

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To date, the exact mechanism underlying bacterial serum resistance is unknown. For *Klebsiella*, two hypotheses have been propounded. First, capsule polysaccharides may cover and mask the underlying LPS and exhibit a surface structure that does not activate complement. On the other hand, the O side chains of the LPS may reach through the capsule layer and be exposed to the exterior milieu in certain *Klebsiella* capsule types, thus C3b become far away from the bacterial cell membrane. Thus, the formation of the lytic membrane attack complex (C5b-C9) is prevented, and subsequent membrane damage and cell death do not take place (Merino *et al*., 1992; Tomas *et al*., 1988; Merino *et al*., 1992).

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Serum resistance does not seem to be a stable characteristic; environmental factors affect the composition and effect of LPS. ~~Recently,~~ ~~T~~the influence of different osmolarity conditions on LPS was demonstrated in which the same bacterial strain may be serum resistant at host body sites with a high-osmolarity milieu, such as the urinary tract, and serum sensitive at low-osmolarity body locations like the respiratory tract (Aguilar *et al*., 1997).

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2-12-4 Siderophores

The growth of bacteria in host tissue is limited not only by the host defense mechanisms but also by its supply of available iron. Iron is an essential factor in bacterial growth, functioning mainly as a redox catalyst in proteins participating in oxygen and electron transport processes (Griffiths, 1987). The supply of free iron available to bacteria in the host milieu is extremely low, since this element is bound intracellularly to proteins such as hemoglobin, ferritin, hemosiderin, and myoglobin and extracellularly to high-affinity iron-binding proteins such as lactoferrin and transferrin. The level of free, bioavailable iron (10^{-18} M) is several thousandfold too low for normal bacterial growth (Bullen *et al*., 1978). The marked effect of the iron supply in the host body on the pathogenesis of infections has been demonstrated for *Klebsiella*. After parenteral administration of iron in a guinea pig model, the susceptibility to *K. ~~pneumonia~~pneumoniae* infections increased dramatically (Khimji and Miles, 1978).

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). Under iron-deficient conditions, e.g., in the host milieu, enterobacteria synthesize a variety of siderophores, which belong to two different chemical groups, one consisting of the phenolate-type siderophores ~~and~~and the other one consisting of the hydroxamate-type siderophores (Podschun and Ullmann, 1998).

Klebsiella are synthesizing two different types of siderophores: first type is phenolate-type siderophores which known as enterobactin (also known as enterochelin), is a cyclic trimer of 2,3-dihydroxy-benzoyl-serine. 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).

Enterobactin synthesis are expressed by chromosomal gene, which found in most of the enteric bacteria.

The second type is hydroxamate-type siderophores which known as aerobactin and its found in most of pathogenic bacteria (Pyen, 1988).

~~Most of aerobactin are synthesized by genes carried on plasmid (180 kDa) which was isolated from *Klebsiella* (Nassif and Sanonetti, 1986), while in *Yersienia* aerobactin genes were carried on chromosome (Heesemann, 1987).~~

~~Molecular study showed that there are four genes encoded for aerobactin synthesis: iuc A, iuc B, iuc C and iuc D, while there is another gene called iut A encode for iron receptor at the aerobactin.~~

~~Aerobactin differ from enterobactin in the following points:~~

1. ~~Enterobactin is much higher affinity for Fe (III) than that of ferric aerobactin (K_s 10^{52} and 10^{23}, respectively).~~
2. ~~Aerobactin is more effective that enterobactin because the greater stability and better solubility.~~
3. ~~After delivery of iron aerobactin can be recycled after each turn of iron transport, while enterobactin becomes hydrolyzed by an esterase (Wooldridge *et al*., 1993).~~

Data on the incidence of aerobactin-producing *Klebsiella* indicates that this siderophore does not play a central role in the pathogenicity of the genus *Klebsiella*. However, clinical *K. ~~pneumonia~~pneumoniae~~e~~* isolates, which do not

synthesize aerobactin themselves, are entirely capable of using exogenously introduced aerobactin as their sole source of iron (Williams *et al*., 1989). By synthesizing only the intrinsically expressed aerobactin receptor, such strains could derive an advantage over other aerobactin-synthesizing bacteria in mixed infections. The aerobactin-mediated iron uptake system would thus be an indirect contributor to the pathogenicity of the genus *Klebsiella* (Podschun and Ullmann, 1998).

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2-13 Antibiotics Resistance Mechanisms:

Klebsiella have different types of antibiotic resistance mechanisms:

i. **The production of detoxification enzyme**: like β-lactamase which distroy β-lactam antibiotics, or the production of chloramphenicol acetyltransferase (Reish *et al*., 1993; Pfeifle *et al*., 2000).

ii. **Alteration in the target for the drug**: include both reduction of receptor affinity and the substitution of an alternative pathway (Jacoby and Archer, 1991). For example, quinolones antagonize bacteria by inhibiting DNA gyrase, most *Klebsiella* resistant to ciprofloxacin have a gayrase target altered by changing one amino acid (Ser-83 with Tyr and Pha) (Brisse *et al*., 2000).

iii. **Decreased antibiotic uptake**: by diminished permeability of cell wall. *Klebsiella* become resistance to β-lactam antibiotic through either the loss of an outer-membrane protein (porin) or synthesizing a new one with less permeability that prevent β-lactam from entry to the cells (Antonio, 1999).

iv. **Over expression**: the chromosomal β-lactamase of bacteria is made in low amounts that it dose not contribute appreciably to β-lactam resistance. Most of pathogenic strains have a promoter with efficient expression

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which carried either on plasmid or inserted into chromosome. This increase the amount of β-lactamase produced by the cells (Smith *et al*., 1990).

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2-14 ~~A~~ Molecular Perspective of Microbial Pathogenicity

~~*Genes Responsible for the Virulence of Klebsiella*~~

Virulence factors refer to the properties (i.e., gene products) that enable a microorganism to establish itself on or within a host of a particular species and enhance its potential to cause disease. Pathogenicity is not a microbial trait that has appeared by chance. Instead, particular microbial strains and species have evolved to carry very specific arrays of virulence-associated genes (Relman and Falkow, 2000——). Although, most of the genes responsible for the virulence of microbe (such as the capsule, adhesion pili and antibiotic resistance) are carried on chromosomes, a number of separate observations indicate that microbes frequently carry virulence-associated genes on mobile genetic elements (Finally and Falkow, 1997). Bacteriophages and extrachromosomal elements such as bacterial plasmids are supplements to the bacterial genome that allow a microbe to maintain the integrity of its chromosome and still increase its genetic diversity (Cheetham and Katz, 1995).

Some of these mobile elements are able to enter a wide variety of host organisms and may facilitate the transfer of genes that have been selected for their ability to function in diverse genetic backgrounds (Campbell, 1981). The presence of virulence factors in pathogenic bacteria is also associated with the presence of plasmids, transposons, and bacteriophages (Elwell and Shipley, 1980; Cheetham and Katz, 1995).

~~Often, the mobile element carries multiple virulence-associated genes as a coadapted block, accompanied by a separate self-regulatory system. Some transmissible elements such as bacteriophages prefer specific chromosomal sites~~

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~~for integration, for example, tRNA genes. This specificity may explain the chromosomal distribution of some virulence genes (Cheetham and Katz, 1995).~~

Pathogenic bacteria have distinct blocks of DNA carrying closely linked virulence genes called Pathogenicity Islands (PAIs) (Hacker *et al*., 1990).

These PAIs can be part of plasmid, bacteriophages or may represent particular fragments of the genome~~)~~ (Hacker *et al*., 1990, 1999~~0; Hacker *et al*., 1999~~).

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PAIs are 35- to 200-kilobase segments of chromosomal DNA flanked by insertion or repeat elements, with a nucleotide composition quite unlike the surrounding bacterial genome. Pathogenicity islands contain clusters of virulence-associated genes that encode specialized secretion systems, secreted effector molecules, adhesins, and regulatory proteins (Groisman and Ochman, 1996; Hacker *et al*., 1997)

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α-hemolysin and P fimbrial adhesin gene clusters were the first determinants which were shown to be closely linked on PAIs in pathogenic *E. coli* (High *et al*., 1988). The presence of at least one virulence gene cluster is one of the main features of PAIs. In addition they may be characterized as follows:

1. they occupy large, genomic DNA regions,
2. they are inserted near or within tRNA genes
3. they contain direct repeats and mobility sequences
4. they have a G+C content different from that of the host bacterium (Hacker *et al*., 1990).

~~The major PAIs are that which contains antibiotic resistance gene, these are found either on the chromosomes or carried on plasmid.~~

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2-15 Bacterial Plasmids and Their Association with Pathogenicity

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Plasmids are extrachromosomal circular DNA molecules found in most bacterial species and in some species of eukaryotes. Most of known plasmids are DNA ~~molecules~~molecules; only with one exception is ~~that~~ the killer-plasmid of yeast which is an RNA molecule (Friefelder, 1987). They have their origin~~—~~ of replication, autonomously replicate with respect to chromosomal DNA and stably inherited (Satta *et al.*, 1987; Frie~~i~~felder, 1987). 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 (Jawets *et al.*, ~~1995~~2001).

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Some plasmids are conjugative (transmissible) and others are non conjugative (non transmissible) plasmids. The best known example of conjugative plasmids is F-element which ha~~ves~~ a molecular weight of 62.5~~*~~x10^6 dalton, and contains at least 19 genes necessary~~needed~~ for transfer process called *tra* genes (Goodenough, 1984; Freifelder, 1987).

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

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Normally, plasmids are dispensable to its host cell, though many plasmids contain genes that may be essential in certain environment. R-plasmids carry genes that confer resistance to numerous antibiotics, Environmentally,~~so in nature~~ a cell containing such plasmids had a better chance to survive~~can better survive~~. ~~Resistance genes to the drugs tetracycline, penicillin, ampicillin, chloramphenicol, streptomycin, kanamycin and sulfonamide appears~~Resistance genes to the drugs tetracycline, penicillin, ampicillin, chloramphenicol, streptomycin, kanamycin and sulfonamide are located ~~commonly~~ on R-plasmids (Jacoby, 1994; Jawets *et al.*, ~~1995~~2001). Plasmids may also encode resistance to wide variety of agents that are toxic to bacteria such as

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bacterophages, bacteriocins, heavy metals, detergents, serum components and other environmental poisons (Molnar, 1988; Jawets *et al*., 19952001).

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Predictably, tThese virulence factors have been found in plasmids that also contain drug resistance determinants (Gowal *et al*., 1985)

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Genes responsible for the production of the CFA/I pili in some *Klebsiella* serotype are found on chromosomchromosome al andor on conjugative plasmid. DNA hybridization methods found that DNA sequence of thieses genes are the same as resample that found in other genus in the Eenterobacteriaceaeenterobacteriaceae family (Gerlach *et al*., 1989b).

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Also plasmid mightay carrying genes that enhance the production of the chromosomal genes products for example, there are two chromosomal genes play a role in the active expression of capsular polysaccharide synthesis: ***rcsA*** (regulation of capsule synthesis) which encoded a polypeptide of 23 kDa that iswas required for the induction of a mucoid phenotype and ***rcsB*** which encodesed no apparent polypeptides and wais not capable by itself of causing the overproduction of colanic acid, but when present in the same cell with ***rcsA***,, *either in cis or in trans*, ***rcsB*** caused expression of mucoidy (McCallum and Whitfield, 1991)Allen *et al*., 1987).

There isare another two genes which are carried on plasmid (180 kb) which is are responsible ofor the higher production of mucoid, these two genes are: ***rmp A*** (regulation of mucoid phenotype gene) which encoded a polypeptide of 15.5 kDa, and ***rmp B*** which encoded for a polypeptide of 37 kDa. This plasmid also is carrying genes encoded for aerobactin production (Nassif and Sansonetti, 1986).

Nassif and Sanonetti, (1986) found that aerobactin genes are carried on large plasmid (180 kb) in *Klebsiella*. Moreover,Also aerobactin encoding plasmid was isolated from *E. coli* (Williams *et al*., 19798).

Vernet *et al*., (1995) found that aerobactin production represents an important virulence factor for the pathogenesis of *Klebsiella* and specially K1

and K2 serotype which are encoded by genes located on large plasmid, and when this plasmid is lost, pathogenicity is decreased in mice.

Most of aerobactin are synthesized by genes carried on plasmid (180 kDa) which was isolated from *Klebsiella*, while in *Yersienia* aerobactin genes were carried on chromosome (Nassif and Sanonetti, 1986). Molecular study showed that there are four genes resposible for aerobactin synthesis: ***iuc A, iuc B, iuc C*** and *iuc D*, while there is another gene called ***iut A*** which encodes for iron receptor at the aerobactin (Heezemann, 1987).

~~Vernet *et al*., (1995) found that aerobactin production represent an important virulence factor for the *Klebsiella* and specially K1 and K2 serotype which carried aerobactin genes on large plasmid, and when they lose this plasmid its pathogenicity decreased in mice.~~

In *E. coli*, ~~Some times genes for the same trait are found on chromosome and on plasmid such as most of β-lactamase enzymes are encoded by chromosomal and plasmid genes (Livermore, 1995). Also the genes responsible for the production of the CFA/I pili are found on chromosomal and on conjugative plasmid. DNA hybridization methods found that DNA sequence of this genes are resample that found in other genus in the *Enterobacteriaceae* family (Gerlach *et al*., 1989b)~~

~~Some traits are encoded by chromosomal genes, but another genes which encoded by plasmid genes are enhance the production of the chromosomal genes products for example, there are two chromosomal genes play a role in the active expression of capsular polysaccharide synthesis: rcsA (regulation of capsule synthesis) which encoded a polypeptide of 23 kDa that was required for the induction of a mucoid phenotype and rcsB which encoded no apparent polypeptides and was not capable by itself of causing the overproduction of colanic acid, but when present in the same cell with rcsA, either in cis or in trans, rcsB caused expression of mucoidy (J Gen Microbiol (1987-02-01)).~~

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~~Genes for the various urovirulence factors are often duplicated in uropathogens and also frequently linked as large multigene chromosomal segments called *pathogenicity islands* and are absent in coliforms found in normal fecal flora (Podschun and Ullmann, 1998).~~

~~There is another genes which carried on plasmid (180 kb) are responsible of the higher production of mucoid, these two genes are: rmp A (regulation of mucoid phenotype gene) which encoded a polypeptide of 15.5 kDa, and rmp B which encoded for polypeptide of 37 kDa. This plasmid carrying genes encoded for aerobactin production.~~

the genetics of hemolysins are probably best ~~worked out~~studied ~~for the~~ alpha type variety, which are encoded by both plasmid and chromosomal genes (Cavalieri *et al*., 1984). There is little structural homology between the plasmid-encoded and the chromosomally encoded hemolysins, which has led investigators to believe that these hemolysins serve different virulence functions (Muller *et al*., 1983).

In some strains of *E. coli* that contain the colicin V plasmid, a particular genetic determinant, *iss*, has been shown to encode resistance to the bactericidal action of serum and complement (B~~e~~inns *et al*., 1982).

Even in the absence of the drug resistance determinants, plasmids found in certain strains of virulent bacteria have been found to carry multiple virulence factors. For instance, plasmids found in enterotoxigenic strains of *E. coli* have been shown to carry genes both for enterotoxin production and for the colonization factor antigen found to be important in colonization of the human gastrointestinal tract (Evans *et al*., 1975).

2-~~13~~ 15-1 Role of Plasmid in Antibiotic Resistance:

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~~The wide spread and misuse of antimicrobial agents resulted in the development of antimicrobials resistant strains of bacteria due to the evolution of genetic materials that encoding for such resistance (Waller *et al*., 1997).~~

~~More often r~~Resistance genes are carried on plasmids that may be transferable from organism to organism by conjugation, transformation or transduction (Jawetz *et al*., ~~1995~~2001). Resistant members of enterobacteriacea frequently contain multiple plasmids, the larger of which can carry for resistance to 10 or more antimicrobial agents (Jacoby and Han, 1996). In part this is the result of having survived in a nosocomial environment in which multiple antibiotics are employed. This multi-resistance is a problem in treating infected patients since it limits available options (Jacoby and Archer, 1991).

Large multi-resistance plasmid encoding linked resistance to β-lactam, all aminoglycosides, chloramphenicol, sulfonamides, tetracyclines and trimethoprim was found in *Klebsiella* strains isolated from UTI (Sirot *et al*., 1991).

Klebsiella show β-lactam resistant due to its synthesis of β-lactamase enzyme which destroy the β-lactam ring before reaching the target (Pfeifle *et al*., 2000).

The most common plasmid encoded β-lactamases in *Klebsiella* are TEM-1 and SHV-1 which are also frequent in *E. coli*. These enzymes confer resistance to ampicillin, carbencillin, ticarcillin and cephalothin (Jacoby and Medeiros, 1991).

Plasmid borne β-lactamases capable of hydrolyzing penicillins was the principal stimulus to the development of the compounds that inhibit β-lactamase, e.g. clavulanic acid, sulbactam and tazobactam (Cormican *et al*., 1996).

In Japan, transmissible resistance to carbapenems has been described in isolates of *Klebsiella pneumonia, P. aer~~o~~uginosa and S. marcescens* by plasmid acquisition of a metallo- β-lactamase of alarmingly broad specificity. These

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strains were resistant to carbapenems, cephalosporins and β-lactamase inhibitors (Senda *et al*., 1996).

