A Bacteriological Study of Local Strains of *Enterobacter cloacae* Isolated from Urine Samples

A Thesis

Submitted to the Council of the College of Medicine,

University of Babylon, in Partial Fulfillment of the Requirements for the Degree of Master in Medical Microbiology

By

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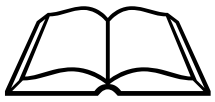
١٤٢٧ A.H **٢٠٠٦ A.D**



بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ ﴿١﴾ خَلَقَ الْإِنسَانَ مِنْ عَلَقٍ ﴿٢﴾
اقْرَأْ وَرَبُّكَ الْأَكْرَمُ ﴿٣﴾ الَّذِي عَلَّمَ بِالْقَلَمِ ﴿٤﴾ عَلَّمَ
الْإِنسَانَ مَا لَمْ يَعْلَمْ ﴿٥﴾

صَدَقَ اللهُ الْعَلِيُّ الْعَظِيمِ

سورة العلق (١-٥)

دراسة بكتريولوجية لعزلات محلية لبكتريا

Enterobacter cloacae

المعزولة من عينات الإدرار

رسالة

مقدمة إلى مجلس كلية الطب في جامعة بابل كجزء من متطلبات نيل

درجة الماجستير في علم الأحياء المجهرية الطبية

من قبل

هالة داود سلمان

١٤٢٧ هـ ٢٠٠٦ م

الخلاصة

من بين مجموعة ١٥٠ عينة إدرار تم جمعها من المركز الإستشاري لمستشفى الحلة الجراحي إستحصلت من المرضى الذين يعانون من أمراض في المجاري البولية ومن كلا الجنسين ،تم عزل وتشخيص سبع عزلات من بكتريا *Enterobacter cloacae* في حين لم يتم عزل البكتريا من الأشخاص غير المصابين . وقد خضعت جميع العزلات إلى الفحوص الزرعية والبايوكيميائية لغرض التأكد من تشخيصها.

ومن ثم درس تأثير بعض المضادات الحياتية على جميع العزلات البكتيرية وقد أظهرت النتائج أن العزلات جميعها كانت مقاومة لكل من الاموكسيسيلين والتتراسايكلين والدوكسيسايكلين بنسبة(١٠٠%)وبدرجة اقل لكل من التراي مثبرم ٨٥.٧% والسيفيكزيم٧١.٤% . أما بالنسبة للسيفوتاكسيم والجنتامايسين فقد كانت المقاومة لكل منها ٥٧.٦%.

على حين كانت معظم العزلات حساسة للسبروفلاكسين بالدرجة الأساس حيث كانت نسبة المقاومة له ١٤.٣% وكانت العزلات حساسة بدرجة اقل لكل من النالديكسك والاميكاسين بنسبة مقاومة٢٨.٦% بينما كانت نسبة مقاومة العزلات للنايتروفيوران ٤٢.٨%.

درست بعض عوامل الضراوة لهذه البكتريا وقد وجد أن جميع العزلات حاوية على المحفظة، وأظهرت النتائج بان جميع العزلات لا تمتلك عامل الاستعمار الثاني(CFA/II) colonization factor antigen في حين أن معظمها استكملت عامل الاستعمار الأول(CFA/I), وان عزلتين فقط امتلكت عامل الاستعمار الثالث(CFA/III).وكذلك أظهرت نتائج الدراسة الخاصة بقابلية العزلات على إنتاج الهيمولايسين والسايدروفورات وانزيمات البروتيز الخارجية أن عزلة واحدة فقط كانت قادرة على إنتاج الهيمولايسين وكان من النوع(α)وان جميع العزلات عدا واحدة فقط قادرة على إنتاج السايدروفورات ,في حين لم تكن هنالك عزلة قادرة على إنتاج

أنزيمات البروتيز الخارجية وكذلك قابلية العزلات البكتيرية على إنتاج البكتريوسين (bacteriocin) وقد أظهرت النتائج أن جميع العزلات غير قادرة على إنتاج البكتريوسين .

وقد تمت دراسة تأثير مركب الاستيل سستين N-actyl-L- cysteine على جميع العزلات وقد لوحظ أن هنالك تأثير واضح على النمو حيث كان هنالك نقصا في معدل الامتصاص الضوئي عند استخدام الطرق اللونية بطول موجي(٦٠٠) نانوميتر كذلك درس تأثير مزج هذه المادة بتركيز ٠.١ ملغم/مل مع بعض المضادات الحياتية التي كانت بكتريا الـ *E.cloacae* عالية المقاومة لها وهي التيتراسايكلين والاموكسيلين والتراي ميثوبرم وقد لوحظ أن هذه المقاومة لم تتغير بوجود مادة الاسيتيل سيستين.

في محاولة للكشف عن احتواء بكتريا الـ *E.cloacae* على عناصر لا كروموسومية فقد اظهرت نتائج العزل للدنا البلازميدي التي أجريت لعزلتين فقط ، احتواء كل عزلة على بلازميد واحد ،يمتلك نفس الحجم تقريبا في كلا العزلتين .

ABSTRACT

This study included (١٥٠) urine sample that collected from patients admitted to Hilla Teaching Surgical Hospital Department of Urology, These patients were of both sexes.

It was found that only seven isolates of *E.cloacae* were identified, whereas no isolates of *E.cloacae* were found in the healthy controls.

All isolates were tested biochemically ,then the effect of some antibiotics on *E.cloacae* was tested, and the results showed that all the isolates were resistant (١٠٠٪) to Amoxicillin, Tetracycline and Doxycyclin, whereas some isolates showed resistance to lesser degrees to Trimethoprim - Sulfamethoxazole (٨٥.٧٪) to Cefexime (٧١.٤٪) while to Cefotaxime and Gentamicin the resistance for both of them was (٥٧.١٪).

On the contrary, most *E.cloacae* isolates were resistant to Ciprofloxacin at the ratio of (١٤.٣٪), while these isolates were resistant to Nalidixic acid with low higher degree (٢٨.٦٪) and to Nitrofurantoin (٤٢.٨٪).

Some virulence factors of bacteria were also studied, and the results showed that all bacterial strains have capsule ,the results also showed that most of the isolates have Colonization

Factor Antigen type I (CFA/I) while no isolates have the (CFA/II), and only two isolates have (CFA/III).

In addition to that , the results of Haemolysin, Siderophore and extracellular protease production tests were showed that only one isolate can produce alpha haemolysin, where as six isolates can produce siderophores, and all the seven isolates were unable to produce extracellularly protease enzymes .

The ability of this bacterium to produce bacteriocin was also tested and it was found that all these isolates have no ability to produce the bacteriocin .

The effect of N-Acetyl L-Cysteine (NAC)on the bacterial isolates were also studied. it was found that this chemical material (NAC) have the detectable effect on the growth of bacteria which had been indicated by through the decreased absorption by using the optical density method at the long wave of ٦٠٠nm.in addition this study also tested the effect of the combination of NAC at the concentration of ٠.١ mg/ml with some antibiotics that the *E.cloacae* isolates were highly resistant to it ,which represent Amoxicillin, Tetracycline and Trimethoprim - Sulfamethoxazole, and it was found that the resistance of *E.cloacae* to these antibiotics was not affected by the presence of NAC.

An attempt was carried out to see whether *E.cloacae* was possessed extra chromosomal elements or not. The results of plasmid extraction for only two isolates showed that these two isolates harbor one plasmid; these two plasmids in both isolates had nearly the same size.

Acknowledgments

Praise to " ALLAH" and to his prophet " Mohammed" . This research has been completed under their benediction.

I am deeply indebted to my supervisor, Assistant Prof. **Dr. Mohammed Sabri Abdul-Razzak** the Head of Microbiology Department, College of Medicine, University of Babylon for his guidance patience and supervision. This thesis would not have been possible, without his sincere help and assistance.

I am also grateful to Dr.Mohammed Kadhum Assistant Prof in Microbiology Department, College of Medicine, University of Babylon and to Dr. Rabah Najah (Biotechnology Department, University of Al-Nahrain) for their generous co-operation and their kind help.

My thanks are also due to all the members of the Department of Microbiology for their help.

Halah

٢٠٠٦

Dedication

To . . .

My Mother. . .and . . .My Father

To . . .

All the Members of My Family

Halah

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List of Abbreviations

Abbreviations	Meaning
ATP	Adenosin Tri Phosphate
CFA	Colonization Factor Antigen
CFU	Colonization Factor Unite
DNA	Deoxyribose Nucleic Acid
EDTA	Ethylen Diamine Tetra acetic Acid
EMB	Eosin Methylene Blue
EPS	Extracellular Polysaccharide
ESBL	Extended Spectrum Beta-Lactamase
MR	Methyl Red
NAC	N-Acetyl L-Cysteine
NICU	Neonatal Intensive Care Unite
O.D.	Optical Density
RBC	Red Blood Cell
SDS	Sodium Dodecyl Sulphate
TBE	Tris base-Boric acid-Na_2 EDTA
TCA	Trichloro Acetic Acid
TE	Tris base-EDTA
TES	Tris base - EDTA -Sucrose
TMP-SMX	Trimethoprim-Sulfamethoxazole
TSA	Tryptic Soy Agar
TSI	Triple Sugar Iron
UTI	Urinary Tract Infection
VP	Voges - Proskauer
(-)	Negative
(+)	Positive

Certification

We certify that this thesis was prepared under our supervision at the College of Medicine, University of Babylon, as a partial requirement for the Degree of Master in Medical Microbiology .