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~~ESBLs are enzyme usually plasmid mediated. Since these plasmids are easily transmitted among different members of the *Enterobacteriaceae* by conjugation, transduction and transformation, accumulation of resistance genes results in strains that contain multiresistant plasmids. For this reason, ESBL-producing isolates are resistant to a variety of classes of antibiotics. Moreover, the emergence of these multiply resistant *Klebsiella* strains is unfortunately accompanied by a relatively high stability of the plasmids encoding ESBLs. Even years after discontinuation of ceftazidime and other extended-spectrum cephalosporins, continued colonization of patients by ESBL-producing *Klebsiella* strains has been observed (Hibbertrogers *et al*., 1995).~~

There are too many type of β-lactamase enzyme, but the most important is TEM-1, TEM-2, SHV-1 and OXA-1. These enzymes are encoded by genes carried on plasmid (Livermoere, 1995). Most of these enzymes are resulted due to a mutation for example: TEM-1 is different from TEM-2 by replacing two amino acids (Sougakoff *et al*., 1988). *Klebsiella* resist the ampicillin, pipracillin and cephalothin antibiotic by the synthesizing of SHV-1 and LEN-1 (Itokazu *et al*., 1996). CAZ-7 (which is TEM-1) derivative is ESBLs enzymes encoded by 85 kb plasmid which confers the resistance for amikacin, sulfanomides, tetracyclines (Sirot *et al*., 1991).

ESBLs-producing isolates are resistant to a variety of classes of antibiotics. Moreover, the emergence of these multiply resistant *Klebsiella* strains is unfortunately accompanied by a relatively high stability of the plasmids encoding ESBLs. Even years after discontinuation of ceftazidime and other extended-spectrum cephalosporins, continued colonization of patients by ESBL-producing *Klebsiella* strains has been observed (Hibbertrogers *et al*., 1995).

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Some *Klebsiella* strains have a conjugative plasmid which confer it the ability to resist the ciftazidim, cifoxtin and aztronam (Pornull *et al*., 1993).

~~Reich~~ Reish *et al*., (1993) found that *Klebsiella* resistant to chloramphenicol and other antibiotics due to the synthesis of Chloramphenicol acetyl transferase which encoded on plasmid.

PMG 252 plasmid which is multiresistance plasmid, increase the *Klebsiella* resistance to quinolones antibiotics. This plasmid also express in other Enterobacteriaceae~~s~~ family and *the Pseudomonas aeruginosa* and confer them quinolones resistance (Martinez-Martinez *et al*., 1998).

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~~12~~-~~14~~ 16 Plasmid Curing:

In nature plasmid can be lost spontaneously from a very few cells, but the probability of this loss is extremely low, ranging form 10^{-5} to 10^{-7} (~~Trevors, 1985;~~ 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 (~~Groves, 1979;~~ 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 dodecyl sulphate (SDS) affect plasmid replication (~~Hirota, 1960; Bouanchaud *et al*., 1969;~~ Tom~~o~~eda *et al*., 1968).

Elevated temperature and thymine starvation also affect on plasmid replication (Groves, 1979; 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; Molnar, 1988).

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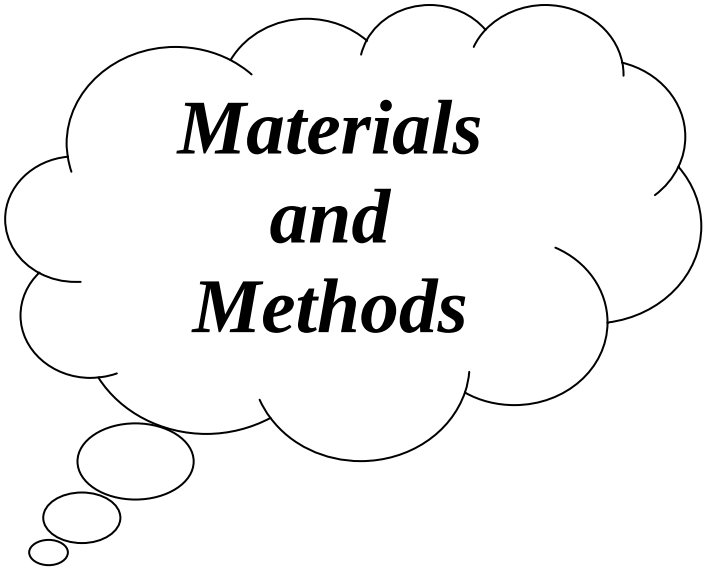
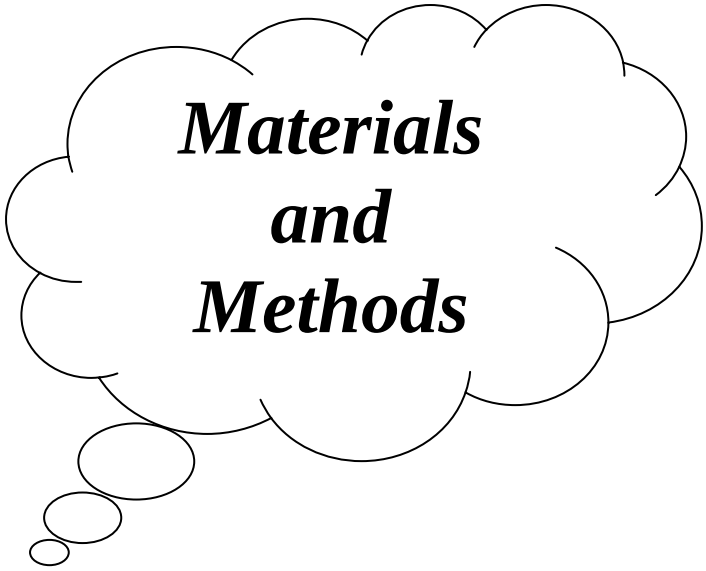


Chapter Three



Chapter Three

3.1 Materials:

3.1.1 Equipments

The following equipments were used in this study:

Equipment	**Company (Origin)**
Autoclave	Gallenkamp (England)
Balance	Ohans (France)
Compound Light Microscope	Olympus (Japan)
Distillator	Gallenkamp
Oven	Memmert (Germany)
Hot plate with magnetic stirrer	Gallenkamp
Incubator	Gallenkamp
Micropipette	Witeg (Germany)
Millipore filters	Millipore and Whatman (England)
pH-Meter	Metter-GmpH Tdedo (U.K.)
Portable Centrifuge	Hermle labortechnik (Germany)
Refrigerator Centrifuge	Harrier (U.K.)
Sensitive balance	Delta Range (Switzerland)
Shaker Incubator	GFL (Germany)
Spectrophotometer	Aurora instruments Ltd. (England)
Vortex	Buchi (Switzerland)
Water bath	GFL (England)

3.1.2 Chemicals

The following dyes and chemicals were used in this study:

Material	**Company (Origin)**
Acetic acid, glucose, Mannitol, Glycerol, HCl, Isoamyle alcohol, K_2HPO_4, Na_2HPO_4, Methylene blue, Urea, $MgSO_4$, Peptone, KH_2PO_4, Gurr R600 Giemsa, KNO_3, KCN, casamino acids, $CaCl_2$, absolute alcohol, KI, Ammonium oxalate, Iodine, Safranine O, Methyl red, Dimethyl-α-naphthylamine, $BaCl_2$, Tannic acid, α-naphthol	BDH (England)
Agar, Lactose, Tetramethyl-p-Phenylene diamine dihydrochloride, L-Phenylalanine, thyamine	Difco (U.S.A)
Hydrogen peroxide, Mannose, KOH, Tryptone, Yeast extract, beef extract	Fluka (Switzerland)
Indian Ink, bromcresol purple	Oxoid (England)
NH_4Cl, KOH, ethidium bromide	Merck (Germany)
2-2dipyridyl, Crystal violate,	Sigma (England)
Ethanol, Methanol, NaCl, p-dimethyl-amino-benzaldehyde	Riedel-DeHaeny (Germany)

3.1.3 Culture Media:

3.1.3.1 Ready to Use Media:

Medium	Company (Origin)
Brain heart infusion agar	Difco (U.S.A)
Brain heart infusion broth	Difco
MacConkey agar	Oxoid (England)
Nutrient agar	Oxoid (England)
Nutrient broth	Oxoid (England)
Simmon citrate media	Difco (U.S.A)
Triple sugar iron (TSI) agar	Difco (U.S.A.)
Tryptic soy agar	Biolife (Italy)
Urea agar base	Biolife

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

3.1.3.2 Laboratory Prepared Media

-Blood Agar (Atlas *et al*., 1995)

It was prepared by dissolving 37g of blood base agar (Mast diagnosis, England) in 950 ml of D.W. and autoclaved. After cooling to 50ºC, 5% of the blood was added to it, mixed well and distributed into petri-dishes.

- Peptone Water Medium (Atlas *et al*., 1995)

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

Peptone	20 g
Sodium chloride	5 g

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

- Methyl Red and Voges-Proskauer (MR-VP) Medium (Atlas *et al*., 1995)

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

Peptone	5 g
K_2HPO_4	5 g
Glucose (10% solution)	50 ml

Peptone and phosphate were dissolved, pH was adjusted to 7.6 and sterilized by autoclaving. Glucose was sterilized by filtration and added.

-Urea Agar Medium (Collee *et al*., 1996):

It was prepared by adjusting pH of the urea agar base (Christensens media) to 7.0 and autoclaved. Then it was cooled to 50˚C. Then 50 ml of 40% urea (sterilized by filtration).

- Nitrate Medium (Atlas *et al*., 1995)

This medium composed of peptone 5g supplemented with 0.2g KNO_3 in 1L of D.W. Distributed into tubes and sterilized by autoclaving.

- KCN Medium (Collins and Lyne, 1985)

It was prepared by adding 0.5% of KCN to nutrient broth. pH was adjusted to 7.0, and sterilized by autoclaving.

-Phenylalanine Deaminase (PAD) Medium (Holt *et al*., 1994)

It was prepared by dissolving trypton (15g), L-phenylalanine (10g) in 1L D.W. pH was adjusted to 7.0 and sterilized by autoclaving.

-Carbohydrate Fermentation Medium (Atlas *et al*., 1995)

It was prepared by dissolving peptone (10g), sodium chloride (5g), beef extract (3g) and bromcresol purple (0.04g) in D.W. pH was adjusted to 7, the volume was brought to 990 ml and sterilized by autoclaving. Ten ml of carbohydrate solution (lactose) (3.1.5.6) was added and mixed thoroughly and distributed into test tubes that contain an inverted Durham tube.

-Semi Solid Agar Medium (Collee *et al*., 1996)

It was prepared by dissolving 0.4% of agar in nutrient broth or peptone water, in which the final medium should be quite clear and transparent. Ten ml were dispensed in test tubes and left to set in the vertical position.

- Luria Broth, Luria Agar (Cruickshank *et al*., 1975)

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

Trypton	10
Yeast extract	5
NaCl	5
Glucose	1

Components were dissolved in 1L of D. W. pH was adjusted to 7.5 then sterilized by autoclaving. To prepare Luria agar, 20 g of agar powder was added and sterilized by autoclaving.

-Modified M9 Minimal Medium (Sambrook *et al*., 1989)

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

$Na_2HPO_4.7H_2O$	12.8
KH_2PO_4	3
NH_4Cl	1
NaCl	0.5

Components were dissolved in D.W.; pH was adjusted to 7.2 and sterilized by autoclaving.

The following components were prepared as stock solutions. Sterilized by filtration and added to the medium.

Casamino acids (50%)	10 ml
Glucose (20%)	10 ml
Thymine (0.5%)	10 ml
$MgSO_4$ (2 M)	5 ml
$CaCl_2$ (0.5 M)	5 ml

- Siderophore Production Medium (Nassif and Sansonetti, 1986)

It was prepared by adding 2 g of agar to 100 ml of modified M9 medium followed by adding 0.2 M of 2-2 dipyridyl (which was sterilized by filtration).

- Mannitol Salt Agar Medium (Collee *et al*., 1996)

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

Peptone	10
Mannitol	10
Sodium chloride	7.5
Phenol red	0.025
Agar	20

pH was adjusted to 7.4, mixed thoroughly, sterilized by autoclaving, then cooled to 50ºC and poured into petridishes.

3.1.4 Dyes and Reagents

3.1.4.1 Gram Stain (Atlas *et al*., 1995)

A- Primary Stain:

Composition per 100 ml	
Solution A	20 ml
Solution B	80 ml

Solution A:

Composition per 20.0 ml:	
Crystal Violet	2 g
Absolute Alcohol (Ethanol)	20 ml

Solution B:

Composition per 80.0 ml:	
Ammonium oxalate	0.8 g
D.W.	80 ml

Twenty millimeter of solution A were mixed with 80 ml of solution B, allowed standing for 24 hr, and filtrated.

B- Gram's Iodine Mordant Solution:

Composition per 300 ml:	
KI	2 g
Iodine	1 g

KI was grinded with iodine by mortar and pestle, and added to D. W. with continuous grinding until the iodine is completely dissolved. The volume was brought to 300 ml, then stored in dark bottles.

C- Counter Stain:

Composition per 110 ml:	
Safranin O	0.25 g
Ethanol (95% v/v)	10 ml

Safranin O was dissolved in 10 ml of 95% ethanol. Then 100 ml of D.W. was added and mix thoroughly. Allow standing for several days and it was filtrated.

3.1.4.2 Giemsa Stain (BDH Chemical Ltd. England)

It was prepared by adding 2 g of Gurr R_{66} Giemsa powder to 100 ml of absolute methanol, stirred for two hours at 50ºC on the hot plate, incubated at 37ºC for 24 hours and filtrated before used.

The stain was diluted before use by adding 1 volume of filtered stain in four volumes of Sorenson's buffer for 5-15 min. The stain stored in a dark place.

3.1.4.3 Oxidase Reagent (Atlas *et al*., 1995)

One percent of tetramethyl-*p*-phenylenediamine dihydrochloride in D.W. was prepared freshly.

3.1.4.4 Hydrogen Peroxide (Atlas *et al*., 1995)

A solution of 3% hydrogen peroxide was prepared.

3.1.4.5 Methyl Red Indicator (Collee *et al*., 1996)

This indicator was prepared by mixing the following components:

Methyl red	0.05 g
Ethanol (95% v/v)	150 ml
D. W.	100 ml

3.1.4.6 Barritt's Reagent (Collee *et al*., 1996)

It consist of two solutions

Solution A: Potassium hydroxide (40%)

Solution B: It was prepared by dissolving 5 g of α-naphthol with 100 ml of absolute ethanol.

3.1.4.7 Nitrate Test Reagent (Atlas *et al*., 1995)

Solution A:

It was prepared by adding 0.8 g of sulfanilic acid to 100 ml of 5*N* acetic acid.

Solution B:

It was prepared by adding 0.5 g of Dimethyl-α-naphthylamine to 100 ml of 5*N* acetic acid.

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

3.1.4.8 Kovac's Reagent (Atlas *et al*., 1995)

Isoamyl alcohol	150 ml
ρ-Dimethyl-aminobenzaldehyde	10 g
Concentrated HCl	50 ml

Aldehyde was dissolved in alcohol and acid was added slowly. The reagent was prepared in small quantities and stored in the refrigerator.

3.1.5 Solutions

3.1.5.1 Antibiotic Solutions

They were prepared as follows:

1. Ampicillin, gentamicin, aztreonam, chloramphenicol and streptomycin were prepared as stock solutions by dissolving 10 mg / ml of antibiotic powders in D. W., sterilized by filtration and stored at -20ºC.
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 in a dark bottle at -20ºC.

3.1.5.2 Ethidium Bromide Solution 10 mg / ml (Bounchaud *et al*., 1969)

Prepared by dissolving 0.2 g of ethidium bromide in 20 ml distilled water and stirred on magnetic stirrer for few hours to ensure that the ethidium bromide has been dissolved then it was filtrated, and stored in a dark bottle at 4ºC.

3.1.5.3 Physiological Saline (Collee *et al*., 1996)

Prepared by dissolving 0.85 g of NaCl in 100 ml D.W. and sterilized by autoclaving.

3.1.5.4 Phosphate Buffer Saline (PBS) (Collee *et al*., 1996):

NaCl	8 g
K_2HPO_4	1.21 g
KH_2PO_4	0.34 g

Components were dissolved in 950 ml of D. W., pH was adjusted to 7.3 and the volume was completed to 1L then sterilized by autoclave.

3.1.5.5 Sorenson's Buffer

It was prepared by dissolving 9.47 g of Na_2HPO_4 and 9.08 g of KH_2PO_4 in 1L of D.W., the pH was adjusted to 6.8. The stock solution was stored at 4ºC in dark place.

3.1.5.6 Carbohydrate Solution:

It was prepared by dissolving 5g of carbohydrate (lactose) into 10 ml D.W. Mixed thoroughly and sterilized by filtration.

3.1.6 Api 20E kit (Api Bio Merieux, Lyon, France):

Api 20E kit consists of:

a) Galleries: the gallery is a plastic strip with 20 microtubes containing dehydrated reactive ingredients.

b) Api 20E Reagents:

- Oxidase reagent (1% tetramethyl-*p*-phenylenediamine dihydrochloride)
- Kovac's reagent (as in 3.1.4.8)
- Voges-Proskauer reagent:

  -Vp1 (40% potassium hydroxide).

  -Vp2 (5% solution of α-naphthol in absolute ethanol).
- Ferric chloride 10%.

3.1.7 Antibiotics Disks:

The antibiotic disks used in this study were:

Antibiotic	**Code**	**Concentration (µg)**	**Source (Origin)**
Penicillin	P	10 U	Bioanalyse LTD (Turkey)
Ampicillin	AM	10	Bioanalyse LTD
Aztreonam	ATM	30	Bioanalyse LTD
Cefotaxime	CTX	30	Bioanalyse LTD
Cephalexin	CL	30	Bioanalyse LTD
Chloramphenicol	C	30	Bioanalyse LTD
Gentamicin	CN	10	Bioanalyse LTD
Imipenem	IPM	10	Bioanalyse LTD
Norfloxacin	NOR	10	Bioanalyse LTD
Streptomycin	S	10	Bioanalyse LTD
Tetracycline	TE	30	Bioanalyse LTD

3.2 Methods:

3.2.1 Urine Samples Collection:

Mid stream urine samples were collected in sterile tubes from patients visiting AL-Kadhemia and AL-Yarmoq hospitals in Baghdad during the period from 30/3/2004 to 30/6/2004. A total of 160 samples were aseptically collected and transported to the laboratory within 30 minutes of collection.

3.2.2 Determining the Number of Microorganisms in Urine Samples (Jawetz *et al*., 2001)

Platinum loops that deliver 0.01 ml was used to streak urine samples onto agar plates. After incubation at 37°C for 24 hours, the numbers of colonies were counted, and the total number of organisms originally present in the specimen is estimated by multiplying the colony count by 10^2.

3.2.3 Sample Preparation

Urine samples were first centrifuged at 6000 rpm. Supernatant were discarded and only 0.5 ml were kept. Sediments were resuspended in PBS. One loopfull of the resuspended sediment was streaked on nutrient agar, blood agar and MacConkey agar plates. Plates then were incubated over night at 37˚C.

3.2.4 Identification of Isolates

Microscopical and biochemical tests were made for the identification of bacterial isolates.

3.2.4.1 Morphological Characteristics

The first step in the identification of bacteria was by doing gram stain. A loopfull of bacterial 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).

3.2.4.2 Cultural Characteristics

Bacterial isolates were studied depending on the colony size, shape, edge, color, and odor.

3.2.4.3 Biochemical Tests

–Catalse 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.

– Oxidase Test (Atlas *et al*., 1995)

This test was done by using moisten paper with few drops of a freshly prepared solution of tetramethyl-p-phenylene diamine dihydro-chloride. Aseptically a clump of cells was picked up from the slant growth with a sterile wooden stick and smeared on the moisten paper. The development of a violet or purple color within 10 seconds indicates a positive results.

– Indole Test (Collee *et al*., 1996)

Peptone water was inoculated and incubated at 37˚C for 48 hours. A quantity of 0.05 ml of kovac's reagent was added and mixed gently. Positive result was recorded by the appearance of a red ring on the surface.

–Methyl Red Test (Collee *et al*., 1996)

MR-VP broth tubes were inoculated and incubated at 37˚C for 48 hours. Then 5 drops of methyl red reagent was added and mixed. Positive results are bright red and negative are yellow.

–Voges-Proskauer Test (Collee *et al*., 1996)

MR-VP broth tubes were inoculated and incubated at 37˚C for 24 hr. Then 1 ml of Barritt's reagent A and 3 ml of Barritt's reagent B were added to 5 ml of cultured broth and shaked for 30 seconds. The formation of pink to red color indicates positive test for acetoin.

–Citrate Utilization Test (Atlas *et al*., 1995)

Simmon's citrate agar slants were inoculated with bacteria, and incubated for 24 hr. The changing of color to royal blue indicates positive result while the green color indicates a negative result.

– Urease Test (Atlas *et al*., 1995)

Urease activity was detected by inoculating the surface of Christensen urea agar slants with the bacterial growth and incubated at 37˚C for 24 hours. The appearance of a red-violet color indicates a positive result while a yellow-orange color indicates a negative result.

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

Isolates were cultured on TSI agar slants by stapping into the butt and streaking on surface, and then incubated for (24-48) hours at 37˚C. Results are as following:

Slant / Butt	Color	Utilization
Alkaline / acid	Red / Yellow	Glucose only fermented; peptones utilized
Acid / acid	Yellow / Yellow	Glucose fermented; lactose and / or sucrose fermented
Alkaline / alkaline	Red / Red	No fermentation of glucose, lactose or sucrose/ peptones utilized

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

– Phenylalanine Deaminase Test (PAD) (Collee *et al*., 1996)

Phenylalanine deaminase slant was inoculated with heavy inoculums. After incubation at 37˚C for 24 hours, a few drops of a 10% solution of ferric chloride were added over the growth. Positive results were recorded if a green color developed on the slop.

-Mannitol Fermentation (Collee *et al*., 1996)

Mannitol salt agar medium was inoculated and incubated at 37˚C for 24 hr. The changing of medium color to yellow indicates positive results for mannitol fermentation.

–Nitrate Reduction Test (Atlas *et al*., 1995)

Nitrate media (5 ml) were inoculated with bacterial cultures. All tubes were incubated at 37˚C for 24 hr. After incubation, 0.1 ml of the test reagent was added to each tube. The immediate formation of red color indicates positive results; that nitrate reduced to nitrite.

-Motility Test (Collee *et al*., 1996)

Semi solid agar medium was inoculated with a straight wire, making a single stab sown the center of the tube to about half the depth of the medium. Motile bacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque.

-Coagulase Test (Collins and Lyne, 1985)

It was done by emulsifying one or two colonies in a drop of water on a slide. If no clumping occurs in 10-20 s, a straight wire was dipped into human plasma (EDTA) and stirred with the bacterial suspension. Agglutinates, causing visible clumping in 10 s, indicates positive results.

3.2.4.4 Api 20E Identification for *Klebsiella* Isolates:

Identification of the isolates was carried out by sub-culturing representative colonies from MacConkey Agar plates on API 20 E microtubes systems. This system is designed for the performance of 20 standard biochemical tests from a single colony on plate medium. Each test in this system is performed within a sterile plastic microtube which contains the appropriate substrates and is affixed to an impermeable plastic strip (gallery). Each gallery contains 20 microtubes (each of which consists of a tube and a couple selection.

The biochemical tests included in this system are the following:

l - Beta- galactosidase test ONPG.

2- Arginine dihydrolase test ADH.

3- Lysine decarboxylase test LDH.

4- Omithine decarbxylase test ODC.

5- Citrate utilization test CIT.

6- Hydrogen sulphide test PLS.

7- Urease test URE.

8- Tryptophane deaminase test TDA.

9- Indole test IND.

10-Voges-Proskauer test VP.

11 - Gelatin Liquefaction test GEL.

12- Glucose Fermentation test FLU.

13- Manitol Fermentation test MAN.

14- Inositol Fermentation test INO.

15- Sorbitol Fermentation test SOR.

16- Rhamnose Fennentation test RHA.

17- Sucrose Fermentation test SAL.

18- Melibiose Fennentation test MEL.

19- Amygdalin Fermentation test AMY.

20- Arabinose FenTientation test ARA.

2 l - Oxidase test OX1.

-Preparation of the Galleries:

Five ml of tap water dispensed in to the incubation tray to provide a humid atmosphere during incubation.

-Preparation of Bacterial Suspension:

By using a flamed loop, a well isolated colony from plating medium was picked. The inoculum was emulsified in 5 ml suspending medium (sterile distilled water) by rubbing against the side of the tube

and mixed thoroughly with the water.

-Inoculation of the Galleries:

With a sterile Pasteur pipette, the twenty microtubes were inoculated. According to the manufactures instructions both the tube and couple section of CIT, VP and GEL microtubes were filled. After inoculation couple section of the ADH, LDC, ODC, H_2S and URE microtube were completely filled with sterile mineral oil.

-Incubation of the Galleries:

After inoculation, the plastic lid was placed on the tray and the galleries were incubated for 18 to 24 hrs. at 37°C.

-Reading of the Galleries

All the reactions not requiring reagents were recorded first, then the following reagents were added to the corresponding microtubes:-

1- One drop of 3.4% ferric chloride to the TDA micortube.

2- One drop of kavoc's reagent to the IND micortube.

3- One drop of voges- proskauer reagent to VP micortube.

4-One drop of the oxidase reagent to either H_2S or ONPG micortube.

The biochemical reactions performed by the API 20 E and their interpretations are listed in table (3-1).

Table (3-1):- Interpretation of Reactions Performed by API20E.

Microtube	**Positive**	**Negative**
ONPG	Yellow	Colorless
ADH	Red/Orange	Yellow
LDC	Orange	Yellow
ODC	Red / Orange	Yellow
CIT	Blue-Green	Pale green / Yellow
H_2S	Black deposit	Colorless / Grayish
URE	Red / Orange	Yellow
TDA	Dark brown	Yellow
IND	Red Ring	Yellow Ring
VP	Pink / Red	Colorless
GEL	Diffusion of Black Pigment	No diffusion
GLU	Yellow	Blue / Blue green
MAN	Yellow	Blue / Blue green
INO	Yellow	Blue / Blue green
SOR	Yellow	Blue / Blue green
RHA	Yellow	Blue / Blue green
SAC	Yellow	Blue / Blue green
MEL	Yellow	Blue / Blue green
AMY	Yellow	Blue / Blue green
ARA	Yellow	Blue / Blue green
OX	Violet / Dark purple	Colorless / Light purple

-Identification of the Isolates:

Identification of the isolate using the analytical profile index (Numerical coding) for rapid identification at species and biotype level were done as supplied by the manufacturer.

For using the index, the biochemical profiles obtained have to be transformed into a numerical profile and to compare it with those listed in the index by transform all 21 biochemical results into a seven- figure numerical profile (seven-digit number) by placing them into groups of three and consigning a specific value for each of the positive as follows:

Group 1			**Group 2**			**Group 3**			**Group 4**		
ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU
1	2	4	1	2	4	1	2	4	1	2	4
Group 5			**Group 6**			**Group 7**		
MAN	LNO	SOR	RHA	SAC	MEL	AMY	ARA	OXI
1	2	4	1	2	4	1	2	4

Each positive reaction is given a value equal to l, 2 or 4 according to the position of the test in its group. The sum of these three values was given the corresponding figure. Thus, the figure can have a value from 0 to 7 (zero for negative reaction) the seven digits numerical profile is then looked up in the index and the identification is determined

3.2.4.5 Additional Tests for Identification of *Klebsiella*

-Growthe at 10˚C

Nutrient agar plate was inoculated and incubated at 10˚C for 48 hr.

-The Ability of Bacteria to Produce Gas from Lactose at 44˚C

Tubes of carbohydrate fermentation media were inoculated with a loopfull of liquid culture, then incubated at 44.5˚C for 24 hr. Medium color changing indicates the presence of acid and the presence of bubbles inside Durham tube indicates gas production (Collee *et al*., 1996).

3.2.5 Maintenance of Bacterial Isolates:

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

3.2.5.1 Short-Term Storage:

Bacterial isolates were maintained for few weeks on nutrient agar plates. The plates were tightly wrapped with parafilm, and then stored at 4˚C.

3.2.5.2 Medium-Term Storage:

Bacterial isolates were maintained as stab cultures for few weeks. Such cultures were prepared in small screw-capped bottles containing 2-3 ml of nutrient agar medium and stored at 4˚C.

3.2.5.3 Long-Term Storage:

Single colonies were cultured in BHI broth and incubated for 24 hr, and then 8.5 ml of bacterial culture are mixed with 1.5 ml of glycerol, then stored for long time at -20˚C.

3.2.6 Sterilization Methods:

Three methods of sterilization were:

3.2.6.1 Moist Heat Sterilization:

Media and solutions were sterilized by the autoclave at 121˚C (15 Ib/in^2) for 15 minutes, except heat some sensitive solutions.

3.2.6.2 Dry Heat Sterilization:

Electric oven was used to sterilize glasswares and others at 160-180 ˚C for 2-3 hours.

3.2.6.3 Membrane Sterilization (Filtration):

Millipore filtering was used to sterilize heat sensitive solutions by using millipore filters (0.22 μm) in diameter.

3.2.7 Sensitivity of *Klebsiella* Isolates to Antibiotics:

From primary isolation medium 4-5 colonies (that showed similar morphology) were taken by flamed loop and suspended in 4-5 ml of Muller-Hinton broth. Broth was incubated at 37˚C for several hours until a visible turbidity was observed. The turbidity was adjusted against Mac Farland Standard Tube (0.5) ml 1.175% $BaCl_2.2H_2$ + 99.5 ml 0.36 N (1%) H_2SO_4) with physiological saline.

One hundred microliter of inoculum was transferred by micropipette on Muller-Hinton agar plat surface and spread in three different planes (by rotating the plate approximately 60˚ each time to obtain an even distribution of the inoculums). The inoculated plates were incubated at 37˚C or at room temperature for 30 minutes to allow absorption of excess moisture. With a sterile forceps the selected antibiotic disks (5 disks / plate) were placed on the inoculated plates and incubated at 37˚C for 18 hours in an inverted position. After incubation, the diameter of inhibition zones in (mm) was measured by a

ruler. Results were determined and compared according to the National Committee for Clinical Laboratory Standards (NCCLs, 1991).

3.2.8 Detection of Some Virulence Factors

3.2.8.1 Detection the Presence of Capsule (Atlas *et al*., 1995)

One drop of Indian ink was mixed with a loopfull of bacterial suspension on a clean glass slide. Then a cover slide was placed on the drop and examined under oil-immersion lense. The background will be gray and capsules surrounding the cells will be colorless.

3.2.8.2 Detection the Presence of Adhesion Pili

Hemagglutination test of red blood cells was conducted according to Gerlach *et al*., (1989) and Langstraat *et al*., (2001) as follows:

A- Preparation of Guinea Pig Erythrocytes Suspension

- Three ml of guinea pig blood (Biotechnology Research Center) were taken using heart puncture and mixed with 20 µl of heparin (5000 U/ml), to prevent the formation of clot, then it was centrifuged at 3000 rpm for 5 min.
- The precipitate was washed out three times with normal saline.
- Three hundred microlitre were taken from the precipitate and mixed with 9.7 ml of normal saline to have suspension with final concentration of 3% (volume / volume) and stored at 4ºC.

B- Preparation of Tanned Ox Erythrocytes Suspension

- The suspension of ox erythrocytes (Biotechnology Research Center) was prepared as described above, then 10 ml of instantly prepared tannic acid (12.5 mg of tannic acid with 250 ml of normal saline) were added to the ox suspension.
- The suspension was incubatd in shaker incubator (70 rpm) at 37ºC for ten min.

- The suspension was centrifuged at 3000 rpm for 5 min. Precipitate were washed two times with normal saline to remove the acid.
- The precipitate of red cells was resuspended with 10 ml of normal saline to have a final concentration of 3%.

C- Preparation of Bacterial Suspension

Brain heart infusion broth was cultured with bacterial cells and incubated at 37ºC for 24 hr. Broth were centrifuged at 3000 rpm for 10 min, precipitate were washed with normal saline, washing were repeated for three times, then the precipitate was resuspended in normal saline to have suspension with concentration of $3x10^9$ cells / ml (Sahly *et al*., 2000).

D- Detection of Adhesion Pili Type One (CFA / I)

Twenty microliter of bacterial suspension as described in (3.2.7.2.C) were placed on a clean glass slide, then it was mixed with 20 µl of (0.1 M) mannose solution (which was prepared by dissolving 0.1802 g of mannose in 10 ml normal saline). On the same slide another sample (20 µl of bacterial suspension with 20 µl of normal saline) were placed, and then 20 µl of guinea pig erythrocyte suspension (3.2.7.2.A) were mixed with the two samples. The appearance of hemagglutination in the absence of mannose during 1-3 min indicates the presence of adhesion pili type one (Sahly *et al*., 2000).

E- Detection of Adhesion Pili Type Three (CFA / III)

It was the same as in the (3.2.7.2.D) except the use of ox erythrocyte cells which were treated with tannic acid instead of the guinea pig erythrocytes. The appearance of hemagglutination in the presence of mannose refers to the presence of adhesion pili type three (Old *et al*., 1985)

3.2.8.3 Detection of the Ability of Bacteria to Produce Hemolysin and Siderophore

Bacterial samples were cultured on blood agar medium and incubated at 37ºC for 24 hr. The appearance of hemolysis zone around the bacterial colonies refers to production of hemolysin. Also bacterial samples were cultured on the modified M9 medium which was supplemented with 2,2 dipyridyl (3.1.3.2) and incubated at 37ºC for 48 hr. The appearance of bacterial growth refers to the ability of bacteria to produce siderophore (Nassif and Sanosonetti, 1986).

3.2.9 Bacterial Adhesion to Epithelial Cells Test (Iwahi *et al*., 1982)

3.2.9.1 Preparation of Bacterial Suspension:

Ten milliliter of nutrient broth medium were inoculated with bacterial growth, the culture was then incubated at 37˚C over night until an optical density of OD_{600} equals to 1 was obtained which gives $1x10^9$ cells / ml. Cultures of bacteria were washed twice in PBS and centrifuged at 6000 rpm for 20 min and resuspended in PBS.

3.2.9.2 Preparation of Epithelial Cells:

Uroepithelial cells were isolated from the urine of healthy females by centrifugation at 6000 rpm for 5 minutes, washed three times with PBS and centrifuged at 6000 rpm for 10 minutes before resuspended in PBS. Precipitate was resuspended in PBS to have a suspension of $1x10^5$ epithelial cells / ml.

3.2.9.3 Adhesion Test:

- A mixture of 0.2 ml of the bacterial suspension, 0.2 ml of the epithelial cell suspension and 0.1 ml of PBS was incubated at 37˚C for one hour.
- Unfixed bacteria to uroepithelial cells were removed by centrifugation at 6000 rpm for 10 minutes.

- The pellet was resuspended in PBS then a drop of it was put onto a microscope slide, air-dried fixed with methanol : acetic acid (3:1) and stained with methylene blue.
- The adherent bacteria to epithelial cells were observed by the compound light microscope.
- Epithelial cells alone were included as a control test.