Signature ……

Name: Dr. Mohammed Sabri Al-Saeed

Assistant Professor of Microbiology

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In view of the available recommendation , I forward this thesis for debate by the Examining Committee.

Signature ……

Name: Dr. Mohammed Sabri Al-Saeed

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Head of Department of

Microbiology

Appendix (١): The Effect of NAC on the Growth of *Enterobacter cloacae* (cfu/ml)

Concentration of NAC mg/ml	O.D at ٦٠٠ nm	Number of colonies
Without NAC	٢٠.١	١.٧ x ١٠^{٧}
٠.٠٥	١٩.٩	١.٦ x١٠^{٧}
٠.١	١٦.٤	١.٤ x١٠^{٧}
٠.٢	١٦.٠	١.٣ x١٠^{٧}
٠.٤	١٥.١	١.٠٦ x١٠^{٧}
٠.٦	١٣.٧	٦.٥ x ١٠^{٦}
٠.٨	١٢.٠	٥.١ x ١٠^{٦}
١.٠	١١.٨	٤.٤ x ١٠^{٦}

Chapter One: Introduction and Literatures Review

١.١ Introduction

Enterobacter cloacae **is gram - negative , rod shaped bacterium , and facultatively anaerobic , it belongs to the family enterobacteriaceae (Salas and Geesey,١٩٨٣).**

It has been recognized as a nosocomial pathogen , and sometimes as a primary pathogen mainly due to its ability to develop resistance to antibiotics (Neto, *et. al.*, ٢٠٠٣).

E.cloacae **causes urinary tract infections(UTIs)in human and the spectrum of illness ranges from a symptomatic bacteriuria to pyelonephirtis and urosepsis (John, *et.al.*,١٩٨٢). However, Urinary tract infections due to resisant bacteria such as *E. cloacae* are usually associated with complicated UTIs of about (٥-١٠٪)and lower rates of eradication of bacteriuria (Romolo, *et.al.* ,٢٠٠٤).**

Other diseases caused by *E. cloacae* are extraintestinal and tend to involve the pulmonary system, blood stream, central nervous system, soft tissues, and the genitourinary tract. *E. cloacae* strains are frequently resistant to many antibiotics, making treatment of disease difficult (Karam and Heffer , ٢٠٠٠).

E.cloacae **has an intrinsic resistance to Ampicillin and narrow spectrum cephalosporins and exhibit a high frequency mutation to resist the expanded spectrum and broad-spectrum cephalosporins. *E.cloacae* commonly resistant to all cephalosporins, variable to aminoglycosides and rarely to flouroquinolones (Filius, *et.al.*, ٢٠٠٥).**

There is no local independent study on this bacterium had been conducted; so this study is aimed to :

١. **Isolate and identify some isolates of *E. cloacae* which are associated with UTIs in patients aged between (٧-٨٠) years old from both sexes.**

٢. **Detect some virulence factors such as (capsule, haemolysin, colonization factor antigen (CFA) , siderophore , and bacteriocin).**

٣. **Study the effect of some antibiotics on *E.cloacae* isolates.**

٤. **Describe the plasmid profile of this bacterium.**

٥. **Investigate the effect of N-Acetyl L -cysteine (NAC) on the growth or capsular polysaccharide of *Enterobacter cloacae* isolates, and to study the effect of the combination of (NAC) with some antibiotics on these isolates.**

١.٢ Literature review

١.٢.١ *Enterobacter cloacae* :-

Taxonomically from higher order taxa : Bacteria; Proteobacteria; Gamma proteobacteria; Enterobacteriales; Enterobacteriaceae (Hoffmann ,*et.al.*,٢٠٠٣).

It is a gram-negative, non spore-forming, rod -shaped bacterium that has peritrichous flagella and small polysaccharide capsule , this bacterium measures ٠.٣ - ٠.٦ × ٠.٨ - ٢.٠ µ m, is oxidase - negative , catalase - positive , and facutatively anaerobic . Biochemically this bacterium is positive of Beta-galactoisidase , arginine dihydrolase , ornithin decarboxylase, citrate utilization, nitrate reduction and voges-proskauer reaction . Production of hydrogen sulfide, tryptophan deaminase and indole are all negative . Acid is produced from many carbon sources . Other differentiating features for *E.cloacae* , it grows well on bacteriological media on which yellow pigment and purple stain are not produced, colonies are creamy tan on yeast extract dextrose-calcium carbonate agar (Nishijima, *et.al.*, ١٩٩٣) .

In addition to that, urease production , malonate utilization are both variable for *E . cloacae* growth (Collee , *et.al.*, ١٩٩٦) .

The important biochemical tests that are useful for differentiating *E. cloacae* from other *Enterobacter* species are methyl red, lysine decarboxylase , esculin hydrolysis and gelatin liquefaction, all these tests collectively are negative for *E. cloacae* (MacFaddin, ٢٠٠٠).

The only important confounding bacteria is *Klebsiella* but *Enterobacter* can be easily distinguished from it because *Enterobacter* is motile and positive for ornithine decarboxylase and that unlike *Klebsiella* (Emori and Gaynes, ١٩٩٣), moreover *Enterobacter* colonies are somewhat mucoid as a result to small capsule, while *Klebsiella* species generally have large capsules , therefore the colonies are quite mucoid and tend to coalesce into a mass with prolonged incubation (Roman-Marchant , *et. al.*, ٢٠٠٤) .

There are ١٤ species or biogroups of *Enterobacter*, not all of them have been implicated as causes of disease in humans , among those the mostly

important species that are more commonly recovered from clinical specimens are *E.cloacae* and *E.aerogenes* (Farmer, *et .al.*, ١٩٨٥).

***E. cloacae* is widespread throughout the environment and also carried by humans, it is well - recognized as community and nosocomial pathogen that cause significant infections (Bouza and Cercenado,٢٠٠٢).**

***E.cloacae* causes different diseases in humans,this bacterium usually infects hospitalized patients, people who have used many antimicrobial agents and those who have serious underlying conditions such as diabetes, malignances, burns , mechanical ventilation , etc) and those using foreign device such as intravenous catheters and immunosuppression people (Roberts , *et. al* . , ١٩٩٩).**

In the UTIs, *E.cloacae* is less frequent, and generally more resistant species than *E . coli* , and there are several parameters influence the respective distributions including a previous history of UTI , antibiotic therapy or hospitalization(Cornaglial, *et.al.*, ٢٠٠٤).

:- ١.٢.٢ Physiopathology of *Enterobacter cloacae*

Urinary infections occur as a result of the interaction of bacterial virulence, host biologic 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 , ascending , hematogenous and Lymphatic

pathways . However , infection of kidney with gram negative bacilli rarely occurs by the hemotogenous route (Roberts and Phillips , ١٩٧٩) .

***E.cloacae* colonizes the intestinal tract of healthy humans as normal flora. The source of infection may be endogenous via colonization of the**

gastrointestinal tract or exogenous resulting from the ubiquitous nature of this bacterium, although *E.cloacae* is the most common *Enterobacter* spp. causing nosocomial infections , little is known about its pathogenic potential (Falkiner,١٩٩٢).However , this potential of *E.cloacae* is higher than that of some other members of enterobacteriaceae such as *Klebsiella oxytoca, K. terrigena* , some *Serratia* spp. and

Hafnia alvei **, therefore it is significantly better at invading bladder epithelial cell than these species (Sahly ,*et.al.*,٢٠٠٤).**

Motile bacteria can ascend in the ureter against the flow of urine and the endotoxins of gram - negative bacilli have been shown to decrease uretral peristalsis and possibly contribute to the renal parenchymal inflammatory response by the phagocytic cell activation (Eden and Svennerholm,١٩٧٨).

١.٢.٣ Prevalence of *Enterobacter cloacae* :-

Enterobacter **species infections in human have expanded exponentially, the incidence of infection has increased and new clinical syndromes have been**

recognized (Scriver, *et.al.*, ١٩٩٥). *E . cloacae* among the species that responsible for major health problems world wide in the last decades and has become increasingly important as a nosocomial pathogen (Toltzis and Blumer , ١٩٩٥), also the patients morbidity and mortality due to *E . cloacae* has increased (Cosgrove , *et. al* ., ٢٠٠٢).

E. cloacae **is part of the normal flora of the gastrointestinal tract of ٤٠ to ٨٠ % of people and rarely cause infection in these individuals, they infect those with risk factors such as serious underlying illness and immunosuppression (Lindh and Ursing ,١٩٩١).**

Since ١٩٨٠ there is significance increase in the number of bacteriological isolates of *E . cloacae* and this increasing mainly due to increased use of antibiotics especially cephalosporins and aminoglicosides (Anderson and Hieber,١٩٨٣).

E.cloacae **accounted for ٥ % to ٦ % of the total pathogens causing various types of infections . Its infections have the highest mortality rate among all *Enterobacter* infections . *E .cloacae* is a serious nosocomial pathogen and it is the third most prevalent bacterium isolated in Intensive Care Unite (ICU) settings (Sohn , *et. al.*, ٢٠٠١).**

In adults *E . cloacae* can cause severe and complicated UTI_s such as

pyeloonephritis moreover, it cause catheter associated UTI, anatomic abnormalities UTI in diabetics , UTI in renal transplant recipients , UTI in neutropenic patients and UTI in AIDS patients (Brown,

٢٠٠٢ and Patterson and Andriole , ١٩٩٥) . In addition to that *E . cloacae* can cause serious pyelonephritis (Evanoff, *et. al.*,١٩٨٧) .

***E.cloacae* considered one of the most common causal organisms for acute**

bacterial prostatitis and prostatic abscess also it can cause chronic prostatitis. *E.cloacae* represented ٢.٥٪ in the cases of chronic prostatitis due to bacterial

cause from a total of seventy five patients in Washington in ١٩٨٨ (Lipsky, ١٩٩٩)

Fifty eight (٥٨) isolates of atypical *E. cloacae* that sharing the common

O antigen were responsible for noscomial outbreak of UTIs (Mummery, *et .al.*,

١٩٧٤), while it represent ٢٪ in asymptomatic bacteriuria among pregnant women in Philippine in ٢٠٠٢(Sescon, *et .al.*, ٢٠٠٣).

On the other hand *E. cloacae* had the ratio of (١.٩٪) in the outpatient urinary isolates obtained in various geographic regions in the USA and Canada in ٢٠٠٥ (Zhanel , *et. al.*, ٢٠٠٥).

***E.cloacae* had been isolated as two cases (٢.٢%) from the urine samples of ٩٠ women with symptomatic and asymptomatic UTI in India ٢٠٠١ (Ethel, *et. al.*, ٢٠٠٦).**

In addition to UTI_s, *E. cloacae* can cause other infections in adults such as lower respiratory tract infections (ventilator associated pneumonias) which in some studies *E.cloacae* may account for up to one quarter of this infection, and it can cause bronchitis (Weber , *et .al* ., ١٩٩٩).

***E .cloacae* does not exist as skin flora of human and soft tissue infections caused by *E .cloacae* have been reported quick rarely(Ganelin and Ellis ,١٩٩٢).**

however, recently it had been isolated from the case of prolonged cellulites as the causative agent(Cengiz ,*et .al.*, ٢٠٠٥) .

No report of periostitis , spondylodiscitis or osteomyelitis cases due to *E . cloacae* invasion (Solans , *et. al.*, ١٩٩٢).

Besides , neutropenic patients with malignances, and patients with burns, wounds , respiratory and UTI_s , also those who have invasive procedures, and indwelling catheters are the groups at highest risk of *E .cloacae* bacteremia and sepsis (Singh and Yu Vl , ٢٠٠٠).

In elderly patients aged ٧٠ years or more *E . cloacae* can cause UTIs, pneumonia and pleural effusion (Knockaert,١٩٨١).

A greater resistance to disinfectants and antimicrobial agents of *E. cloacae* than that of other members of enterobacteriaceae is likely to play a role in the increasing prevalence of *E . cloacae* as nosocomial pathogens (Wade , *et .al* ., ١٩٩١).

١.٢.٤ Epidemiology of *Enterobacter cloacae* :-

Nosocomial infections caused by *Enterobacter* spp. are by far the most frequent than community acquired infections which are occasionally observed. The source of hospitalized UTIs caused by *E. cloacae* either endogenously or from common source, such as medicaments , food , apparatus ; fecal carriage, but hand spread less common (Fryklund, *et.al.*, ١٩٩٤).

Several factors has been caused and aggravated outbreaks of *E.cloacae* nosocomial infections , patient - to - patient transmission , understaffing ,

overcrowding , and poor hygiene practices (Harbarth ,*et.al.*,١٩٩٩).

The use of broad- spectrum antibiotics was considered to be the risk factor for the outbreak of multiresistant clones of *E. cloacae* in the neonatal intensive - care unit (NICU), in addition medical personnel also transmit the infection (Carmen ,٢٠٠٥).

***E.cloacae* among the organisms that have the relative frequency of recurrent UTIs , especially in the presence of structural abnormalities of urinary tract**

(such as obstructive uropathy , congenital abnormalities , neurogenic bladder, and fistulous communication involving the urinary tract) (Bronsema, *et.al.*,

١٩٩٣).

Certain properties of *E.cloacae* may be important in the epidemiology of hospital acquired infections , and may increase the resistance to antiseptic and disinfectants (Schaberg, *et.al.*, ١٩٩١).

***E. cloacae* is unusual organism among bacteria in their ability to proliferate in acidic, nitrogen - poor, and (٥٪) dextrose - containing fluids (Matsaniotis, *et.al.*,١٩٨٤). In addition, it capable of proliferating at room temperature and it can grow and reproduce in a restricted saline environment for extended periods of time (Young, *et. al.*, ١٩٩٧).**

Clinical awareness of the potential of *E.cloacae* strains to cause disease has been reflected in the increasing number of epidemiological studies of this bacterium (Bodey ,*et.al.*,١٩٩١).

١.٢.٥ Urinary tract infection (UTI) :-

Urinary tract infections (UTIs) are defined as an inflammatory syndrome caused by microbial invasion of the urinary tract. They affect a variety of patients ranging from young children to elderly, and from healthy men and women to compromised patients. UTIs are the most common occurring bacterial infection and have a significant social and economic burden (Nicolle, ٢٠٠٢).

The etiology of UTI is affected by underlying host factors that complicate UTI, such as age , spinal cord injury , consequently ,organisms that rarely cause

disease in healthy patients can cause significant disease in hosts with anatomic,

metabolic ,or immunologic underlying disease(Ronald, *et.al.*,٢٠٠١).

Most infections of the urinary tract are caused by gram - negative enteric

organisms , of which *E . coli* is the most common , particularly in first and

uncomplicated infections, *Enterobacter* spp. among other organisms that can cause UTI (Heptinstall,١٩٩٢).

***Enterobacter* spp. UTI is indistinguishable in (signs and symptoms) from**

a UTI caused by other gram-negative bacilli, other *Enterobacter* species that can cause UTI in addition to *E. cloacae* including *E. aerogenes* (which is

with *E. cloacae* are the most commonly *Enterobacter* species isolated from UTIs), *E.amnigenus* has been occasionally isolated from urine specimens,

***E. gergoviae* and *E .asburiae* have also been isolated from a UTI , but have the lower importance (Grimont and Grimont , ١٩٩١). However , while *Enterobacter* spp. are most often recognized as opportunistic pathogens of hospitalized patients especially those treated with antibiotics and associated with UTIs and other infections, typically, nosocomial *E.cloacae* strains are often multiple antibiotic resistant(Goldmann and Huskins,١٩٩٧),monitoring this antimicrobial resistance is important, because resistance has been reported to be associated with increased patient morbidity and mortality , prolonged hospitalization and increased hospital expenditure (Cosgrove ,*et.al.*,٢٠٠١).**

١.٢.٦ Virulence factors of *Enterobacter cloacae* :-

The factors that determine the initiation ,development, and outcome of an infection involve a series of complex and shifting interaction between the host and the parasite which can vary with different infecting microorganisms (Brogden , *et.al.*,٢٠٠٠).

E.cloacae **has the ability to produce the virulence associated properties**

and specific virulence determinants that play a definitive role in the infections,

some authors suggested that *E.cloacae* may be more virulent than *E. aerogenes* (Gaston, ١٩٨٨).

١.٢.٦.١ The Capsule :-

Some strains of bacteria may have a capsule around the bacterial wall which is a polysaccharide material and it is an important virulence factor in

some bacterial species such as *Streptococcus* and *Klebsiella* . It is contributed to adhesion and prevention from phagocytosis . Most of these antiphagocytic

surface structures show much antigenic heterogeneity that the antibodies against one type of the antiphagocytic factor protect the host from disease caused by bacteria of that type but not from those with other types of the same factor (Robbins, *et.al.*,١٩٨٠).

Although , the slime layer is enclosed around the bacterial wall and it consisted of polysaccharide and proteins, it has toxic effect on the phagocytic cells and T-lymphocytes that leading to deficiency in the immunity of the host. The slime layer is commonly presented in most enterobacteriaceae (Burrell and Rylander ,١٩٨٢).