3.2.10 Protocol for Determination the Percentage of Phagocytic Cells

3.2.10.1 Bacterial Suspension Preparation

Klebsiella was cultured on nutrient agar media, incubated for 24 hours at 37˚C then colonies were collected in normal saline or PBS. The bacterial cells were washed for three times and reconstituted in concentration of $1x10^6$ cells / ml (by comparing with Mac Farland tube) and stored at 4˚C until used.

3.2.10.2 Phagocytic Test

The method described by Furth *et al*., (1985) was followed:

1. One ml of heparinized human blood was mixed with 1 ml of bacterial suspension (1×10^6 cells / ml).
2. Then incubated at 37˚C for 30 min with slow movement.
3. A blood film was prepared on dry clean slide.
4. The slide was covered with methanol for 5-10 min.
5. The slide was covered with Geimsa stain and left for 5-10 min.
6. The stain was washed with Sorenson's solution.
7. The buffer was tripped off and the slide was dried.
8. The percentage of phagocytic cells was calculated as follows:

$$\% \text{ Phagocytos is} = \frac{\text{Phagocytic cells}}{\text{Total of Phagocytic and Non Phagocytic Cells}} x100$$

3.2.11 Serum Resistance Assay

The Serum resistance assay was performed by the method described by Kumer *et al*., (1997) as follows:

1. Polled normal human serum (PNHS) was taken and inactivated at 56˚C for 30 min for the test.
2. The test isolates grew overnight at 37˚C in nutrient broth and, were diluted in 5 ml of fresh nutrient broth and incubated at 37˚C for 2 hr.
3. The cultures were centrifuged at 6000 rpm for 5 min and the deposit was resuspended in 5 ml PBS (pH 7.2).
4. Equal volumes (0.2 ml of each) PNHS and bacterial suspension were mixed and incubated in water bath at 37°C.
5. The viable counts were made on nutrient agar at 60, 120 and 180 min.
6. If viable count dropped to less than 1 percent of initial value, the isolates were termed as sensitive and if more than 90 percent of organisms survived after 180 min they were termed resistant. The score obtained between 1 and 90 percent was considered as intermediate.

3.2.12 Curing of Plasmid DNA

Curing experiments were performed on the multiresistant *Klebsiella* isolate. Ethidium bromide was used in these experiments according to Trevors (1986) and as follows:

Cells of the selected isolate were grown in 5 ml of Luria broth to mid log phase ($O.D._{600}$ about 0.4–0.5). A 0.1 ml samples of each culture were inoculated in a series of 5 ml fresh L broth tubes containing various concentrations of ethidium bromide (0, 20, 50, 100, 200, 250, 300, 400, 800, 1600 and 3200 µg / ml). All tubes were incubated at 37ºC for 24-48 hrs.

The growth density of the different tubes was measured visually and compared with the control to determine the effect of each concentration of curing agent on bacterial growth. The lowest concentration of curing agent that

inhibited the growth of the bacterial isolate was considered as the minimum inhibitory concentration (MIC).

Samples were taken form tubes containing the highest concentration of ethidium bromide that still allows bacterial growth and diluted appropriately. Then 0.1 ml samples from suspected dilutions were spread on brain heart infusion agar plates and incubated overnight at 37ºC to score the survived colonies.

3.2.13 Selection of Cured Cells

After treatment of bacterial isolates with curing agent and the isolation of survivors on brain heart infusion, survivors were analyzed for the presence or absence of drug resistance as a result of eliminating the plasmid by selecting 150 colonies of the bacterial isolate. These colonies were replica plated (using toothpick) on brain heart infusion plates (master plates) and on brain heart infusion plates containing an antibiotic to which the original isolate was resistant (Trevors, 1986).

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 Four

4-1 Isolation and Identification of Bacterial ~~Strains~~Isolates

4-1-1 Isolation

One hundred and sixty urine samples were collected from patients in Al-Kadhumia Hospital and AL-Yarmook ~~hospital~~ Hospitals during the period from 30-3-2004 to 30-~~4~~6-2004. One hundred and fifty ~~one~~ of these samples were considered as true bacteriur~~ia~~ea (i.e. more than 10^5 bacteria / ml of urine were detected). All the UTI samples were caused by single infectious agent~~s~~.

4-1-2 Identification

One hundred and fifty isolates were identified, these isolates were characterized as: *E. coli* (7~~1~~0 isolates), *Klebsiella* (20 isolates), *Proteus* (18 isolates), *Pseudomonas* (12 isolates), *Enterobacter* (13 isolates), *Staphylococcus aureus* (8 isolates) and *Staphylococcus epidermid~~e~~is* (9 isolates). The results of morphological, physiological and biochemical tests for isolates (table 4-1, 4-2) were in agreement with Holt *et al*., (1994) and Forbes *et al*., (1998).

Moreover, identification of the *Klebsiella* isolates was confirmed by using Api system (Api 20E) as shown in ~~plate~~figure ~~-~~(4-1), the findings obtained by the conventional biochemical tests.

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~~Figure~~Table (4-1) Colony appearance and characteristics of bacterial isolates.

Organism	Medium	Appearance
E. coli	Mac	LF; flat, dry, pink and non viscous colonies
Klebsiella	Mac	LF; mucoid, large, pink and viscous colonies
Proteus	Mac BA	NLF; pale colonies swarming motility after 24 hr incubation
Enterobacter	Mac	LF; raised, pink colonies
Pseudomonas	Mac NA	NLF; pale colonies Small colonies, greenish pigment
Staphylococcus aureus	BA	medium to large; smooth, entire slightly raised translucent; they are yellow to creamy colonies and beta hemolytic
Staphylococcus epidermideis	BA	small to medium translucent gray white colonies most colonies nonhemolytic slime-producing strains are extremely sticky and adhere to the agar surface

Mac: MacConkey agar; BA: Blood agar; NA: Nutrient Agar; LF: Lactose fermenter; NLF: Non-lactose fermenter

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Table (4-2) Morphological, physiological and biochemical characteristics of the locally isolated UTI bacteria.

	E. coli	*Klebsiella pneumoniae*	*Klebsiella oxytoca*	*Enterobacter spp.*	*Pseudomonas spp.*	*Proteus spp.*	*S. aureus*	*S. epidermidies*
Cell shape	bacilli	bacilli	bacilli	bacilli	bacilli	bacilli	cocci	cocci
Motility	+	–	–	+	+	+	–	–
Gram stain	–	–	–	–	–	–	+	+
Oxidase	–	–	–	–	+	–	–	–
Catalase	+	+	+	+	+	+	+	+
Indole Production	+	–	+	–	–	+	–	ND
Methyl red	+	–	–	–	–	+	ND	ND
Voges-Proskauer	–	+	+	+	–	–	+	ND
Citrate (Simmon)	–	+	+	+	ND	–	ND	ND
KCN, growth	–	+	+	+	ND	+	ND	ND
Malonate Utilization	–	+	+	+	ND	–	ND	ND
H_2S	–	–	–	–	–	+	ND	ND
~~Lactose, acid production~~	~~ND~~	~~+~~	~~+~~	~~ND~~	~~ND~~	~~ND~~	~~ND~~	~~ND~~
Lactose, gas production at 44.5˚C	ND	+	–	ND	ND	ND	ND	ND
Urease	–	+	+	–	ND	+	ND	ND
Nitrate reduction	+	+	+	+	ND	ND	ND	ND
Phenylalanine deaminase	–	–	–	–	ND	+	ND	ND
Mannitol fermenter	+	+	+	+	+	–	+	–
Coagulase	ND	ND	ND	ND	ND	ND	+	–

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Growth at 10˚C	ND	–	+	ND	ND	ND	ND	ND
No. of isolates	7~~1~~0	18	2	13	12	18	8	9

+ = positive results; – = negative results; ND = Not determined

Escherichia coli was the major causative agent of UTIs in collected samples and represent 4~~7~~6.7% of all cases (figure 4-2). This is in agreement with what is known about ~~the~~ *E. coli* as the major cause of UTIs world wide (Malkawi and Youssef, 1996; Al-Bayati, 1999).

The frequency of infection with *Klebsiella* was 13.3% (20 isolates). Ninety percent of these isolates (18 isolates) were belonged to *Klebsiella pneumoniae* in which they were indole negative, while 10% (2 isolates) of *Klebsiella* ~~isolates~~ were belonged to *Klebsiella oxytoca* which were indole positive and cannot produce~~s~~ acid and gas from lactose at 44°C. This result was in agreement with Al-Mulla (2003) who isolated 17 *Klebsiella* from 100 urine samples.

Proteus represents 12~~.0~~ % (18 isolates) of all isolates, this result~~s~~ was agreed with Al-Jeboury (2005) who found that 13.7% of all UT1 isolates were belonged to *Proteus*.

Klebsiella and *Proteus* represent the second causative agent of UTIs after *E. coli* (Twaij, 1998). This is probably because *Klebsiella* and *Proteus* were able to produce a potent urease which acts on urea to produce ammonia, rendering the urine alkaline.

Enterobacter and *Pseudomonas* represent the third causative agents of UTIs about 8.7% (13 isolates) and 8% (12 isolates) respectively. *Enterobacter* and *Pseudomonas* are more frequently found in hospital acquired UTI because their resistance to antibiotics favors their selection in hospital patients (Mims *et al.*, 1987).

Seventeen cases were caused by *Staphylococcus*. Nine ~~isolates~~ of all *Staphylococcus* isolates were belonged to *Staphylococcus epidermid~~e~~is* while 8 isolates were belonged to *Staphylococcus aureus*. Gram-positive bacteria are known to be involved in the UTI. However the vast majority of UTI are caused

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by gram-negative bacteria originating from the gut before entering the urethra (Stamey, 1973).

A- Cultural Characteristics:

Escherichia coli, *Enterobacter* and *Klebsiella* gave pink to red colonies on MacConkey agar. *Klebsiella* colonies are lager and mucoid. While *Proteus* gave pale colony appearance on MacConkey agar as non lactose fermenters, and swarming motility on blood agar after 24 hr incubation. *Pseudomonas* appeared small, low-convex, rough and often oval and they were pale (non lactose fermenter). They have the same morphology on the nutrient agar but they produce a greenish pigment.

Staphylococcus aureus colonies on blood agar medium are medium to large; smooth, entire slightly raised translucent; they are yellow to creamy colonies and beta hemolytic. While *Staphylococcus epidermides* are small to medium translucent gray white colonies most colonies nonhemolytic slime-producing strains are extremely sticky and adhere to the agar surface.

B- Morphological Characterization

The oil immersion lence objective of the compound light microscope, Gram staining examination, showed the following results:

- *Escherichia coli*, *Enterobacter*, *Klebsiella* and *Proteus*: are Gram-negative bacilli, motile (except *Klebsiella* which is non motile), non-spore former. *Klebsiella* are encapsulated.
- *Pseudomonas*: are Gram-negative medium sizes, straight rods, motile, non-spore former.
- *Staphylococcus*: are Gram-positive cocci, usually in clusters, non-motile, non-spore former.

C- Biochemical Characterizations:

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Several biochemical tests were done to characterize the bacterial isolates (Table 4-1) and as follows:

- *E. coli*: Indole positive, methyl red (MR) positive, Voges Proskauer (VP) negative, fails to grow in citrate and KCN media, malonate negative. H_2S negative. Produces acid and gas from lactose at 44ºC and at lower temperatures.
- *Klebsiella*: Oxidase negative, catalase positive. MR and VP negative. Urease and KCN positive. H_2S negative. Reduce nitrates. Malonate and citrate positive. *Klebsiella pneumonia* was indole negative, while *Klebsiella oxytoca* which was indole positive and cannot produces acid and gas from lactose at 44ºC.
- *Enterobacter*: Indole and MR negative, VP and citrate positive, lactose fermenting, oxidase negative, catalase and KCN positive, malonate positive, urease negative, H_2S negative.
- *Proteus*: Phenylalanine deaminase (**PAD**), catalase and urease positive. Oxidase negative, H_2S positive.
- *Pseudomonas*: Oxidase and nitrate positive
- *Staphylococcus aureus*: Oxidase negative, catalase positive, mannitol fermenter, coagulase positive.
- *Staphylococcus epidermides*: Oxidase negative, catalase positive, non-mannitol fermenter and coagulase negative.

Table (4-1) Biochemical tests for characterization of bacterial isolates of UTI.

	E. coli	*Klebsiella pneumonia*	*Klebsiella oxytoca*	*Enterobacter*	*Pseudomonas*	*Proteus*	*S. aureus*	*S. epidermides*

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Gram stain	–	–	–	–	–	–	±	±
Oxidase	–	–	–	–	±	–	–	–
Catalase	±	±	±	±		±	±	±
Indole Production	±	–	±	–		V		
Methyl red	±	–	–	–		±		
Voges-Proskauer	–	±	±	±		–		
Citrate (Simmon)	–	±	±	±		–		
KCN, growth	–	±	±	±				
Malonate Utilization	–	±	±	±				
H_2S	–	–	–					
Lactos, acid production	±	±	±					
Lactos, gas production	±							
Urease	–	±	±	–		±		
Nitrate reduction	±	±	±	±				
Phenylalanin deaminase	–	–	–	–				
Mannitol fermenter	±	±	±	±	±	–	±	–
Coagulase	ND	ND	ND	ND	ND	ND	±	–

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Moreover, identification of the isolates was confirmed by using Api system (Api 20E) as shown in (plate 3-1) the findings obtained by the conventional biochemical tests.

Depending on Mmorphological (appearance of colonies on the culture media), microscopically, and biochemical tests and identification by the the use of API 20E system (figure fig. 4-1) showed that the causative agent of UTI were belong to five different species of gram-negative bacteria and to two genera of gram-positive bacteria (figurefig. 4-2)

Escherichia coli was the major causative agent and represent 47.4% of all cases (figure 4-2). *E. coli* was the major causative agent and represent 41% of all

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cases (figure fig. 4-12). This is in agreement with what is known about the *E. coli* as the major cause of UTIs world wide (Malkawi and Youssef, 1996; Al-Bayati, 1999).

The frequency of infection with *Klebsiella* was 13.3% (20 isolates). Ninety percent of these isolates (18 isolates) were belonged to *Klebsiella pneumonia* subspecies *pneumonia* in which they were indole negative, while 10% (2 isolates) of *Klebsiella* isolates were belonged to *Klebsiella oxytoca* which were indole positive and cannot produces acid and gas from lactose at 44ºC. This result was in agreement with Al-Mulla (2003) who isolated 17 *Klebsiella* isolates from 100 urine samples.

Proteus represents 12.0 % (18 isolates) of all isolates, this results was agreed with Al-Jeboury (2005) who that 13.7% of all UTI isolates were belonged to *Proteus*.

Klebsiella and *Proteus* represent the second causative agent of UTIs after *E. coli* (Twaij, 1998). This is probably because *Klebsiella* and *Proteus* were able to produce a potent urease which acts on urea to produce ammonia, rendering the urine alkaline.

Enterobacter and *Pseudomonas* represent the third causative agent of UTIs about 7.3% and 8.7% respectively. *Enterobacter* and *Pseudomonas* are more frequently found in hospital acquired UTI because their resistance to antibiotic favors their selection in hospital patients (Mims *et al.*, 1987).

Seventeen cases were caused by *Staphylococcus* which represented 11.3% of all the isolates (fig. 4-2). Six isolates (35.3%) of all *Staphylococcus* isolates were belonged to *Staphylococcus epidermides* while 5 isolates (64.7%) were belonged to *Staphylococcus aureus*. Gram-positive bacteria are known to be involved in the UTI. However the vast majority of UTI are caused by gram-negative bacteria

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originating from the gut before entering the urethra (Stammy, 1973). The frequency of infection with *K. pneumoniae* and *P. mirabilis* have approximately the same rate, they represent compared with previous studies that showed that *P. mirabilis* was the second causative agent of UTIs after *E. coli* (1998; Twaij, 1998). This is probably because *Klebsiella* and *Proteus* were able to produce a potent urease which acts on urea to produce ammonia, rendering the urine alkaline. *Enterobacter* and *Pseudomonas* species represent the third causative agent of UTIs. *Enterobacter* and *Pseudomonas* are more frequently found in hospital acquired UTI because their resistance to antibiotic favors their selection in hospital patients (Mims *et al*., 1987).

Twenty positive cases were caused by gram-positive bacteria, which represented 11.33 % of all positive cases (figurefig. 4-2). Gram-positive bacteria are known to be involved in the UTI. However the vast majority of UTI are caused by gram-negative bacteria originating from the gut before entering the urethra (Stammy, 1973)

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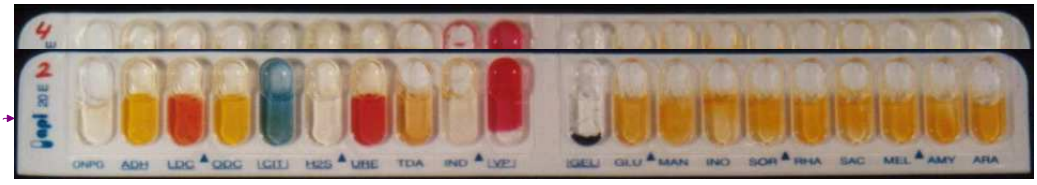

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~~Klebsiella~~Klebsiella ~~pneumonia~~pneumoniae

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~~subspecies *pneumonia*~~

Klebsiella oxytoca

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~~Plate~~Figure (~~34~~-1)~~.~~ Api 20E system for characterization of different ~~bacterial~~*Klebsiella* isolates showing the results of reactions includes~~d~~ in the system.