The ability of *E.cloacae* to cause a disease is not clear but the presence of capsule makes it more virulent and through this polysaccharide, the bacteria become able to resist the action of phagocytosis (Mannhardt, *et.al.*, ١٩٩٦).

١.٢.٦.٢ Adhesion and/ or colonization factors :-

The first host barrier for many invading pathogens is usually a mucosal surface , and since epithelial cell turnover is around ٤٨ hours in these , the

environments bacterium must attach and replicate sufficiently to avoid being

swept away, therefore many have evolved motile or attachment elements like flagella and pilli to cross the barrier and invade (Sauer, *et. al.* , ٢٠٠٠) .

Bacterial adhesion to epithelial surfaces is thought to be one of the most important virulence factors, playing a significant role in the initiation of UTI.

The bacterial adhesion capacity is most frequently associated with the presence of fimbriae on bacterial cells. It has been show that fimbriae are responsible for the attachment of bacteria to uroepithelial cells (Silverblatt and Ofek , ١٩٨٨).

The mannose - specific fimbriae which named type I fimbriae are wide

spread in most species of enterobacteriacea. This colonization factor antigen

(CFA/I)causes the agglutination of red blood cell for human which is group (A). Another colonization factor called (CFA / II) has been found to cause the agglutination of chicken blood . The two factors are non - inhibited in the

presence of mannose sugar and these two factors have specialized host (Al - Zaag, ١٩٩٤).

The third colonization factor (CFA/III) causes the agglutination of red blood cell in the presence of Tannic acid . This factor underlyies control of two

genetic chromosome : one of them is responsible for piliation , and the other for adhesion (Hornick , *et .al.* , ١٩٩٥) .

E. cloacae **can produce all the three types of fimbriae(I, II, and III), type I fimbriae of it have combining sites differ from those of *E.agglomerans* at the species level and from those of *Salmonella* spp. and *E. coli*(Firon , *et.al.*,١٩٨٤).**

E.cloacae **CFA / I or type I fimbriae have comprised a distinct serological group, and have considerable heterogeneity existing in its gene cluster (Steven and Gerlad , ١٩٨٧).**

In addition, *E. cloacae* can express type III fimbriae, which are serologically related to *Klebsiella* spp. fimbriae III , the gene encoding CFA /III(mrkA)

is conserved within *Enterobacter* spp. and *Klebsiella* spp. among the

enterobacteriaceae (Clegg and Gerlach, ١٩٨٧).

١.٢.٦.٣ Siderophores production :-

Iron is critical to growth and metabolism of nearly all living organisms, prokaryotic and eukaryotic. Limiting the concentration of free extracellular iron is a strategy of host defense against pathogenic microorganisms that practiced

by many animal species. Many bacterial pathogens secrete highly efficient low- molecular -weight iron chelating agents, termed siderophores (Bradley,

et .al. **, ٢٠٠٠).**

These agents compete for and bind available iron . The siderophore -iron complexes are recognized by the bacteria , which then internalize the iron . It is a high -affinity iron assimilation system , this system has the ability of induction of multivalent cationic metals and it is unaffected when the cells are depleted of ATP or when receptor - mediated endocytosis is inhibited with dihydrocytochalasin. (Weinberg and Weinberg, ١٩٩٥) .

The biological importance of siderophore has been demonstrated for

a number of species , the siderophore - deficient mutants of pathogenic bacteria are invariably less virulent in disease models thus , siderophores , are compounds secreted under low iron stress, that act as a specific ferric iron chelate agent and due to their potentialities it is used in the biological control of phytopathogenic fungi and bacteria (Lamont, *et. al.*, ٢٠٠٢).

Clinical strains of *E. cloacae* produce one or more types of siderophores (Loper, *et. al.* , ١٩٩٣), certain strains of *E.cloacae* can produce enterochelin

and hydroxmate compound which defined as aerobactin and these two compounds represent siderophores (Oudega, *et.al.*,١٩٧٩).

The outer membrane protein receptor for ferric aerobactin in *E. cloacae*

strains that produce bacteriocin also act as the binding site for this bacteriocin (cloacin) (Crosa, *et. al.* ,١٩٨٨).

١.٢.٦.٤ Haemolysin production :-

It is a metabolic virulence factors that allows the colonizing bacteria to

survive. The synthesis of cytotoxic haemolysins is common between both gram positive and gram-negative bacteria. The production of α- haemolysin or β- haemolysin has lyses the host cells by creating transmembranous pores.

The haemolytic factor seems to be strongly cell associated and not stable , since not haemolytic activity was observed in cell- free supernatants and filtrates of bacterial cultures (Nassif and Sansonetti, ١٩٨٧) .

Generally, *E.cloacae* dose not produce haemolysin(Rubinstien, *et.al.*,١٩٩٣).

However, some strains of *E.cloacae* can produce haemolysin and this production was tested with specific monoclonal antibody and by DNA hybridization with an α-haemolysin specific gene probe (Prada and Beutin, ١٩٩١).

The haemolysin facilitates tissue invasion and causes renal tubular epithelial and parenchymal cell damage (Mobley, *et.al.*, ١٩٩٠).

١.٢.٦.٥ Bacteriocin Production :-

Microorganism naturally produce a range of protein components from simple polypeptides to very complex macromolecules such as toxins, pilin, adhesions , siderophores , flagellin, etc. Bacteriocins are grouped under the

term toxins and provide a means of defense against other microorganism in the same environment (Leslie , *et.al.*,١٩٩٨).

Bacteriocin is a subgroup of bacterial toxins , as proteinacious compound that kill closely related bacteria .Although this is true for most bacteriocins, it is evident that these molecules take many forms and may have bactericidal action beyond closely related species. Frequently, bacterial species carry genes that encode both the production of one or more bacteriocin and immunity to them on chromosome or on plasmids (Frank , ١٩٩٤) .

Bacteriocins have a spectrum of size ; generally proteinacious agents , they are sometimes complexed with lipids , carbohydrates or other distinctive proteins (Jimines- Diaz , *et. al.*,١٩٩٣) . There are three general classes:-

١- The microcins , which are small molecules produced in stationary phase by

gram-negative bacteria .

٢- The lantibiotics are small molecules produced by gram - positive bacteria.

٣- Bacteriocins , which are a group encompassing medium to large phage – like structure .

These proteins share many characteristics . Many have a low molecular weight , are cationic , tend to aggregate and are benign to the producing

organism (kim,١٩٩٣)

E. cloacae **can produce the bacteriocin which is mediated by clo DF١٣ bacteriocinogenic plasmid (Francia, *et. al.*, ٢٠٠٤).**

E. cloacae **produces susceptible diffusing, non - sedimentable, protease-resistant bacteriocins . Bacteriocins, mainly of the protease-stable type , might be one of the factors determining the composition of mixed bacterial populations colonizing or infecting patients . Bacteriocin typing of *E. cloacae***

could be useful tools for epidemiological analysis of nosocomial infections caused by this bacterium (Adolf and Christl, ١٩٨٤).

١.٢.٧ Bacterial resistance to antibiotics :-

The choice of appropriate antimicrobial agents is complicated by the fact that the majority of bacteria in the genus *Enterobacter* are either very resistant to these agents or it can develop resistance during antimicrobial therapy.

E. cloacae **has emerged as an important nosocomial pathogen that cause UTIs and other infections(Cunha, *et. al* .,١٩٩٩) .**

Moreover, resistance to antimicrobial agents and reports for multi- drug resistant isolates of *E. cloacae* have increased during the last decade, probably as a result of extensively use of broad spectrum antibiotics (Tzelepi,

***et. al.*, ١٩٩٢).**

With rare exceptions , *E . cloacae* is highly resistant to Amoxicillin which is narrow spectrum Penicillin that is generally effective for treatment UTIs, *E .cloacae* also highly resistant to first and second generation cephalosporins (Ristuccia and Cunha , ١٩٨٥) .

Resistance of *E .cloacae* to these antibiotics is due to production of inducible chromosomal group-١ β- lactamases also called cephalosporinases, *E. cloacae* is the most commonly isolated member of enterobacteriaceae that posses these enzymes. Over expression of these enzymes is the usual mechanism of β- lactam resistance in *E. cloacae*, but recently it has also become apparent that this species can acquire and express genes encoding Extended Spectrum β - lactamases (ESBLs) (Canton ,*et. al.*,٢٠٠٢) .

Bacteria producing ESBLs considered resistant to all generations of cephalosporins,all Penicillins, and to the Aztereonam(De champs, *et.al.*,٢٠٠٠).

Cefotaxime and Cefixime which are third generation cephalosporins have been affected on bacterial UTIs, However, resistance of *E.cloacae* to

these broad spectrum cephalosporins which can develop during therapy with these agents is a consequence of the selection of the mutants stably derepressed for the production of the group ١-β-lactamases, therefore, *E.cloacae* under intensive selective pressure from broad - spectrum β-lactam usage (Doucet- Popularie ,*et. al.* , ٢٠٠٠) .

The proportion of *E . cloacae* isolates that resistant to third generation cephalosporins including Cefotaxime and Cefixime increased from ٢٤.٣ % to ٢٩.٦ % between ١٩٩٩ and ٢٠٠٢, this increasing has been associated with increased use of the more newly cephalosporins (Muller , *et.al.*, ٢٠٠٤).

Gentamycin and Amikacin has good activity against *E.cloacae* , however resistance of *E.cloacae* to them has been increased during the last decade in several countries with some geographical variation(Jones , *et .al* . ,٢٠٠٤).

Resistance of *E.cloacae* to Gentamycin is much fewer than that of Amikacin this increasing has appeared to be related to an increase in aminoglycosides usage (Struelens ,١٩٩٨) .

The mechanisms of *E . cloacae* resistance to these aminoglycosides

is the production of one or more aminoglycosides - inactivating enzymes

(Peyret, *et. al.*, ١٩٩٣).

Ciprofloxacin is the most frequently prescribed flouroquinolones for UTIs, *E.cloacae* has high susceptibility rate to Ciprofloxacin and resistance

to it is relatively rare . About ٨٠٪ of *E.cloacae* isolates are susceptible to Ciprofloxacin (Janicka, *et.al.*, ٢٠٠٠).

In certain study Ciprofloxacin was the most active antibiotics (>٩٠٪ susceptibility) against *E.cloacae* strains, with no observed reduction in activity over ٨ years (Patzer, *et.al.*, ٢٠٠٥). However, Resistance of *E. cloacae* to

quinolones increased from (١٦.٧٠٪) to (٢٤.١٠ %) in a ten-year period (١٩٩٣-٢٠٠٢) (Rodriguez , *et. al.*, ٢٠٠٤).

Regarding to Nalidixic acid, the resistance to it markedly increased from ٢.٠٪ to ١٧.٥٪ in *E. cloacae* strains between ١٩٨٤ and ٢٠٠١ according to the susceptibility data from three central European countries (Kresken and Hafner, ٢٠٠٥).

However, this resistance has increased markedly due to inappropriate prescribing of antibiotics and poor infection control strategies (Astal ,*et al.*, ٢٠٠٢). The penetration of quinolones through the *E.cloacae* outer membrane

has not been extensively studied , two entry mechanisms were possible:

(١) gliding of the antibiotic molecule at the lipid porin interface.

(٢) diffusion of the antibiotic molecule through the porin channel(Chevalier,

***et. al.*, ٢٠٠٠).**

In clinical isolates of *E . cloacae* , DNA gyrase is a primary target of

quinolones, that only a single amino acid changing is sufficient to generate high-level resistance to Nalidixic acid and to decrease susceptibility to Ciprofloxacin, and that the accumulation of amino acid changes in *gyr* A play a central role in developing high-level resistance to Ciprofloxacin .The plasmid - mediated quinolones resistance determinant *gyr* A and the transfer of this resistance was discovered in multidrug - resistant *E. cloacae* isolates (Corkill, *et. al.*, ٢٠٠٥).

About the effect of TMP/SMX on *E.cloacae*, Barsic, *et. al.*,(١٩٩٧) showed that these bacteria have high resistance to TMP/SMX . The mechanisms of *E .cloacae* resistance to TMP/SMX like its resistance to quinolones is mediated

through modification of the antibiotic target and prevention the access to the target (Tsakris, *et. al.*, ١٩٩١) .

Concerning to Nitrofurantion , this antibiotic interferes with bacterial

carbohydrate metabolism by inhibiting acetyl - Co-A enzyme, Nitrofurantion activity against *E. cloacae* like other species of *Enterobacter* is less predictable than that of other organisms (Stamey ,*et.al.*,١٩٨٧).The mechanism of *E. cloacae* resistance to Nitrofurantoin may be due to the decreased Nitrofrantoin reductase enzyme (Sprenger, *et. al.*, ١٩٩٩).

Carbapenems are the only reliable beta lactam drug for the treatment of severe *E .cloacae* infections , however, resistance to it has also been

documented, and fourth generation cephalosporins (Cefepime) are a distant second choice, other drug for treatment *E.cloacae* infections are aminoglycosides and quinolones (Donati ,١٩٩٣).

From this highly resistance of *E.cloacae* to commonly used antibiotics, one can anticipate that these bacteria will remain a significant problem for the coming decade (Neu,٢٠٠٢).In addition , the highly resistance of *E.cloacae* to β-lactam antibiotics often complicates the treatment of the infections caused by this bacteria (D'Agata , *et.al.*, ١٩٩٩).

١.٢.٨ Genomic composition :-

Most genetic informations of the bacterial cell resides in a single circular double stranded DNA molecule commonly referred to as the bacterial chromosome. Some bacteria may also contain extrachromosomal , small circular DNA molecules that replicate autonomously of the bacterial chromosome called plasmids and are incapable of integration in the bacterial chromosome, plasmids often carry genes for antibiotic resistance that confer selective advantage upon their host cells when antibiotics are in their environment. Most plasmid between (١/١٠) to (١/١٠٠) of the size of the bacterial chromosome. In addition there is other genetic elements called jumping genes or transposone which can mediate their own movement from one location to another on the genome(Stansfield , ١٩٩١) .

***E. cloacae* is a heterogeneous and comprises at least six genomic groups, however the groups could not be differentiated phenotypically and that prevented its systemic classification or an attempt to split *E . cloacae* into two or more species (Tang, *et .al.*,١٩٩٨) .**

Gerard and Hendrik (١٩٧٦) showed that certain strains of *E cloacae.* harbor at least five different classes of plasmids, among them the self-transmissible R factor determining resistance against

some antibiotics , others are bactericinogenic factor and has the largest size, and the low molecular weight

plasmids defined as cryptic plasmids in general have unknown functions, but it has been ruled out that the cryptic plasmids represent free antibiotic- resistance determinants derived from the multiple R factor present in *E . cloacae*. A common feature of all these five plasmids is their application kinetics, which follows the anarchical model , i.e. maintain a fixed number of molecules per chromosome .

Sherly, *et .al.* (٢٠٠٣) had found that approximately ٧٠ % of *E. cloacae* carried plasmids , the number of these plasmids ranges from ١ to ٥ with

the size mean range centered around the(٦٤ - ١٢٨) Kbp and skewed a little

towards the higher weights plasmids .

The high incidence of multi resistance in *E.cloacae* and the fact that the resistance is due to enzymatic inactivation of antibiotics indicate that this

species might act as a gene pool for spread the resistance to other bacteria of clinical relevance (Vatopoulos , *et. al.*, ١٩٩٢).

Vrabelova , *et .al.*, (٢٠٠٤) observed that the *E . cloacae* among the bacteria that have nosocomial plasmid of ١١٦ Kbp which had the prevailing incidence at different wards including urological ward of the hospital through

out the three year period . This nosocomial plasmid harbor multi resistance for the β-lactam, aminoglycosides and flouroquinolones agents, restriction analysis suggested a dissemination and persistence of a single nosocomial plasmid at all these hospital units.

The role in transfer some genetic determinants not depend only on the plasmids , some studies were proved that *E . cloacae* has additional genetic elements such as transposones and Integrons, the arm A gene (aminoglycoside resistance methylase) were discovered in clinical isolates of *E . cloacae* and they were observed that this gene was part of composite transposone together with other genes responsible for resistance to Streptomycin,

Sulfonamides and Trimethoprime (TMP) (Galimand ,*et. al* ., ٢٠٠٥).

In molecular epidemiologic study performed by Belkum , *et. al.* (٢٠٠١) they described the frequent occurrence of integron - encoded Gentamicin resistance among nosocomial isolates of Ciprofloxacin resistant *E . cloacae* strains, among these strains , two different integron types were

encountered against a diverse background of chromosome , this could be indicative to intraspecies dissemination of theses particular elements .

Chapter Two: Materials and Methods

٢.١ Materials :-

٢.١.١ Laboratory Instruments :-

The laboratory instruments used in this study are shown in(Table ٢.١).