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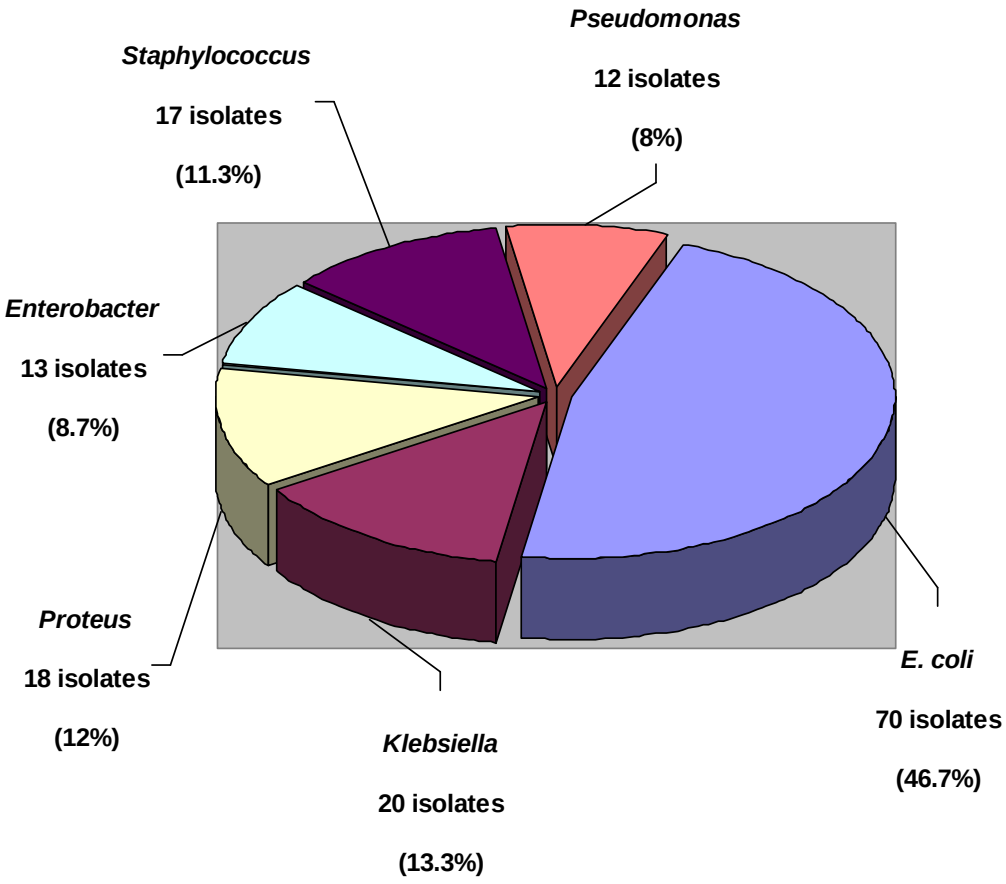

~~Figure~~Figure~~.~~ (4-2) Percentage of bacterial ~~species~~ genera isolated from urinary tract infections.

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34.-2 Prevalence of Urinary Tract Infections in Male and Female

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Out of the 150 1510 positive cases, 96 910 (6460 60 .6%) were from female patients while 5460 (363940.4 %) of the cases were from the males (figurefig. 4-3). It is known that the incidence of UTIs is generally higher in females than the males world wide and for several reasons (Mims *et al.*, 1987; Al-Bayati, 1999). The shorter female urethra is a less effective deterrent to infection than the male urethra. Sexual intercaurseintercourse facilitates the movement of organisms up the urethra, particularly in females, so that the incidence of UTIs is higher among sexually active than celibate women. However, the antibacterial properties of prostatiic fluid may also account for the increased resistance to UTI observed in men. In male infants, UTIs are more common in the uncircumcised and this is associated with colonization of the inside of the prepuce and urethra with faecal organisms (Glauser, 1986; Mims *et al.*, 1987).

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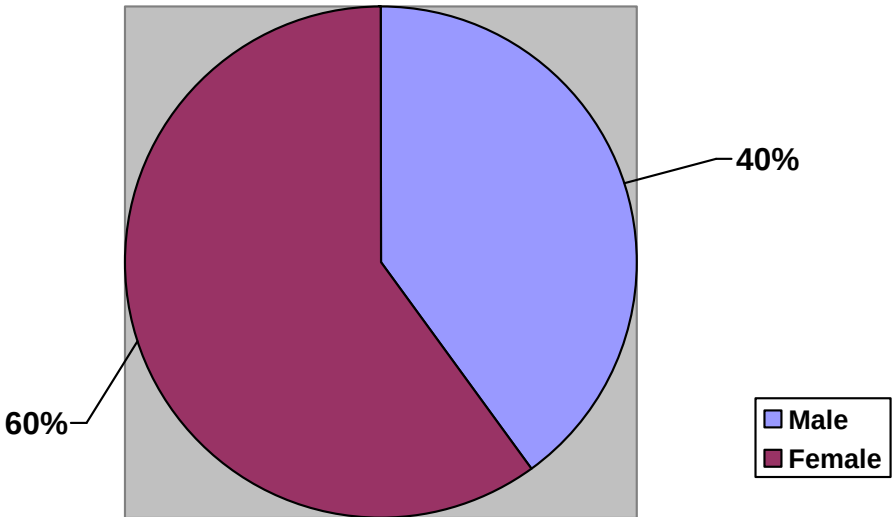

~~Figure~~Fig~~.~~ure (4-3) Prevalence of urinary tract infections in males and females.

~~Sex~~	~~Number of patients~~	~~Percentage~~
~~Female~~	~~96~~	~~64%~~
~~Male~~	~~54~~	~~36%~~
~~Total~~	~~150~~	~~100%~~

Percentages of infected female with ~~*Klebsiella* were~~*Klebsiella* were more than that in male. Twelve isolates (60%) were ~~isolated~~obtained from female while in male 8 isolates (40%) ~~was isolated~~were found. ~~This results~~These results were in agreement with Podschun *et al*., (1993) who found that from 146 UTI patients infected with ~~*Klebsiella* 69.8~~*Klebsiella* 69.8 % were females.

~~4-3 Study the Relationship between Virulence Factors and Antibiotic Resistance Plasmid~~

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~~Percentage of infected female with *Klebsiella* were more than that in male, in which that female were 12 isolate (60%) while in male was 8 isolates (50%) this results were in agreement with Podschun *et al*., (1993) who found that from 146 UTI patients infected with *Klebsiella* 69.8 % were females.~~

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~~3.3~~3.4~~-3~~3~~-1~~ Antibiotic ~~Sensitivity~~ Resistance of ~~*Klebsiella*~~*Klebsiella* Isolates

The standard disk diffusion method was used to determine the sensitivity of ~~*Klebsiella*~~ *Klebsiella* ~~strains~~ isolates to several antibiotics. Results ~~are shown in table~~ (table ~~3~~4~~-3~~3~~2~~3) showed that ~~.~~ 100% of the ~~strains~~ isolates were ~~resistance~~ resistant to penicillin. While 16 (~~86~~80%) isolates ~~of all strains~~ were resistant to ampicillin. Hostackai and Klokocnkovai, (2001) and ~~These results are in agreement with~~ Al-Mulla, (2003) ~~and Hostackai and Klokocnkovai, (2001) who~~ found that 100% of isolates were resistant to ~~ampicilline~~ampicillin. The ampicillin is one of the common antibiotics used for the treatment of UTI (Rice *et al.*, 1996), so the wide spread of resistance in the Iraqi ~~strains~~ isolates represent a major problem in treatment of the infection. It was reported in several parts of the world that the wide spread of ampicillin resistance is attributed to degradation of the antibiotic by β-lactamase enzymes (which is either chromosomally or plasmid encoded~~due to~~ and most of the~~i~~se plasmids are self transmissible plasmids) (Pre~~S~~scott *et al*., 1999).~~,~~ Thomson and Am~~y~~es (1993) showed that 88.2% of ~~*Klebsiella* isolated~~*Klebsiella* isolated from UTIs in Scotland were resistant to ampicillin due to the production of TEM-1 β-

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lactamase enzyme and 64% of these isolates were able to transfer resistance feature to *E. coli* J626.

Also resistance to penicillins is due to the lack of penicillin binding proteins (PBPs) for a specific antibiotic or the microorganisms could change their permiabiliypermeability to the drug (carrying ampicillin resistance gene (Davies, 1994; Hamett *et al.*, 1996; Malkawi and youssefYoussef, 1996; Bermudes *et al.*, 1997).. 75% of strians (the leading causative agent) isolated during this study were resistant to ampicillin (table 4-1). Thomson and Amyes (1993) showed that 88.2% of *Klebsiella* isolated from UTIs in Scotland were resistant to ampicillin due to the production of TEM 1 βbeta-lactamas lactamase enzyme and 64% of these isolates were able to transfer resistance feature to *E. coli* J62.6. In another study, Al-Gazrawi (1996) was found that 100% of *E. coli* isolated from UTI were resistant to ampicillin.

Most of the isolates were resistant to aminoglycosides, that resistant results were 15 (75%) and 16 (80%) for the gentamicin and streptomycin respectively.

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Table (4-423) TheAntibiotic resistant of *Klebsiella* isolated*Klebsiella* isolated from urinary tract infection to antibiotics.

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Antibiotics / Antibiotic Symbol	Penicillin	Ampicillin	Aztreonam	Cefotaxime	Imipenem	Norfloxacin	Cephalexin	Chloramphenicol	Streptomycin	Tetracycline	Gentamicin
	P	Am	ATM	CTX	IPM	NOR	CL	C	S	TE	CN

StrainIsolate												
MR$_1$	*K. p.*MR$_1$	R	R	R	S	S	R	R	R	R	R	R
MR$_2$	*K. p.*MR$_2$	R	R	R	R	S	S	R	S	R	R	R
MR$_3$	*K. p.*MR$_3$	R	R	S	S	S	S	R	R	R	S	R
MR$_4$	*K. p.*MR$_4$	R	S	S	S	S	S	R	R	R	S	SR
MR$_5$	*K. p.*MR$_5$	R	R	S	S	S	R	R	R	R	IntS	R
MR$_6$	*K. p.*MR$_6$	R	R	S	R	S	S	SS	S	S	S	R
MR$_7$	*K. p.*MR$_7$	R	R	S	S	S	IntS	S	R	R	S	SR
MR$_8$	*K. p.*MR$_8$	R	R	S	S	S	R	S	S	R	S	SR
MR$_9$	*K. p.*MR$_9$	R	R	R	R	S	S	S	R	S	R	SR
MR$_{10}$	*K. o.*MR$_{10}$	R	R	R	S	S	R	S	RS	R	R	SR
MR$_{11}$	*K. p.*MR$_{11}$	R	S	S	R	S	S	S	R	R	S	IntR
MR$_{12}$	*K. p.*MR$_{12}$	R	R	S	R	S	S	S	R	R	S	Int
MR$_{13}$	*K. p.*MR$_{13}$	R	R	S	Int	S	R	S	R	S	R	SR
MR$_{14}$	*K. p.*MR$_{14}$	R	R	R	S	S	S	S	RS	R	S	R
MR$_{15}$	*K. o.*MR$_{15}$	R	S	S	S	S	IntS	S	R	S	S	S
MR$_{16}$	*K. p.*MR$_{16}$	R	R	R	R	S	S	S	RS	R	R	S
MR$_{17}$	*K. p.*MR$_{17}$	R	R	S	R	S	S	R	R	R	IntS	S
MR$_{18}$	*K. p.*MR$_{18}$	R	R	R	R	S	R	S	R	R	R	S
MR$_{19}$	*K. p.*MR$_{19}$	R	R	R	S	S	S	S	R	SR	S	R
MR$_{20}$	*K. p.*MR$_{20}$	R	S	R	S	S	S	R	R	R	R	SR

R = resistant

S = Sensitive

Int = intermediateintermediate

K. p. = Klebsiella pneumoniae

K. o. = Klebsiella oxytoca

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Most of the isolates were resistant to aminoglycosides, that ~~resistant results were~~ 15 (75%) and 16 (80%) isolates were resistant for ~~the~~ gentamicin and streptomycin respectively. These results were in agreement with Akindele and Rotilu (2000) who found that about 79% of ~~*Klebsiella*~~*Klebsiella* strains in their study were resistance to gentamicin, while Reish *et al*., (1993) and Roilides *et al*., (2000) found that all the ~~*Klebsiella*~~*Klebsiella* strains in their stud~~y~~ies were resistant to gentamicin.

~~These results were in agreement with Akindele and Rotilu (2000) who found that about 79% of strains in their study were resistance to gentamicin, while Reish *et al*., (1993) and Roilides *et al*., (2000) found that all the strains in their study were resistant to gentamicin.~~

Table (4-~~1~~123) showed that ~~50% of isolate were resistant to Norfloxacin~~, 14 (70%) isolates were resistant to chloramphenicol while resistance to tetracycline, ~~C~~cephalexin, ~~gentamicin, C~~cefotaxime, ~~and~~ aztreonam and norfloxacin ~~are~~were nearly the same in which resistance were 8 (~~(~~40%), 7 (~~35%,~~ 35%), 8 (40%), 9 (~~and~~ 45%)~~)~~ and 6 (30%) isolates ~~respectiv~~respectively. However, ~~ely. However,~~

~~Aall strains (100%) were sensitive to Imipenemthe rate of resistance to trimethoprim, sulfamethoxazed and tetracycline are higher than streptomycin and chloramphenicol and less than ampicillin. This result is similar to that of Chowdhury *et al*., (1994), when they found that the rate of resistance of gram-negative rods to trimethoprim is less than ampicillin and more than tetracycline, chloramphinicol and streptomycin. Such resistanct was carried on conjugative plasmids that spread in *Enterobacteriaceae* causing UTIs.~~

~~All tested isolates were sensitive to nitrofurantion, it is an effective antibiotic in the treatment of UTI all over the world (Damjano vic and whitfleld, 1986; Hannan *etal*., 1993). The second most effective antibiotic agent for all isolates was nalidixic acid (25% of all isolates were resistant). Gentamycin was the third most effective antibiotic (39% of all isolates were resistant), while the~~

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~~cephalixin was the fourth (48% of all isolates were resistant) (table 3-3). Generally speaking, the percentage of resistance to antibiotics reported in this study are higher than those reported in some other parts of the world (Damjanovic and whitfield, 1986; Hannan *et al*., 1993; Bermudes *et al*., 1997), and this is probably a reflection for the misuse of antibiotics in Iraq.~~

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. ~~all~~All ~~strains~~isolates (100%) were sensitive to Imipenem.

The ability of bacterial cell to resist chloramphenicol is either due to their ability to produce chloramphenicol acetyl ~~transferase which modify~~transferase which modifies the antibiotic or due to mutation that happened to the ~~S~~23S rRNA which ~~result~~results in decrease the sensitivity of cells to the chloramphenicol (Prescott *et al*., 1999). While resistance to cephalexin, cefotaxime and norfloxacin mostly are due to plasmid encoding enzymes, which ~~mostly~~ carried on large plasmid with or without β-~~beta~~lactam resistance genes (Li and Lim, 2000; Shen *et al*., 2001). As shown in table (4-~~3~~4) all *Klebsiella* isolates were multiresistant for different types of antibiotics. Most of these isolates (90%) were resistant to ~~8-~~4-~~8~~antibiotics.

~~Initially most of the antibiotic resistance gene are found on chromosomal DNA, such as genes responsible for penicilines and cephalosporins (Eggman *et al*., 1997), but these~~ Many of the antibiotic resistance genes were found to be carried also on self transmissible or mobilizable plasmids, and the transfer of such plasmids 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 belong to the family *Enterobacteriaceae* which represent the major causative agents for UTI (Broda,1979; Pedler and Bint, 1985; Hamett *et al*., 1996; Livrelli *et al*, 1996; Rice *et al*., 1996).

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All tested gram-negative bacterial isolates showed multiresistance for antimicrobial agents used in this study (table 3-4). 18.05% of the gram-negative isolates were resistant to nine antibiotics out of twelve. 14.29% were resistant to eight antibiotics and 19.55% were resistant to seven antibiotics out of twelve. The resistant gram-negative isolates for six antibiotics are about 14.29%, for five antibiotics are 8.27% and finally for four and less antibiotics are about 25.56% (table 3-4).

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Antibiotic / Strain	Penicillin P	Ampicillin Am	Aztreonam ATM	Cefotaxime CTX	Imipenem IPM	Norfloxacin NOR	Cephalexin CL	Streptomycin S	Tetracycline TE	Gentamicin CN
MR_1	R	R	R	S	S	R	R		R	R
MR_2	R	R	R	R	S	S	R		R	R
MR_3	R	R	S	S	S	S	R		S	R
MR_4	R	S	S	S	S	S	R		S	S
MR_5	R	R	S	S	S	R	R		Int	R
MR_6	R	R	S	R	S	S	S		S	R
MR_7	R	R	S	S	S	Int	S		S	S
MR_8	R	R	S	S	S	R	S		S	S
MR_9	R	R	R	R	S	R	S		R	S
MR_{10}	R	R	R	S	S	R	S		R	S
MR_{11}	R	S	S	R	S	R	S		S	Int
MR_{12}	R	R	S	R	S	R	S		S	Int
MR_{13}	R	R	S	Int	S	R	S		R	S

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MR14	R	R	R	S	S	S	S		S	R
MR15	R	S	S	S	S	Int	S		S	S
MR16	R	R	R	R	S	S	S		R	S
MR17	R	R	S	R	S	S	R		Int	S
MR18	R	R	R	R	S	R	S		R	S
MR19	R	R	R	S	S	R	S		S	R
MR20	R	S	R	S	S	S	R		R	S

Table 34 4 Percentages of different multiresistance gram-negative isolates to antibiotics

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Gram-negative rods resistant to	Nine	Eight	Seve	Six	Five	Four and less	Total
	Antibiotic						
Number of isolates	24	19	26	19	11	34	133
Percentage	18.05%	14.29 %	19.55 %	14.29 %	8.27%	25.56 %	100%

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As shown in table (4-23) all *Klebsiella* isolates were multiresistant. Most of the tested isolates showed multiresistance for antimicrobial agents used in this study (table 4-2). 18.05% of the *gram-negative* isolates were resistant to nine antibiotics out of twelve. 14.29% were resistant to eight antibiotics and 19.55% were resistant to seven antibiotics out of twelve. The resistant gram-negative isolates for six antibiotics are about 14.29%, for five antibiotics are 8.27% and finally for four and less antibiotics are about 25.56% (table 4-2).