Table (٢.١): Laboratory Instruments Used

No.	Instruments	Company
١	Sensitive electronic balance	A & D, Japan
٢	Autoclave	Stermite, Japan
٣	Incubator	Memmert, Germany
٤	Distillator	GFL- Germany
٥	Centrifuge	Hermle, Japan
٦	Oven	Memmert, Germany
٧	Refrigerator	Concord, Italy
٨	Milipore filter type ٠.٤٥µm	Satorius membrane filters Gm BH, W.Germany.
٩	Light microscope	Olympus, Japan.
١٠	Micropipette	Oxford, USA.
١١	PH meter	Hoeleze &Cheluis, KG, Germany
١٢	Inoculating loop	Japan
١٣	Inoculating needle	Japan
١٤	Benson burner	Germany
١٥	Spectrophotometer	Bausch & Lomb

١٦	Electrohoresis apparatus Mini sub(TM) DNA cell/١٠٠٠ VDC	LKB Sewedian
١٧	Shaking Incubator	Gallenkamp, England
١٨	Water bath	Memmert, Germany
١٩	Eppendrof Centrifuge	Hermle, Germany
٢٠	Ultra Violet Transilluminator	San.Gabriel,USA
٢١	Balance	Sartorius, Germany
٢٢	Sensitive electronic balance	Mettler, Switzerland

٢.١.٢ Chemical and Biological Materials :-

A- Chemical Materials :-

The Chemical and Biological materials used in this work are shown in Table (٢.٢).

Table (٢.٢): Chemical and Biological Materials Used

No	Chemicals	Company
١	NaCl, $MgSO_4$, KH_2Po_4, Na_2HPO_4, NaOH, KOH,K_2HPO_4.	Merk-Darmstad
٢	Alpha-naphthol, esculin, Trichloroacetic ac Tetramethyl-***p***-paraphylenediamine dihydr -loride, Tannic acid, Chloroform . Meth Boric acid ,EDTA-Na_2,EDTA , Sodium Dodecyl sulphate (SDS) , Glycerol, Inositol, Manitol, Sucrose, Phenol ,Bromothymol blue, Isoprobanol alcohol ,Agarose, , Peptone, Phenol red .	B.D.H.
٣	Glucose , ٩٩٪ and ٧٠٪ alcohol (Ethanol), solution, Kovac's reagent, D-mannose, I Ornithine, Yeast extract , H_2O_2, Dipyridyl.	Fluka chemika-Switzerlan.
٤	Ethidium Bromide, M	Ajax
٥	Antibiotics discs	Oxiod-England

B- Culture media :-

The culture media used in this work are shown in (Table ٢.٣).

No.	Media	Company
١	Blood base agar, Brain heart infusion agar, Brain heart infusion broth,agar-agar, Mulller-Hinton infusion agar, MacConkey agar, Manitol salt agar, peptone broth, Yeast extract, Eosin Methylene Blue agar.	Mast
٢	Nutrient agar media, Nutrient broth , Peptone water medium , Gelatin , liquefaction medium .	Oxiod
٣	Urea base agar, Simon citrate agar, Triple sugar iron agar, MR-VP broth . Tryptic soy agar .	Diffco-Michigan

Table (٢.٣): Culture Media used

٢.٢ Bacterial strains that used in this study :-

The Bacterial strains that used in this study are shown in (Table ٢.٤).

Table (٢.٤): Bacterial strains used

Bacterial strains	Source
١-Bacterial strains used in the detection of bacteriocin production including: *E.coli , Klebsiella*	Microbiological laboratories College of Science Baghdad University

٢.٣ Patients and Methods :-

٢.٣.١ Patients :-

One hundred fifty (١٥٠) samples are collected from patients who suffering from UTIs with the age range between(٧-٨٠)years old from both sexes. The period extended from October ٢٠٠٤ to June ٢٠٠٥.

٢.٣.٢ Collection of specimens :-

The specimens were generally collected from patients suffering from UTIs, mid stream urine sample was collected which allowed the first drops of urine to pass; the samples of urine were collected in sterilized screw-capcontainer, then the urine samples had been inoculated on the culture media(MacConkey, Blood, EMB agar) and

incubated aerobically at ٣٧C° for٢٤ hr.then biochemical tests were done to identify the bacteria.

٢.٣.٣ Preparation of Reagents :-

١. Oxidase reagent:-

It was prepared directly by dissolving ٠.١ gm of Tetra-*p*-paraphenylene diamine dihydrochloride in ١٠ ml of distilled water and stored in a dark container (Baron, *et.al.*,١٩٩٤).

٢.Catalase reagent:-

It was prepared by adding ٣٧ ml of H_2O_2 to ١٠٠ ml of distilled water and stored in dark container (Collee, *et. al.*, ١٩٩٦).

٣. Methyl Red reagent:-

٠.١ gm of methyl red was dissolved in ٣٠٠ ml of ٩٩٪ ethanol and

then the volume was completed to ٥٠٠ ml by distilled water. It was used

to detect the acidity of the medium, which was produced by complete fermentation of carbohydrates (MacFaddin, ٢٠٠٠).

٤.Voges-Proskauer reagent:-

A. ٥ gm of α-naphthol was dissolved in ١٠٠ ml of ٩٩٪ ethanol then stored in a dark container away from light.

B. ٤٠ gm of KOH was dissolved in ١٠٠ ml of distilled water(Collee, *et., al.*, ١٩٩٦).

٥. Kovac's reagent:-

It was prepared by dissolving ١٠gm of *p*-Dimethyl amino benzaldehyde (DAMB) in ١٥٠ ml amyl alcohol; and then ٥٠ml of concentrated HCL was gradually added to this mixture. This solution was stored in a dark bottle, and gently shaken before use. It was used in the demonstration of indole production (MacFaddin, ٢٠٠٠).

٦. Phenol Red Reagent:-

It has been prepared by dissolving ٠.١gm from phenol red stain in ٣٠٠ml of ethyl alcohol (٩٥٪) then the volume was completed to ٥٠٠ ml by distilled water it was used to detect the acidity of the media, which was produced by complete fermentation of carbohydrates (MacFaddin, ٢٠٠٠).

٢.٣.٤ Preparation of Media :-

١. M٩ media:-

٦gm of Na_2HPO_4 ,٣gm of KH_2PO_4, ٠.٥ gm of NaCl , ١gm of NH_4CL

are dissolved in ٩٥٠ ml of distilled water with ٢٪ agar and then sterilized into autoclave at ١٢١ C° for ١٥ min. after cooling the mixture to ٥٠ C°, ٢ ml of ١M of $MgSo_{٤}$, ١٠ ml of ٢٠٪ glucose and ٠.١ ml of ١M of $CaCl_{٢}$ (all of them were sterilized separately by filtrations) were added to it, then the volume was completed to ١٠٠٠ ml (Miniatis, *et.al.*,١٩٨٢).

٢. Esculin media:-

The esculin was ٦,٧-dihydroxycoumarin٦-glucoside which had inhibitory effect on xanthin oxidase enzyme (Holt,*et.al.*,١٩٩٤). This media was made by preparation of nutrient agar supplemented with ٠.٤ gm ferric citrate and ٤ gm esculin added to it and the volume was completed to ١٠٠٠ ml. After that the media was poured into tubes and all were sterilized into autoclave at ١٢١ C° for ١٥ minutes then the slants of media were made (MacFaddin, ٢٠٠٠).

٣.Peptone Water Media:-

It had been prepared by dissolving ٨ gm peptone in ١ litter of distilled water, then it distributed into test tubes, and autoclaved at ١٢١ C° for ١٥ minutes It was used for the demonstration of the bacterial ability to decompose the amino acid tryptophan to indole (MacFaddin, ٢٠٠٠).

٤. Gelatin Liquefaction Media:-

It had been prepared by adding ١٢٪ gelatin to nutrient broth . This medium was used for testing the ability of bacteria for gelatin liquefaction (Collee, *et. al*., ١٩٩٦).

٥. Urea Agar Media:-

This medium had been prepared by adding ١٥ ml of urea solution (sterilized by filtration ٠.٤٥ μ m) into ١٠٠ ml of urea agar base sterilized by autoclaving at ١٢١ C° for ١٥ minutes and cooling to ٥٠ C°, the pH was adjusted to ٧.١ and the medium was distributed into sterilized test tubes and allowed to solidify in a slant form. It was used to test the ability of bacteria to produce enzyme urease (MacFaddin, ٢٠٠٠).

٦. Sugar Fermentation Media:-

Medium is composed of :

A. Basal medium:-

It was prepared by dissolving ١٠ml peptone, ١gm meet extract (Difco), ٥gm Sodium chloride (NaCl) and ٠.٠٨ gm phenol red (BDH) in one liter distilled water, then the pH was adjusted to ٧.٤. The medium was distributed in test tubes and Durham tubes were added

to each test tube then sterilized by an autoclave at ١٢١ C° for ١٥ min. (MacFaddin, ٢٠٠٠).

B. Sugar solutions :-

Sugar solutions were prepared by dissolving ١gm sugar in ١٠٠ ml distilled water and sterilized by filtration , then ٠.١ml sugar solution was added to each test tube (Item-١) containing ٥ml from the basal medium . The medium was used for diagnosis pathogenic bacteria that have the ability of the fermentation of sugar (MacFaddin, ٢٠٠٠).

٧. Amino Acids Decarboxylase media:-

This medium was prepared by dissolving ٥gm of peptone, ٣gm of yeast extract and ١gm of glucose in ١٠٠٠ ml of distilled water, the pH was adjusted to (٦.٧) then ١٠ ml of the indicator (Bromothymol blue) which was prepared at the concentration ٠.٢ % was added, all of them were mixed well and sterilized by at autoclave ١٢١C° for ١٥ min. then the amino acid solution (lysine , ornithine) each of them was prepared at a

concentration of ٠.٥ % (sterilized by filtration) were added, then the

medium was dispensed in sterile test tubes (Collee, *et. al*., ١٩٩٦).

٢.٣.٥ Preparation of Solutions :-

١- Saline - EDTA solution:-

The solution was prepared by dissolving ٠.١٥ M of NaCl and ٠.١ M of EDTA in distilled water, the pH of solution was adjusted to ٨ then the volume was completed to ١٠٠٠ ml and it was sterilized by autoclave at ١٢١ C° for ١٥ min. (Murmur,١٩٦١).

٢- ٢٥٪ Sodium Dodecyl sulphate (SDS):-

The solution was prepared by dissolving ٢٥ gm of SDS in ١٠٠ ml of saline-EDTA, the pH was adjusted to ٨, then the solution was sterilized by filtration and stored at ٤°c (Brinbiom and Dolly,١٩٧٩).

٣-TE buffer:-

٠.٠١ M of Tris-base and ٠.٠٠١ M of EDTA was dissolved in distilled water, the pH was adjusted to ٨ and the volume was completed to ١٠٠٠ ml, then it was sterilized by autoclave at ١٢١ C° for ١٥ min.(Sambrook, *et.al*.,١٩٨٩).

٤- ١٥ % Sucrose TES:-

This solution was prepared by dissolving ١٠ mM of EDTA and ١٥ mM of Tris - base then the sucrose was added at the percentage of ١٥٪ the pH was adjusted to ٨, then the solution was sterilized by filtration (Brinbiom and Dolly,١٩٧٩).

٥-Phenol solution saturated with Tris buffer:-

The solution was prepared by dissolving the phenol at the degree of ٦٨°c and ٠.١٪ of ٨-hydroxyquinolline which is antioxidant material was added to it, then the same volume of ٠.٥ M Tris-base buffer (pH ٨) was added, then the solution was mixed gently by mechanical blender, after that it was left to precipitate and to separate into two layers, the above was aquatic layer while the lower layer was the phenol, the aquatic layer was removed by aspiration with Pasteur pipette. The above process was repeated but with adding the same volume of ٠.١ M of Tris-base (pH ٨) to the phenol then the last process was repeated until the pH of phenol became more than(٧.٨) the phenol solution was stored under the layer of Tris buffer in a dark container for more than one month (Sambrook, *et.al.,*١٩٨٩).

٦-Phenol:Chloroform :Isoamylalcohol solution:-

Phenol, Chloroform and Isoamylalcohol solution were mixed at the ratio of ٢٥:٢٤:١ and stored under the layer of ٠.١ M of Tris-base

solution (PH ٨) in a dark container at ٤°c, and it was valid for using more than one month (Sambrook, *et.al.,* ١٩٨٩).

٧-TBE buffer:-

It prepared by dissolving ٠.٠٨٩ M of Tris-base and ٠.٠٨٩ M of boric acid and ٠.٠٠٢ M of Na_2-EDTA in distilled water the pH was adjusted to ٨, the volume was completed to ١٠٠٠ ml and the solution was sterilized by autoclave at ١٢١ C° for ١٥ min. (Sambrook, *et.al.,* ١٩٨٩).

٨-Ethidium bromide solution:-

The stock solution was prepared by dissolving ٠.٢٥ gm of Ethidium bromide in ٥٠ ml of distilled water for obtaining the final concentration ٥ mg/ml(Sambrook, *et.al.*,١٩٨٩).

٩-Loading buffer:-

It was prepared from ٤٠٪ of sucrose and ٠.٢٥٪ of bromophenol blue stain in distilled water and stored at ٤C° until using(Sambrook, *et.al.,* ١٩٨٩).

١٠-Phosphate buffer solution:-

٨٠ gm of NaCl, ٠.٣٤ gm of KH_2PO_4 , and ١.١٢ gm of $K_2H\ PO_4$ were all dissolved in ١٠٠٠ ml of distilled water The pH was ٧.٣, then the

solution was sterilized in autoclave at ١٢١ C° for ١٥ min.(Baron, *et.al*., ١٩٩٤).

٢.٣.٦ Isolation and Identification of *Enterobacter cloacae*:-

A single colony was taken from each primary positive culture and it was identified depending on its morphology (shape, color, borders and texture) and then examined microscopically after staining with gram-stain. In addition to that other biochemical tests were done to each isolate according to MacFaddin, (٢٠٠٠) and Collee, *et.al.*(١٩٩٦).

٢.٣.٧ Biochemical tests :-

١. Catalase test:-

A small amount of bacterial growth on the medium at a ٢٤ hour age was transferred by sterile wooden stick onto the surface of a clean,dry glass slide, and one drop of (٣٪ H_2O_2) is added to it . The formation of gas bubbles was indicated the positive result (Collee, *et.al.*,١٩٩٦).

٢. Oxidase test:-

A piece of filter paper was saturated in a Petri dish with oxidase reagent (prepared soon) then a small portion of the colony of bacteria

was spread on the filter paper by wooden stick. When the color around the smear turned from rose to purple that mean the oxidase test was positive(Baron, *et.al*.,١٩٩٤).

٣. Indole test:-

This test was performed by inoculating peptone water medium with

bacterial growth by the loop and it was incubated for(٤٨-٧٢)hours at ٣٧ C˚. Indole test was done by adding ٦-٨ drops of Kovac's reagent (p-dimethylaminobenzaldehyde in amyl alcohol). The positive reaction characterized by the formation of red color ring at the top of the broth while formation of yellow color ring was a negative result(MacFadin, ٢٠٠٠).

٤.Methyl-red test:-

This test was performed by inoculating tubes containing MR - VP

medium with bacterial growth. The tubes were incubated for (٢٤-٤٨) hours at ٣٧ C˚, after that ٥ drops of methyl red reagent were added. The change of color to orange-red after ١٥ minutes indicated a positive result (Collee, *et.al.,*١٩٩٦).

٥.Voges-proskaur test :-

The test was performed by using (MR - VP broth). The tubes were

inoculated with bacterial colonies then they were incubated for ٢٤ hours at ٣٧ C˚. Afterwards, ١٥ drops of ٥٪ alpha-naphthol (reagent A) were added followed by ١٠ drops of ٤٠٪ KOH (reagent B) then shaken well and allowed standing up for ١٥ minutes before considering the reaction as negative, when we have positive result, the culture turned red throughout

٢-٥ minutes.The positive result indicated partial analysis of glucose which produced (Acetyl-Carbonyl) (Collee, *et.al.,* ١٩٩٦).

٦.Simon's test :-

After the sterilization of Simon Citrate slants by autoclave at ١٢١ C˚ for ١٥ min., the bacterial colonies were inoculated and incubated them for (٢٤-٤٨) hours at ٣٧ C˚. The change of color of media from green to blue indicated positive result, while the unchanging of the color indicated negative reaction (Benson, ١٩٩٨).

٧.Urease Test :-

This test was carried out by inoculating urea medium with bacterial growth and the tubes were incubated for (٢٤-٤٨) hours at ٣٧ C˚. The color change of medium into pink indicated positive result while unchanging of

the color mean negative reaction (MacFaddin, ٢٠٠٠).

٨. Esculin test :-

The organisms were grown in an esculin slants for ٢٤ hr. at ٣٧ C˚. The dark brown color was indicated the positive result. The unchanging of the color was a negative result (MacFaddin, ٢٠٠٠).

٩. Triple Sugar Iron (TSI) test:-

The bacterial colonies were inoculated on TSI media slant by stabbing and streaking then they were incubated at ٣٧ C˚ for (٢٤-٤٨) hours. Changing the color of the media from orange - red to yellow was due to carbohydrate fermentation with or without gas formation at the bottom of the slant. In addition, the formation of hydrogen sulfide was given a black color precipitation at bottom (MacFaddin, ٢٠٠٠).

١٠. Eosin Methylen Blue (EMB) agar:-

After culturing the bacteria on this media, bacterial colonies with lactose-fermented were either dark or possessing dark centers with transparent colorless peripheries, while those that did not ferment lactose remain uncolored. This purple color was due to absorption of the eosin -methylene blue complex, which formed in the presence of acid. Certain members of the coliform group, especially *E.coli*,

exhibited a greenish metallic sheen in reflected light (Baron, *et.al.*, ١٩٩٤).

١١. Manitol salt agar test:-

The medium was inoculated with bacterial colonies then incubated at ٣٧ C° for ٢٤ hours . The color changed from pink to bright yellow

when the bacteria was lactose fermented and mean positive result, while unchanging color of the medium was negative result (MacFaddin, ٢٠٠٠).