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for different type of antibiotics. Most of these isolates (90%) were resistant to 8-4 antibiotics about (90%) of all isolates.

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Table (4-34) Percentage of different multiresistance *KlebsiellaKlebsiella* isolates to antibiotics.

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No. of antibiotics / Isolated strains	Nine	Eight	Seven	Six	Five	Four	Three OneTwo
No. of isolates	1	2	3	5	3	5	1
Percentage %	5	10	15	25	15	25	5

No. of antibiotics / Isolated strains	Nine	Eight	Seven	Six	Five	Four	Two
No. of resistant isolates	1	2	34	5	36	51	1
Percentage %	5	10	1520	25	1530	255	5

Initially most of the antibiotic resistance genes are found on chromosomal DNA, such as genes responsible for penicillines and cephalosporins resistance (Eggman *et al*., 1997), but these genes were carried also on self transmissible or mobilizable plasmids, and the transfer of such plasmids 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 *EEnterobacteriaceae* which represent the major causative agents forof UTI (Livrelli *et al*, 1996; Rice *et al.*, 1996).

Some bacterial cells may contain more than one plasmid molecules which in turn might contain different resistance genes. Sirot *et al.*, (1991) found that Some time bacterial cells may contains several plasmids and the same plasmid encoding resistance genes for several antibiotics. Sirot *et al.*, (1991) found that one *KlebsiellaKlebsiella* strain which was isolated from UTI patients was carrying antibiotic resistance plasmid for β-lactams, all aminoglycosides,

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amikacin, sulfonamide, and tetracycline. This plasmid was able to transfer resistance to other ~~*Klebsiella*~~*Klebsiella* strain and also to *E. coli* strain.

The multidrug resistance of bacteria to several antibiotics could be due to certain mutations that occur as a result of overuse and misuse of antibiotics, but antibiotic resistant mutants that arise spontaneously are generally resistant to only one antibiotic (Malkawi and Youssef, 1996; Al-~~shallcm~~Shallc~~m~~hi, 1999; Rasool *et al.*, 2003). In addition to mutations, R plasmids ~~that carrying resistance genes play an important role in spreading the multidrug resistance between bacteria~~offer resistance to antibiotics and are transmissible from one cell to another by direct cell contact (~~Burke and Levy, 1985;~~ Amyes *et al.*, 1989). ~~Conjugation (direct *in vivo* gene transfer) is a convenient method of transferring drug resistant genetic determinants among intra and inter~~Conjugation (direct *in vivo* gene transfer) is a convenient method of transferring drug resistant genetic determinants among intra- and inter~~ss~~ generic bacterial populations (Rasool *et al.*, 2003). This multidrug resistance ~~lead~~led to antibiotic ineffectiveness against bacteria responsible for UTI and ~~"~~ other life threatening diseases.

~~34. 4 5 Minimum Inhibitory Concentrations (MICs)~~

~~The MICs for several multiresistant isolates (MR1 and MR2) were determined. Results in table (3-5) showed that the MR1 strain was resistant to rifampicin (RA) and the MIC was higher than 30 µg /ml. However it was sensitive to all other antibiotics and the MICs were less than 10 µg /ml, which was the lowest concentration, used in the experiment. On the other hand, the MR2 strains were all more sensitive to rifampicine than MR1 but they were~~

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~~resistant to all other antibiotics tested in the experiment. Their MICs to all antibiotics were higher than that of MR1 strain. According to the MIC values obtained, it was decided to add rifampicine (30 µg /ml) and ampicillin (25 /ml) or rifampicine (30 µg/ml) and tetracycline (30 µg/ml) to the media for the selection of transconjugants resulted from conjugation experiment between *E. coli* HB101 recipient strain and multiresistant isolates of *E. coli* (Donor). The MIC values for other *Enterobacteriaceae* donor strains are shown in table (3-5) and accordingly (30 jug/ml) rifampicin and tetracyclin were used for the selection of transconjugants in all conjugation experiments between *E. coliHB* 101 recipient strain and multiresistant *Enterobacteriaceae* donor strains.~~

~~The aim of this experiment was not the determination of the level of resistance of these isolates, instead, the experiment was designed to determine which antibiotic and at what concentration can be used for the selection of the transconjugantscured cells. For this reason, the exact MICs for the resistant strains were not determined.~~

~~In transformation experiment, the selective media used for the selection of the transformant *E.coliHBlOl* with plasmid DNA of the multiresistant *E.coli* isolates contain 15 jug/mi of ampicillin or tetracyclin or streptomycin or neomycin or nalidixic acid. These concentrations of antibiotics prevent the growth of *E.coliHBlOl* but it allow the multiresistant *E.coli* isolates to grow. So, this concentration is so efficient for transformant selection.~~

4-63 24 Detection of the Virulence Factors in *KlebsiellaKlebsiella*

Virulence factors were studied for all the *KlebsiellaKlebsiella* isolates and the results are showingillustrated in (Aappendix (-1).

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4-6-1A- Capsules

Capsule examination was made by using negative stain (with Indian ink) and by observing the shape and appearance of colony which were mucoidyon the culture media.

All the isolates were encapsulated in which Mmicroscopic resultsexamination showed -the presence of white shadow surrounding the cells (Plate 4-3),.

Capsule synthesis is encoded by chromosomal genes (Allen *et al*., 1987) and it representCapsule represents the most important virulence factor, by protecting the cell from the phoagocytosis (Cyze *et al*., 1984), and other bactericidal and helpagent which found in the host serum (Simmons-smit *et al*., 1986) and helps the microbial cell to attaching to the solid surface and the medical apparatus which increases its pathogenecity (Podschunn and Ullmann, 1998).

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B-Adherence Pili

Adhesion pili type one (CFA / I) activity was determined by the assays of hemagglutination of guinea pig erythrocytes in the absence and presence of D-mannose., and a All the isolates showed positive reaction for mannose sensitive hemagglutination (MSHA) which means that all isolates have CFA / I. While adhesion pili type 3 (CFA / III) activity was determined by the same method of type 1 fimbriae but by using tanned ox erythrocytes instead of guinea pig erythrocytes and the results were 80% of isolates showed positive reaction for mannose-resistant *Klebsiellae-like* hemagglutination (MR/K-HA) which means that the 16 isolates have CFA / III (Appendix 1).

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These results were in agreement with Al-Mulla (2003) who found that 100% of *Klebsiella* isolated from UTI were *MSHA* positive, while 81.5% of his

isolates were *MR/K-HA* positive. Podschun *et al*., (1993) found that MSHA were detected in 115 (79%) of the 146 isolates examined while MR/K-HA was observed in 103 (71%) of the 146 isolates.

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~~4-6-2B- Adherence Pili~~

~~Type 1 fimbriae activity was determined by assays of hemagglutination of guinea pig erythrocytes in the absence and presence of D-mannose, and all the isolates were MSHA positive. While type 3 fimbriae activity was determined by the same method of type 1 fimbiea but by using tanned ox erythrocytes instead of guinea pig erythrocytes and the results were 16 (80%) of isolates were MR/K-HA positive.~~

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~~*MSHA* (type 1 pili). MSHA of guinea pig erythrocytes could be detected in 100% of the 20 isolates examined.~~

~~*MR/K-HA* (type 3 pili). MR/K-HA of tanned ox erythrocytes was observed in 80% of the 20 isolates examined.~~

~~These results were in agreement with Podschun *et al*., (1993) and HoriaAl-Mulla (2002) who found that 100% of *Klebsiella* isolated from UTI. were *MSHA* positive, while 81.5% of isolates were *MR/K-HA* positive. Podschun *et al*., (1993) found that MSHA were detected in 115 (79%) of the 146 isolates examined while MR/K-HA was observed in 103 (71%) of the 146 isolates.~~

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These results indicated that type 1 pili ~~are~~were found in all the isolates of the pathogenic ~~*Klebsiella*~~*Klebsiella* ~~in which it found in all the isolates~~, while type 3 pili ~~are~~were thought to be not very important in its pathogenecity. ~~That much l~~Less is known about the role of type 3 pili in UTI, even it has an important role for the persistence of bacteria in catheter-associated bacteriuria (Mobley *et al*., 1988).

The importance of CFA/I and CFA/III are in the first stage of infection, in which it mediate the adherence of bacterial cells on the epithelial tissue of infected site, thus facilitates its colonizing (La Ragion *et al*., 2000). The forming

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of biolayer for *Klebsiella* are affected by the presence of pili in which it increasing its density (Langstraat *et al*., 2001).

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4-6-3C- Hemolysis and Siderophores Production

Hemolysin production was tested on blood agar plate and the results showed that all strains (100%) were unable to produce hemolysin.

All the strains were able to produce siderophore in the modified M9 medium supported with 2,2 dipyridyl compound, that only siderophore producing bacteria can utilize such product (Nassif and Sansonetti, 1986; Vernet *et al*., 1995).

Siderophores are important for the bacterial cells by helping them to chelate the iron compounds from the host tissue, in which urinary tract represents an iron restricted environment to which bacterial pathogens may respond by producing iron-chelating compounds (siderophores). (Poschun *et al*., 1993).

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4-6-4D- Serum Resistance Properties

The responses of the examined isolates to the bactericidal activity of normal human serum were recorded over 3 hrs and arranged into sixthree grades, highly sensitive (grade 1 or 2viable count less than 1 percent of initial value), resistant (viable count more than 90 percent of initial value) or intermediately sensitive (3 or 4score between 1 and 90), or resistant (5 or 6). The majority of the strainsisolates, in this study, 15 (6075%) were highly serum resistant and only 5 (25%) were serum sensitive. These results was were in agreement with Ang-Kücüker Ang-Kucuker *et al*., (2000) who found that 53%

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of the 26 isolates were serum resistant, and th but they is is in agreement with Mine Ang Kucuker *et al*., (2000) and disagreement with Podschun *et al*., (1993) who found that most of isolates (57.5%) were serum susceptibilitye. These variable percentageThese variable percentages This may be relatedrelated to the fact that serum resistance of urinary pathogens is related to the site of infections and the severity of the symptoms (Podschun *et al*., 1991).

Most of the serum resistance strains were multiresistance for many type of antibiotics.

The ability of bacteria to avoid killing by normal serum is considered as a property which co-determines their virulence. Most of pathogenic *Klebsiella*Klebsiella are serum resistance (Ang-Kücüker Ang-Kucuker *et al*., 2000).

E- Adhesion of *Klebsiella* on the Human Epithelial Cells

The adhesion properties of *Klebsiella* on the epithelial cells were studied for all (20) isolates. Average number of adherent was 20 15 20bacterial cells / epithelial ceill.

The adherence of pathogenic bacteria on the epithelial cells is very important and represents the first step in bacterial invasion, and this adherent is due to several factors such as: pili (type 1I and III), KpF-28 fimbriea and adherent protein (CF29K protein). (Podschun and Ullmann, 1998), and some times resistant plasmid are encoded for unpili adherent protein CF29K, and the gene encoded for this protein and genes encoded for resistant are on the same location on conjugated plasmid (185 kb) (Di-Martino *et al*., (1995). Also, Di-Martino *et al*., (1996) found that there is a new pili (KPF28) present in most of the extended spectrum β-lactamase (ESBLs) producing bacteria.

F-Phagocytosis

Phagocytosis percentage was calculated for all *Klebsiella* isolates. Results showed that phagocytosis percentages were ranging from 7.5% - 15.0%. This

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~~decrease~~low level of phagocytosis may be due to that *Klebsiella* have large capsule surrounded the bacterial cell, thus decreasing the ability of white blood cells to phagocytose bacteria. This happened by two mechanisms: first, their polysaccharide composition makes capsules highly hydrophilic, which strongly inhibits phagocytosis by hydrophobic surfaced host-cells. Second, many capsules are relatively poor immunogens poor activators of complement (Eisenstein and Zaleznik, 2000)

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4-5 The Relationship between Plasmid Content of *Klebsiella* and Virulence Factors

4-5-1 Curing of Plasmid DNA

Klebsiella pneumoniae MR1 isolate which was multiresistance to 9 antibiotics was selected to perform curing experiment~~, in which this isolate was multiresistant for 9 antibiotics from 11 ones were tested~~ (table 4-~~2~~3).

Results (table 4-~~4~~5) indicated that the highest concentration of ethidium bromide that allows the bacterial ~~growth,~~growth ~~were~~was 800 µg / ml. From this concentration appropriate dilutions were made and spread on brain heart infusion agar plates, and then 150 colonies were tested on a selective media containing a specific antibiotic (ampicillin, tetracycline, az~~e~~teronam, chloramphenicol, gentamicin and streptomycin) in order to determine the cured strains, which cannot grow~~th~~ on th~~is~~ese antibiotic containing media.

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Table (4-~~4~~5) Effect of ethidium bromide on the growth of *Klebsiella* MR1 isolate.

	Ethidium bromide concentrations (µg/ml)										
Bacterial ~~strain~~isolate	0	20	50	100	200	250	300	400	800	1600	3200
MR1	+++	++	++	++	+	+	+	+	±	–	–

+++ = very good growth

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++ = good growth

+ = moderate growth

± = slightly growth

– = no growth

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4.74 Effect of Ethidium Bromide on Bacterial GrowthEffect of Different Curing Agents on Bacterial Growth

Results (table 4-4) indicated that the highest concentration of ethidium bromide that allows the bacterial growth, were 800 µg/ml. From this concentration appropriate dilutions were made and spread on brain heart infusion agar, and then 150 colonies were tested on a selective media containing a specific antibiotic (ampicillin, tetracycline, azetronam, chloramphenicol, gentamicin and streptomycin) in order to determine the cured colonies, which cannot growth on this antibiotic.

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Numbers and percentage of cured cells obtained from treatment with ethidium bromide are shown in table (4-5). Seventy (46.6%) cured cells were obtained. Two cells (1.3%) lost the resistance to ampicillin, aztreonam, tetracycline and chloramphenicol. Eighteen cells (12%) lost the resistance to chloramphenicol and aztreonam. Five (3.3%) cells lost chloramphenicol and 45 (30%) cells lost resistance to aztreonam, which means that these cells have lost one plasmid carrying the aztreonam resistance marker. While other cured cells have lost different types or different number of plasmid.

None of the cells (0%) were lost the resistance to streptomycin and gentamicin and this may indicate that these resistance genes are either chromosomal genes or carried on mega plasmid which not cured by ethidium bromide.

Table (4-4) Effect of ethidium bromide on the growth of *Klebsiella* MR1 isolate.The minimum inhibitory concentration (MIC) of ethidium bromide used in this study was determined in L- broth using MR1 and MR6 isolates (the most antibiotic resistance isolates).

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The curing concentration used for each isolates was; the highest concentration of curing agentethidium bromide that still allows bacterial growth. Bacterial growth in different concentrations of curing agents was monitored visually, and the lowest concentration that inhibited the growth considered as the MIC.

The minimum inhibitory concentration of ethidium bromide for two differentMR1 strains is shown in table ( ). MR1 strain was very resistant and the MIC was at concentration (1600800 µg/ml). Bacterial sample from this concentration was used for curing experiments. This result was in agreement with AL-Yasiry (2000) who found that the MIC of *Klebsiella* Z35 with ethidium bromide was 800 µg/ml. For all other strains, samples from subinhibitory concentrations (The highest concentration allows bacterial growth) were taken to selecting cured cells.

Results of MIC experiments indicated that the subinhibitory concentration was varies from strain to another. These results agreed with that of Trevors (1986) who postulated that the curing concentration depend on the organism and the curing agent.

Carlton and Brown (1983) reported that the most effective concentration of a particular curing agent can vary considerably, in the range of 100 to 1000 fold depending on the species being treated, curing agent efficiency and the mode of action of curing agent.

4.8 Selection of Cured Cells

After treatment of bacterial strains with curing agents, survivors were analyzed for the loss of resistance to antibiotics by plating them on agar media containing the proper antibiotic. Then the curing percentage and efficiency of each agent was analyzed.

The numbers and percentages of cured cells obtained from treatment with ethidium bromide are shown in table ( ). Form MR1, four cured cells (4%) were resulted. All these cured cells lost the resistance to penicillin, which means that these cells have lost only one plasmid carrying the streptomycin resistance marker.

	Ethidium bromide concentrations (µg/ml)									
Bacterial strain		0	0	00	00	50	00	00	00	600	200
MR1	++	+	+	+						

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Table (4-5) Number of bacterial colonies that lost resistance to antibiotics after treatment with ethidium bromide.

	Amp, Te, C, Atm	C, Atm		Atm	n		Total no. of cured colonies
No. of cured cells	2	18		45	-		70
Percentage %	1.3	12	.3	30	0		46.6

For MR6, 10 (10%) were obtained. Nine cells lost the resistance to , streptomycin, tetracycline and gentamicin, one cell lost [he resistance to streptomycin, tetracycline and chloramphenicol. Ten cells lost the resistance to streptomycin and gentamicin, four cells lost the resistance to streptomycin and tetracycline, and one cell lost the resistance to streptomycin and chloramphenicol. Thirty six cells lost the resistance to streptomycin only, which means that these cells have lost only one plasmid carrying the streptomycin resistance marker. While other cured cells have lost different types or different number of plasmids.

No cured cells were obtained from *K. pneumoniae* MR1 strain, however ten cured cells (10%) were obtained from treatment of *K. pneumoniae* MR6 with ethidium bromide, two cells lost the resistance to both gentamycin and streptomycin while eight cells lost the resistance to gentamycin only. These results indicated that this strain contains a plasmid carrying the gentamycin marker only and probably another plasmid carrying the streptomycin marker only or carrying streptomycin and gentamycin marker together. The present curing results agree with previous results showing that ethidium bromide is a powerful drug in eliminating plasmids in antibiotic resistant Entrobacteria (Bouanchaud *et al.*, 1969).

All previous curing results indicated that the efficiency of curing agent depend on the organism being investigated and on the curing agent.

The results also indicated that the curing efficiency of the curing agent vary from strain to another of the same species. Since there are great differences in the percentages of cured cells obtained from different strains of the same organism treated with the same curing agent, this is probably due to the differences in the nature of the plasmids in different strains.

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AccordinglyAlso, it can be concluded that the nature of the plasmid is a very important factor in the curing process.

The above curing percentages results agreed with Lerman (1963) who postulated that the efficiency of curing generally varies from less than 0.1% to more than 99% depending upon the agent involved, the bacterial strain, and the conditions used and he assumed that curing activity is generally related to the ability of these compounds to intercalate into the DNA molecule. Molnar (1988) in a comparative study on the plasmid curing mechanism of twelve tricyclic compounds in *E. coli* concluded that the antiplasmid effects of the drugs depend partly on the host bacterial cell properties and partly on the incompatibility groups of the plasmids.

In order to determine whether the cured cells have lost other antibiotic resistant markers in addition to those investigated previously. Several cured cells from different strains were selected randomly and their antibiotic sensitivity was examined using the disk diffusion method and compared with that of the wild type strains from which they have been derived.

The antibiotic sensitivity of six cured cells derived from *K. pneumoniae* MR1 after treatment with ethidium bromide is shown in table (4-). Some cells had lost resistance to two markers, others to three or four or five markers out of seven tested antibiotics as a result of curing process. Five of the six cured cells lost the resistance to gentamicin or rifampicin indicating that plasmids carrying these markers are efficiently eliminated by ethidium bromide. There was loss of resistance to ampicillin which might indicated that this marker is located on plasmid.

4-95 The EffectRelationship between β-lactam Encoding Plasmid and other Virulence Factors of Curing on the Virulence Factors of *Klebsiella*

To study the relationship between β-lactama encoding plasmid and other virulence factors, these virulence factors were tested on two cured cells which lost resistant for four antibiotics: ampicillin, tetracycline, chloramphenicol and azetronam, two of these antibiotics were β-lactama: ampicillin and azetronam.

4-9-1A Capsules

After curing, tbacterial growth curve were not affected, even there was is decrease in the density of turbidity and this may happened due to the inhibition of the capsular polysaccharide synthesis and this may be due to the loss of gene which carried on plasmid

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that help in the higher production of capsular polysaccharide and the mucoidy properties of the cell such as rmp A and rmp B which carried on plasmid.

This reduction in capsule result in the decreasinge the pathogenecitysis of bacteria (Domenico *et al*., 1982).

B. Adherence Pili

Adherence pili (CFA/I and CFA/III) were not affected by curing, in which both cured cells were positive for MSHA and MR-K/HA, this indicates that genes encoding for these two pili are carried on chromosomal gene.

C. Siderophore Production

Siderophore production was not affected after curing experiment, and this may be related to that siderophore production are mediated by chromosomal encoded genes.

D. Serum Resistance Properties

Cured strains were examined to the bactericidal activity of normal human serum. Serum resistance was decreased in the cured cells (intermediate to sensitive) compared to the parental non cured cells (resistance).

This decrease may be due to the reduction of capsule and the mucoidy substance (that capsule represent the first line of defense against the killing action of serum) and thus become easiest to the C3 component to attacking the bacterial cells.

Also some investigator found that some plasmids, including R plasmids, have also been found to increase the resistance of *E. coli* to serum bactericidal activity (Eisenstein and Zaleznik, 2000).

4.9.24.6 Adhesion of *Klebsiella* on the Human Epithelial Cells

The adhesion properties of *Klebsiella* on the epithelial cells were studied for all (20) isolates, the multi antibiotic resistant isolates wereisolates were the more adherent than the multisusceptible strains. Number of adherent was 20-15 bacterial cells / epithelial cell. This results according with Di-Martino *et al*., (1995) who found that resistant plasmid are encoded for unpili adherent protein CF29K, and the gene encoded for this protein and genes encoded for resistant are on the same location on conjugated plasmid (185 kb). Also, Di-Martino *et al*., (1996) found that there is a new pili (KPF28) present in most of the extended spectrum β-lactamase (ESBLs) producing bacteria.

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Adhesion properties were studied on After curing, cured cell (Amp^{s}, Te^{s}, C^{s} and Atm^{s}). number of adherent cells wereNumbers of adherent cells were decreased reaching to 2-7 bacterial cells / epithelial cell. Bacterial cell not lost its adherent properties, because they not lost its pili (CFA/I and III), but but with no big difference with non cured cells. That mean the reduction of capsular size notmay affect the adherence properties and thus decrease number of adherent cells. This in agreement with Favre-Bonte *et al*., (1999) who found that the adherent not dependadherent not depends on the presence of capsule and it may increase with unencapsulated cells.

Adhesion of bacteria to the epithelial cell is very important for the colonization of bacteria. This adhesion is pili mediated and some times by adherent protein. Any reduction in one of these factors may affect the adhesion of bacteria. These may happened either by loosing the genes responsible of production or due to special circumstances that bacterial cell may switch off the production of such substances (Eisenstein and Zaleznik, 2000).

4-97-3 Phagocytosis

Phagocytic index and pPhagocytosis percentage werepercentage was calculated after curing for the selected isolates (MR1 and MR6). Results show that phagocytosis was 23, 20.5 for the MR1 and MR6 respectively while after curing phagocytosis were increased 170 and 202 respectively.for all (20) the isolates. Results show that phagocytosis percentages were ranging from 7.5% – 15.0%. This decrease level of phagocytosis may be due that *Klebsiella* have large capsule surround the bacterial cell, thus decreasing the ability of white blood cells to phagocytose bacteria. This happened by two mechanisms: first, their polysaccharide composition makes capsules highly hydrophilic, which strongly inhibits phagocytosis by hydrophobic surfaced host cells. Second, many capsules are relatively poor imunogens poor activators of complement (Eisenstein and Zaleznik, 2000).

Phagocytosis percentages were calculated for the cured cell (Amp^{s}, Te^{s}, C^{s} and Atm^{s}), and they were 53.0% and 60.5%.This increasing in the phagocytosis process may be due to reducing in the capsular size, this reduction in capsule size facilitate the activity of phagocytic cells. This increase in the phagocytosis percentage is due to the reduction of capsules, which facilitate the action of phagocytic cells.

4-9-4 Serum Resistance

Cured strains were examined to the bactericidal activity of normal human serum. There was an obvious reduction in the resistance to the serum and this may be due to decrease in the

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bacterial capsule, and thus become easiest to the C3 component to attacking the bacterial cells.

3.4 Effect of different curing agents on bacterial growth

The minimum inhibitory concentration (MIC) of each curing agent used in this study was determined in L broth using *P.aeruginosa* `& N3, *E.coli* Z53 & Z149, and *K.pneumomae* Z35 & Z29 as assay organisms. The curing concentration used for each bacterial strain was; the highest concentration of curing agent that still allows bacterial growth. Bacterial growth in different concentrations of curing agents was monitored visually, and the lowest concentration that inhibited the growth considered as the MIC.

The minimum inhibitory concentrations of sodium dodecyi sulphate (SDS) for different bacterial strains are shown in table (3-7). The MIC for *P.aeruginosa* N1 & *K.pnezimoniae* Z35 was 10% SDS, so bacterial samples from 8% were plated on media containing the proper antibiotics for scoring cured cells. MICs for other strains were higher than 10%. However the growth on this concentration was very weak, so samples were taken from this concentration for scoring cured cells.

The minimum inhibitory concentration of ethidium bromide for different bacterial strains is shown in table (3-8). *P. aerziginosa* N1 was very resistant and the MIC was higher than the highest concentration (3200 ^ig/ml) used in this experiment. Bacterial sample from this concentration was used for curing experiments. For all other strains, samples from subinhibitory concentrations (The highest concentration . allows bacterial growth) were taken to selecting cured cells.

The minimum inhibitory concentrations ofacridine orange used for • different bacterial strains are shown in table (3-9). Different bacterial strains showed different MIC values. Samples from subinhibitory concentrations were used to select cured cells after plating them on agar media containing the proper antibiotic.

Results of MIC experiments indicated that the subinhibitory concentration for each curing agent varies from strain to another. These results agreed with that of Trevors (1986) who postulated that the curing ' concentration depend on the organism and the curing agent.

Carlton and Brown (1983) reported that the most effective concentration of a particular curing agent can vary considerably, in the range of 100 — to 1000 — fold depending on the species being treated, curing agent efficiency and the mode of action of curing agent.

3.5 Selection of Cured Cells

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After treatment of bacterial strains with curing agents, survivors were analyzed for the loss of resistance to antibiotics by plating them on agar media containing the proper antibiotic. Then the curing percentage and efficiency of each agent was analyzed.

The numbers and percentages of cured cells obtained after treatment with SDS are shown in table (3-10). No cured cells were obtained from *P. aeruginosa* N1 & N3 and *K. pneumoniae* Z35 & Z29. However two cured cells (2%) from E.coli Z53 were obtained that lost the resistance to ampicillin, tetracycline and chloramphenicol while no cured cells were obtained from *E. coli* Z149. Similar result was reported by Al - Amir (1998) who failed to obtain cured *P.aeruginosa* by treatment with SDS. However SDS proved to be effective enough to eliminate R factors present in *E.coli* and at a high frequency (Tomoeda *et al*., 1968). Salisbury *et al*., (1972) believed that non susceptibility to SDS is strongly correlated with failure to produce Pili. The degree, to which a particular compound is able to penetrate the bacterial cell surface, is likely to be of primary importance in determining any potential curing activity (Molnar *et al*., 1978) and this may explain the failure of SDS to show curing activity in our study.

The numbers and percentages of cured cells obtained from treatment with ethidium bromide are shown in table (3 -IT). Form *PMeruginosa* N1, four cured cells (4%) were resulted. All these cured cells lost the resistance to streptomycin, which means that these cells have lost only one plasmid carrying the streptomycin resistance marker.

For *P. aeruginosa* N3, sixty one cured cells (61%) were obtained. Nine cells lost the resistance to , streptomycin, tetracycline and gentamicin, one cell lost [he resistance to streptomycin, tetracycline and chloramphenicol. Ten cells lost the resistance to streptomycin and gentamicin, four cells lost the resistance to streptomycin and tetracycline, and one cell lost the resistance to streptomycin and chloramphenicol. Thirty six cells lost the resistance to streptomycin only, which means that these cells have lost only one plasmid carrying the streptomycin resistance marker. While other cured cells have lost different types or different number of plasmids.

No cured cells were obtained from the treatment of *E. coli* Z53 & Z 149 with ethidium bromide.

• No cured cells were obtained from *K. pneumoniae* Z29, however ten cured cells (10%) were obtained from treatment of *K. pneumoniae* Z35 with ethidium bromide, two cells lost the resistance to both gentamycin and streptomycin while eight cells lost the resistance to gentamycin only. These results indicated that this strain contains a plasmid carrying the gentamycin marker only and probably another plasmid carrying the streptomycin marker only

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or carrying streptomycin and gentamycin marker together. The present curing results agree with previous results showing that ethidium bromide is a powerful drug in . eliminating plasmids in antibiotic resistant Entrobacteria (Bouanchaud *et al*., 1969). Efficient elimination of *P.aerugmosa* plasmids with ethidium bromide has also been reported by Al Kazaz (1985) and Al ^ Amir (1998) with high curing frequency up to 32%. Bouanchaud *et al.* (1969) suggested that differences in DNA polymerase and RNA polymerase sensitivities are responsible for differences in ethidium bromide sensitivity of bacterial strains.

Numbers and percentages of bacterial cells, which lost resistance to antibiotics after treatment with acridine orange, are shown in table (3-12). No cured cells were obtained from *P. aeruginosa* N1 &N3, *E. coli* Z53 & Z 149 or *K. pneumoniae* Z29. Three cured (3%) cells derived from *K.pneumoniae* Z35, two of them lost the resistance to both streptomycin and tetracycline, which might indicate that this strain lost two plasmids, and the third cell lost the resistance to gentamycin in addition to tetracycline and streptomycin.

The present curing results agree with previous results showing that .acridines have little if any effect in elimination of R factor in *E. coli* (Mitsuhashi *et al*., (1961). However they were very effective in curing *E. coli* of sex factor F (Hirota, 1960). Al Amir (1998) also showed that acridine orange have no effect in eliminating the antibiotic resistance plasmids of *P.aeruginosa* even in very high concentration of the dye which reach about 5000 pg/ml.

All previous curing results indicated that the efficiency 'of curing agent depend on .the organism being investigated/since ethidium bromide appeared to be powerful curing agent for *P. aeruginosa* and *K. pneumoniae* but not for *E. coli*. SDS gave low curing efficiency for *E. coli* but neither for *P. aeruginosa* nor for *K. pneiimoniae*. Also acridine orange affects *K. pneumoniae* but not *P. aeruginosa* or *E. coli*.

The results also indicated that the curing efficiency of the curing agent vary from strain to another of the same species. Since there are great differences in the percentages of cured cells obtained from different strains of the same organism treated with the same curing agent, this is probably due to the differences in the nature of the plasmids in different strains. Accordingly it can be concluded that the nature of the plasmid is a very important factor in the curing process.

The above curing percentages results agreed with Lerman(1963) who postulated that the efficiency of curing generally varies from less than 0.1% to more than 99% depending upon the agent involved, the bacterial strain, and the conditions used and he assumed that curing activity is generally related to the ability of these compounds to intercalate into the DNA molecule. Molnar (1988) in a comparative study on the plasmid curing mechanism of

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twelve tricyclic compounds in *E.coli* concluded that the antiplasmid effects of the drugs depend partly on the host bacterial cell properties and partly on the incompatibility groups of theplasmids.

In order to determine whether the cured cells have lost other antibiotic resistant markers in addition to those investigated previously. Several cured cells from different strains were selected randomly and their antibiotic sensitivity was examined using the disk diffusion method and compared with that of the wild type strains from which they have been derived.

The antibiotic sensitivity of two cured cells obtained from treatment of *E.coli* Z53 with SDS is shown in table (3 – 13). The cured strain *E.coli* S3 6 has lost resistant to carbencillin, amoxicillin, Co-trimoxazole and bacitracin in addition to ampicillin, tetracycline and chloramphenicol previously investigated, while strain *E. coli* S 17 has lost resistant to carbencillin and amoxicillin in addition to ampicillin, tetracycline and chloramphenicol. *E.coli* S36 has lost seven antibiotic resistance markers out of nine after treatment with SDS, while *E. coli* S17 has lost five resistance markers, which closely indicated that different types or numbers of plasmids are involved in the process. There was no loss of resistance to nalidixic acid and cloxacillin, which might, indicated that these markers are not located on plasmid while other markers are located on plasmids. However it should be mentioned that failure to cure a plasmid does not necessarily imply that the trait is not plasmid – encoded since many plasmids can not be cured (refractory) (Trevors, 1986).

The antibiotic sensitivity of six cured cells derived from *K. pneumoniae* Z35 after treatment with ethidium bromide or acridine orange is shown in table (3 – 14). Some cells had lost resistance to two markers, others to three or four or five markers out of seven tested antibiotics as a result of curing process. Five of the six cured cells lost the resistance to gentamicin or rifampicin indicating that plasmids carrying these markers are efficiently eliminated by ethidium bromide or acridine orange. The major difference between plasmid curing by ethidium bromide and acridine orange was that the tetracycline resistance had been lost by acridine orange but not by ethidium bromide. This is probably a reflection of the dependence of curing efficiency of the agent on the nature of the plasmid. There was no loss of resistance to ampicillin which might indicated that this marker is not located on plasmid.

The antibiotic sensitivity of eight cured cells derived from *P. aeruginosa* N3 after treatment with ethidium bromide is shown in table 3-15. All tested cells lost the resistance to carbencillin, two cells lost the resistance to tobramycin also in addition to previously investigated antibiotics. These results indicated that plasmids carrying streptomycin and

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~~carbencillin are efficiently eliminated by ethidium bromide. The results also indicated that different numbers and types of plasmids are involved in the process.~~

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~~Detection of Virulence Factors in Klebsiella~~

~~Studies were made on MR1 and MR6 strains~~

~~Capsules~~

~~Capsule detection was made by using negative stain (with Indian ink) and by observing the shape and appearance of colony which were mucoidy.~~

~~Microscopic results show the presence of white shadow surrounding the cells (Plate 4-3), in which the capsule synthesis is made by chromosomal genes (Allen~~

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~~4-9-5 Siderophore Production~~

~~Siderophore production was not affected after curing experiment, and this may be related to that genes encoded for siderophore production are found on chromosome and not on plasmid~~Number and percentage of cured strains obtained from treatment with ethidium bromide are shown in table (4-~~5~~6). Seventy cured ~~strains~~isolates (46.6%) were obtained.

Two strains (1.3%) lost the resistance to ampicillin, aztreonam, tetracycline and chloramphenicol. Eighteen strains (12%) lost the resistance to chloramphenicol and aztreonam. Five strains (3.3%) lost resistance ~~chloramphenicol~~to chloramphenicol and 45 ~~strains~~isolates (30%) lost resistance to aztreonam.

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Table (4-~~5~~6) Number of cured bacterial colonies that lost resistance to antibiotics after treatment with ethidium bromide.

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	Amp, Te, C, Atm	C, Atm	C	Atm	Cn	S	Total no. of cured colonies
No. of cured cells	2	18	5	45	–	–	70
Percentage %	1.3	12	3.3	30	0	0	46.6

Amp: ampicillin; Te: tetracycline; C: chloramphenicol; Atm: aztreonam

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S: streptomycin; Cn: gentamicin; –: no cured cells

These results indicated that chloramphenicol and aztreonam resistance genes may be carried on different plasmids (two plasmids), one of them coded for chloramphenicol and the other coded for aztreonam. Ampicillin and tetracycline resistance genes may be carried on the same plasmid, because no cured isolated were obtained that lost one of them only.

These –.

~~genes are carried on different plasmids (two plasmids), one of them coded for chloramphenicol and the other coded for aztreonam. Ampicillin and tetracycline resistance genes are carried on one plasmid, because no cured strains were obtained that lost one of them only.~~

~~Result~~results ~~in~~ above declared that there are three types of cured ~~strains~~isolates~~,~~. ~~strains~~Isolates that lost one plasmid (the 45 ~~strains~~isolates that lost the ampicillin resistance and the 5 strains that lost the chloramphenicol resistance), ~~strains~~isolates lost two plasmids (the 18 strains that lost the chloramphenicol and aztreonam resistance), and ~~strains~~isolates lost three plasmids (the 2 ~~strains~~isolates that lost the resistance to ampicillin, tetracycline, chloramphenicol and aztreonam).

There was no loss of resistance to streptomycin and gentamicin, which might indicated that these markers are not located on plasmid (may be located on chromosome or on mega plasmid). However, it should be mentioned that failure to cure a plasmid does not necessarily imply that the trait is not plasmid-encoded since many plasmids can not be cured (Refractory) (Trevors, 1986).

The above results indicated that ethidium bromide is a powerful agent in eliminating antibiotic resistance plasmids. Other researchers found also that ethidium bromide powerful agent in eliminating plasmids in antibiotic resistan~~ce~~t enterobacteria (Bouanchaud *et al*., 1969).

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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 ~~are~~were carried on one plasmid in *Klebsiella pneumoniae*. Also Sirot *et al*., (1991) found that *Klebsiella pneumoniae* CF1314 have a 150 kb plasmid which carr~~ying~~ied genes encoded for CAZ-7, amikacin, sulfonamide and tetracycline resistance.

In this study

~~These results are in agreement with Sirot *et al*., (1991) who found that *Klebsiella pneumonia* CF1314 have a 150 kb plasmid which carrying genes encoded for CAZ-7, amikacin, sulfonamide and tetracycline resistance.~~

~~C~~curing percentage (46.6%) was relativity high and this may be attributed to several factors: agent involved, the bacterial ~~strain~~isolates, conditions used and the ability of these compounds to intercalate into the DNA molecule (Lerman, 1963). Molnar (1988) in a comparative study on the plasmid curing mechanism of twelve tricyclic compounds in *E. coli* concluded that the antiplasmid effects of the drugs depend partly on the host bacterial cell properties and partly on the incompatibility groups of the plasmids.

4-5-2 Detection of Other Virulence Factors in the Cured *Klebsiella* ~~Strains~~Isolates

To study the relationship between plasmid DNA content of MR1 an-other virulence factors, ~~these virulence factors~~ were tested on two cured ~~strains~~isolates which lost three plasmids (lost resistant to ampicillin, tetracycline, chloramphenicol and aztreonam). These two ~~strains~~isolates were named as MR1C1 and MR2C2.

A- Capsules

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Cured ~~strains~~isolates (MR1C1 and MR~~2~~1C2) have not lost there capsules but they showed a decrease in the turbidity when they allowed to grow~~n~~ in comparison with the parental MR1 isolate, and this might be attributed to the decrease or inhibition of the capsular polysaccharide synthesis or inhibition of the higher production of mucoid substances and this may be due to the loss of regulatory gene (s) that help in the higher production of the mucoid~~y~~ properties of the cells. So it can conclude that the structural gene(s) responsible for capsule production in *Klebsiella pneumoniae* MR1 are carried on chromosome and regulated by gene (s) located on plasmid.

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Other researchers such as Allen *et al*., (1987) have also shown that capsule synthesis is encoded by chromosomal genes. While Nassif *et al*, (1989) found that *Klebsiella pneumonia* has a plasmid encoding for the mucoidy phenotype and it exerts it effect independently from the capsule production genes. Vernet *et al*., (1995) demonstrated that a large plasmid of *Klebsiella* carrying mucoidy genes ~~and~~which can transfer to other bacteria. ~~Sabri~~Al-Saeed, (1997-) found that mucoidy phenotype in addition to ampicillin and tetracycline resistance genes ~~are~~were carried ~~on a~~on a 75 kb plasmid.

~~It was found that the reduction in capsular size resulted in decreasing the pathogenecity of bacteria (Domenico *et al*., 1982).~~

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B-Adherence Pili

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Cured ~~strains~~isolates (MR1C1 and MR~~2~~1C2) were tested for the detection of adherence ~~pilli~~ pili (CFA I ~~/~~and III) in which both cured ~~strains~~isolates were found to be positive for MSHA and MR-K/HA, and this indicates that genes encoding for these two ~~pilli~~ pili are carried on chromosome~~al genes~~ in *Klebsiella pneumoniae* MR1.

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Gerlach *et al*., (1989) found that genes responsible for the production of the CFA/I pili in some *Klebsiella* serotypes are located on chromosomal fragments and on conjugative plasmid, and he found that DNA sequences of

thiese genes is the same as thatas thatthose found in other genus in the enterobacteriaceae family. Soto and Hultgren, (1999) found that chromosomal *fim*-gene-clusture composed of nine genes encoding for the expression of type 1 fimbrial proteins.

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C-Siderophore Production

Ability for the production of siderophore was investigated in the cured strainsisolates (MR1C1 and MR21C2). Both strainsisolates were not affected after curing, and this might lead to the conclusion that siderophore production in *Klebsiella pneumoniae* MR1 isolates is mediated by chromosomal encoded genes.

SabriAl-Saeed, (1997–) showed that siderophorethat siderophore genes in *Klebsiella pneumonia* -are carried on chromosome.

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D-Serum Resistance Properties

Cured strainsisolates (MR1C1 and MR21C2) were examined to the bactericidal activity of normal human serum. Serum resistance was decreased in both cured strainsisolates (intermediate to sensitive) compared to the parental MR1 isolates which were (resistance.).

This decrease may be due to:

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1- The reduction of capsule and the mucoidy substances (that capsule represents the first line of defense against the killing action of serum) and give promotion to C3 component to attack the bacterial cells. aAlso this result emphasizes the result of capsule experiment.

2- Losing one of the resistance plasmid which may have a prime role in resistance might increase sensitivity. Eisenstein and Zaleznik, (2000) found that some plasmids, including R plasmid, have a role in increasing resistance of *E. coli* to serum bactericidal activity.

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E- Adhesion of *Klebsiella*

Cured ~~strains~~isolates (MR1C1 and MR~~2~~1C2) were also examined for the adhesion properties and results showed that although the bacterial cells have not lost ~~its~~thier adherent properties completely, because they did not lost ~~its~~their pili (CFA I / III), the average of adherent cells for both ~~strains~~isolates was decreased down to 2-7 bacterial cells / epithelial cell. The decreasing number of adherent cells might be due to loss of other factors such as KPF28 pili or loss of unpilated adhesins protein such as CF29K~~.~~, which may be present on isolate MR1.

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Di-Martino *et al*., (1995) have found that gene coding for the adhesion protein CF29K is located ~~in~~on a single conjugative plasmid of 185 Kb in length and Di-Martino *et al*., (1996) have found new adhesion pili called KPF28 in many of β-lactamase producing *Klebsiella* strains isolated from UTI.~~.~~

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Adhesion of bacteria to the epithelial cells is very important for the colonization of bacteria. This adhesion is mainly ~~pilli~~ pili mediated and some times it's mediated by adherent protein. ~~These~~The decrease of adhesion may happened either by losing genes responsible for production of adhesion or due to special circumstances that might enforce bacterial cell to switch off the production of such substances (Eisenstein and Zalezink, 2000).

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D-Phagocytosis

Phagocytosis percentages for cured ~~strains~~isolates (MR1C1 and MR~~2~~1C2) were calculated and they were 53% and 60.5% respectively. This increase in the phagocytosis percentage might be attributed to the reduction of capsules, which facilitate the action of phagocytosis cells; also this result ~~is in agreement with~~ensured the result of capsule experiment.

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Chapter Five

5-1 Conclusions

1. *E. coli* was the major causative agent of urinary tract infections followed by *Klebsiella* and *Proteus*.
2. The incidence of UTI was higher in females than in males.
3. High percentages of isolated Klebsiella spp. were resistance to penicillin, ampicillin, chloramphenicol and streptomycin. While the most effective antibiotic against *Klebsiella spp.* are imipenem.
4. Ethidium bromide demonstrated a powerful activity as a curing agent in elimination of plasmid(s) responsible for antibiotics resistance in *Klebsiella pneumoniae.*
5. Four types of antibiotic resistance markers of *K. pneumoniae* MR1 are located on plasmids, ampicillin and tetracycline resistance markers are located on one intact plasmid. While each of aztreonam and chloramphenicol resistance markers are located on different plasmids.
6. Genes in charge of capsule, adhesion pili (type I and III) and siderophore production are carried on chromosome, but gene(s) encoding capsule could be regulated by plasmid gene(s).
7. Phagocytosis, serum resistance and adhesion properties of *Klebsiella pneumonia* do not depend on plasmid genes and they are correlated with capsule.

5-2 Recommendations

1. New generation of antimicrobial agents must be developed, to cope with the multiresistance patterns of bacteria to antimicrobial agents.
2. Investigation of the plasmid content of *Klebsiella spp*. and its relationship to the pathogenesis of *Klebsiella pneumoniae*.
3. Study of other virulence factors that have a role in pathogenesis of *Klebsiella spp.* for example: enterotoxin, urease production, etc.
4. Further study to investigate the mode of action of different virulence factor *in vivo* and *in vitro.*
5. Further study to investigate the nature of virulence factors genes whether they are carried on chromosome or plasmid or they carried on plasmid but under the regulation of chromosomal gene or vise versa.

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Appendix 1

Virulence factors of the *Klebsiella* isolates

Isolate no.	Capsule	CFA/I	CFA/III	Siderophore Production	Hemolysin production	Phagocytosis percentage %	Serum bactericidal activity	Adhesion on Epithelial cells (no. of bacterial cells / epithelial cell)
MR1	+	+	+	+	–	8.5	R	19
MR2	+	+	+	+	–	7.5	R	20
MR3	+	+	–	+	–	11.5	R	17
MR4	+	+	+	+	–	12.5	R	15
MR5	+	+	+	+	–	8.6	R	15
MR6	+	+	+	+	–	7.9	S	16
MR7	+	+	+	+	–	11.5	S	15
MR8	+	+	–	+	–	13.5	S	15
MR9	+	+	+	+	–	15.0	R	19
MR10	+	+	+	+	–	9.5	R	20
MR11	+	+	–	+	–	10.0	S	17
MR12	+	+	+	+	–	13.1	R	15
MR13	+	+	+	+	–	15.0	R	18

Isolate no.	Capsule	CFA/I	CFA/III	Siderophore Production	Hemolysin production	Phagocytosis percentage %	Serum bactericidal activity	Adhesion on Epithelial cells (no. of bacterial cells / epithelial cell)
MR14	+	+	+	+	–	14.2	R	16
MR15	+	+	+	+	–	14.3	S	16
MR16	+	+	+	+	–	15.0	R	20
MR17	+	+	+	+	–	7.5	R	15
MR18	+	+	+	+	–	7.9	R	18
MR19	+	+	–	+	–	14.0	R	17
MR20	+	+	+	+	–	14.9	R	16

الخلاصة

تم عزل وتشخيص مائة وخمسين عزلة بكتيرية من المصابين بالتهاب المجاري البولية، فوجد انها تعود لخمسة انواع من البكتريا:

E. coli 70 عزلة (46.7%) ، و*Klebsiella spp.* 20 عزلة (13.3%) [*Klebsiella pneumoniae* 18 عزلة و*Klebsiella oxytoca* عزلتان]، *Proteus* 18 عزلة (12%) ، *Enterobacter* 13 عزلة (8.7%) ، و *Pseudomonas* 12 عزلة (8.0%)، و *Staphylococcus spp.* 17 عزلة (11.3%) [*Staphylococcus epidermidis* 9 عزلات و *Staphylococcus aureus* 8 عزلات] . كانت 90 عزلة منها (60%) معزولة من النساء في حين 60 عزلة (40%) معزولة من الرجال.

تم فحص نمط الحساسية للمضادات الحيوية لجميع عزلات الـ *Klebsiella* (20) وبينت النتائج ان جميع العزلات 100% كانت مقاومة للـ Penicillin و16 عزلة (80%) كانت مقاومة للـ Ampicillin و الـ وStreptomycin و 15 عزلة (75%) مقاومة للـ Gentamicin و 14 عزلة (70%) مقاومة للـ Chloramphenicol بينما كانت جميع العزلات (100%) حساسة للـ Imipenem و14 عزلة (70%) حساسة للـ Norfloxacin و13 عزلة (65%) حساسة للـ Cephalexin و12 عزلة (60%) من العزلات حساسة للـ Tetracycline و الـ Cefotaxime و11 عزلة (55%) من العزلات حساسة للـ Aztreonam.

تم التحري عن عدد من عوامل الضراوة لبكتريا الكلبسيلا؛ واظهرت النتائج أن جميع العزلات (100%) محاطة بمحفظة. وتمتلك مستضدات عوامل الاستيطان من النمط الاول (CFA/I) في حين اظهرت 16 عزلة (80%) امتلاكها لمستضدات عوامل الاستيطان النمط الثالث (CFA/III). كما بينت جميع العزلات (100%) قابليتها على انتاج السايديروفور وعدم قابليتها على انتاج الهيمولايسين.

اشارت النتائج الى ان 75% من عزلات الكلبسيلا كانت مقاومة للتاثير القاتل لمصل الدم في حين كانت 25% منها حساسة له. كما بينت النتائج ان جميع العزلات لها قابلية الالتصاق على الخلايا الطلائية وقد كانت العزلات MR1 وMR2 وMR10 وMR16 الاكثر قابلية على الالتصاق اذ كان معدل الخلايا الملتصقة 20 خلية بكتيريا / خلية طلائية. اظهرت جميع العزلات مقاومة عالية لظاهرة البلعمة حيث كانت النسبة المئوية للبلعمة متدنية وضمن مدى 7.5-15%.

وفي محاولة لدراسة علاقة بلازميدات عزلة البكتريا *Klebsiella pneumoniae* MR1 بامراضيتها، عوملت تلك البكتريا بمادة بروميد الاثيديوم واظهرت النتائج الحصول على عدد من العزلات

المحيدة التي فقدت المقاومة لعدد من المضادات الحيوية، وتبين ان صفة المقاومة للـ Chloramphenicol والـAztreonam في هذه البكتريا محمولة على بلازميدين مختلفين في حين ان صفة المقاومة للـAmpicillin و الـTetracycline محمولة على بلازميد واحد، اما صفة المقاومة للـSterptomycin والـGentamicin فربما تكون محمولة على الكروموسوم او على بلازميد كبير لم يمكن تحييده.

اختيرت العزلتين المحيدتين MR1C1 و MR1C2 (فقدتا صفة المقاومة للـ Ampicillin والـ Tetracycline و الـ Chloramphenicol والـ Aztreonam) واختبرت عوامل الضراوة الاخرى فيهما. اظهرت النتائج احتفاظ كلا العزلتين بالمحفظة الا انها كانت مختزلة (اصغر حجما)، كما احتفظتا بقابليتهما على انتاج مستضدات عوامل الاستيطان من النمط الاول (CFA / I) ومن النمط الثالث (CFA III /) وقابليتهما على انتاج السايديروفور. الا ان مقاومة السلالتين المحيدتين للتاثير القاتل لمصل الدم قد انخفضت مقارنة بالعزلة الاصلية (MR1)، كما انخفضت قابلية الالتصاق لخلايا العزلتين بالخلايا الطلائية، اذ اصبح معدل الالتصاق 2-7 خلية بكتريا \ خلية طلائية. فضلا عن قلة مقاومة العزلتين المحيدتين لعملية البلعمة اذ بلغت النسبة المئوية للبلعمة 53% و 60% للعزلة MR1C1 وMR1C2 على التوالي.

مما سبق يمكن الاستنتاج ان الجينات المشفرة لعوامل الاستيطان وانتاج السايديروفور في بكتريا MR1 *Klebsiella pneumoniae* ربما تكون محمولة على الكروموسوم اما الجينات المشفرة للمحفظة.

ان تغاير الصفات الاخرى للعزلة (مقاومة التاثير القاتل لمصل الدم وقابلية الالتصاق ومعدل البلعمة) يمكن ان تعزى لاختزال المحفظة.

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دراسة بكتريولوجية وراثية على عوامل الضراوة لبكتريا *Klebsiella pneumoniae* المعزولة من التهابات القناة البولية

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

مهند رضا مهدي ال شكر

بكلوريوس تقانة احيائية جامعة النهرين ٢٠٠١

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