١٢. Motility test:-

The semi solid media was dispensed in test tubes with ١٠ ml in each

tube and leaving to set in vertical position then the bacterial colonies were

inoculated by stabbing singly down the center of the tube to about half the depth of the medium. The cultured tubes were incubated at ٣٧ C° and the tubes were examined after ٦ hours, ١ and ٢ days. Non-motile bacteria had generally confined to the stab-line and given sharply defined margins and leaving the surrounding medium clearly transparent, while motile bacteria were typically given diffuse hazy

growths that spread throughout the medium rendering it slightly opaque (MacFaddin, ٢٠٠٠).

١٣.Carbohydrate fermentation test:-

Carbohydrate fermentation medium was inoculated with bacterial culture and incubated at ٣٧ C° for (٢٤-٧٢) hours the color change of the medium from red to yellow and gas formation in the Durham tube was indicated that this bacteria may ferment the carbohydrate with gas.The used sugar were (Inositol, mannitol, sucrose, glucose) (MacFaddin, ٢٠٠٠).

١٤- Lysine and Ornithine decarboxylase test :-

This test was carried out by inoculation the media that containing the amino acid (Lysine or Ornithine). The bacteria was inoculated and then incubated at ٣٧ C° for ٤ days, changing the indicator color that found in

the medium from the blue to yellow throughout ١٠-١٢ hr. then backing to the blue color throughout ٢٤-٩٦ hours indicate that the result is positive while the negative result is the remaining of the yellow color (Collee, *et.al.*,١٩٩٦).

٢.٣.٨ Virulence Factors tests :-

٢.٣.٨.١ Capsule stain:-

a-A slide smear was prepared from bacterial suspension on glass slide without heat fixing and was left to dry.

b-Flood gently with ١٪ the crystal violet and leave for about ٤ minutes.

c-The smear was washed with ٢٠٪ copper sulfate, allowed to dry in air, and examined under the microscope.

The organism should be deep purple, and the capsule a faint blue against a light purple background (Cruickshank, *et.al*.,١٩٧٥).

٢.٣.٨.٢ Colonization Factor Antigen(CFA) :-

A-Detection of (CFA/I):-

After culturing the organism on Tryptic soy agar and incubating it for ٢٤ hours at ٣٧C°, the agglutination of RBC with bacteria occurs in presence of D-mannose as follows:-

١- RBC suspension was prepared from the human blood (group A) and washed with phosphate buffer saline (repeated ٣ times). ٣٪ suspension from RBC (v/v) was then prepared.

٢- A bacterial suspension was prepared by taking half of the bacterial growth for each strain from TSA and mixing it with ١ ml

of ٠.١٥ M NaCl, to determine RBC agglutination test and vasticated colonization factor antigen type I.

٣- On a clean slide, one drop of bacterial suspension was mixed with one drop of ٠.١ M D-mannose on one side, and with one drop of ٣٪

suspension on the other side (without D-mannose).

The agglutination of RBC with bacteria was detected after ١/٢-٢ min in room temperature (Smyth, ١٩٨٢).

B- Detection of (CFA/II):-

To determine second colonization factor antigen, the same step was followed with CFA/I by using chicken blood instead of human blood (group A). This factor causes agglutination of chicken blood (Al-Zaag, ١٩٩٤).

C- Detection of (CFA/III):-

To determine third colonization factor antigen the same step CFA/I is followed except for the replacement of Tannic acid instead of D-mannose.

٢.٣.٨.٣ Siderophores production:-

M٩ media was prepared and then supplemented with ٢٪ agar. After sterilization in autoclave at ١٢١ C° for ١٥min. and cooling to ٥٠ C°, ٠.٢٥٪ gm/L glucose (sterilized by filtration) and ٢٠٠ µm of dipyridyl were added to it. Then the organisms were inoculated into this media and incubated for ٢٤ hours at ٣٧ C°. The results were seen if the growth of organism was present or not (Nassif, *et.al*., ١٩٨٩).

٢.٣.٨.٤ Haemolysin production:-

Haemolysis production was shown on blood agar media. The results were obtained after the incubation of the non-cultured plates for ٢٤ hours at ٣٧ C° to exclude any contamination of blood, then the organism was inoculated at this blood agar plates and incubated again for ٢٤ hours at ٣٧ C°. Any haemolysis presence showed be detected around the colonies (either α or β-haemolysin) (De Boy, *et.al*., ١٩٨٠).

٢.٣.٨.٥ Extracellular protease production :-

This method was carried out by using M٩ agar supplemented with ٢٪ agar. After sterilization in autoclave at ١٢١ C° for ١٥ min. and cooling it to ٥٠ C°,٠.٢٥ gm/ L glucose (sterilized by filtration)was

added, and then the medium was supported by ١٪ gelatin. After that this medium was inoculated with bacterial strain and was incubated for (٢٤-٤٨) hours at٣٧C°, and then ٣ml of Trichloroacetic acid (٥٪)was added to precipitate the protein. The formation of transparent area around the colony indicated the positive result (Benson, *et.al.*, ١٩٩٨).

٢.٣.٨.٦ Bacteriocin production :-

The method of Abbot and Graham(١٩٦١) had been used:-

١- A medial streak of the test strain by vertical line was done on TSA and then incubated at ٣٧ C° for ٤٨hours to allow bacteriocin to spread around the growth line.

٢- On the second day, sensitive or indicator strain was inoculated on nutrient agar and incubated at ٣٧ C° to the next day.

٣- On the third day, the Petri-plate cover of the streaked plate was covered by filter paper impregnated with chloroform in an upright position .

٤- Then plate culture was inverted on its cover for ١/٢ hours the culture was scarped by sterilized glass slide into disinfecting vessel, and the

plate culture was exposed to chloroform vapors and then left the plate open for ١ hours to remove the chloroform .

٥- Inoculated sensitive or indicator strain (which has grown on nutrient agar) was streaked crossing the original scraped streak line on TSA plate culture and incubated at ٣٧ C° overnight. The bacteriocin production was scored as growth inhibition at the medial streak line.

٢.٣.٩ Antibiotics sensitivity test :-

Antibiotic diffusion test (The Kirby-Bauer susceptibility test) was used to show the effect of antibiotics on isolated bacteria.

١. It was performed by using a pure culture of previously identified bacterial organism. The inoculum to be used in this test was prepared by adding growth from ٥ isolated colonies grown on a blood agar plate to ٥ ml of broth. This culture was then incubated for ٢ hours to produce a bacterial suspension of moderate turbidity.

A sterile swab was used to obtain an inoculums from the standardized culture. This inoculum was streaked on a Muller-Hinton plate.

٢. The antibiotic discs were placed on the surface of the medium at

evenly spaced intervals with flamed forceps or a disc applicator.

Incubation was usually overnight with optimal time of(١٤) hours at ٣٧C°.

٣. Antibiotics inhibition zones were measured using a caliber. Zone size was compared to standard zones to determine the susceptibility or resistance of organism to each antibiotic(MacFaddin, *et.al.,*٢٠٠٠).

Antibiotics discs potency was supplied from Oxiod Company (Table ٢.٥).

Table (٢.٥) :Antibiotics Discs Potency

No.	Antibiotics	Discs potency µg/ml
١	AMX	١٠
٢	CTX	١٠
٣	CXM	١٠
٤	CTH	٣٠
٥	GM	٣٠
٦	AK	٣٠
٧	TE	٣٠
٨	DOX	٣٠
٩	CIP	٣٠
١٠	NA	٣٠
١١	TF	٣٠
١٢	TMP-SMX	٣٠

***AMX:**Amoxicillin; ***CTX:**Cefotaxime ; ***CXM:**Cefixime ; ***CTH:** Cefalothin ; ***GM:** Gentamicin; ***AK:** Amikacin; ***TE:** Tetracycline; ***DOX:** Doxcyclin; ***NA:** Naldixic acid; ***CIP:**Ciprofloxacin; ***TF:**Nitrofurantoin; ***TMP-SMX:**Trimethoprim-Sulfamethoxazole.

٢.٣.١٠ The Effect of N - Acetyl L - cysteine (NAC) on the bacterial capsule and growth:-

The effect of N-Acetyl L-Cysteine(NAC) on the *Enterobacter cloacae* isolates was tested by the following method:

١. Nutrient broth was prepared in tubes and NAC (sterilized by filtration) was added to each tube at various volume to gain the final concentration (٠.٠٥, ٠.١, ٠.٢, ٠.٤, ٠.٦, ٠.٨ , and ١.٠) mg/ml.

٢. Control tube was prepared by using nutrient broth free from NAC.

٣. The tubes were inoculated with ٠.١ml of freshly grown bacterial suspension, then serial of tenth fold dilutions($١٠^{-١}$, $١٠^{-٢}$, $١٠^{-٣}$, -$١٠^{-٤}$, and $١٠^{-٥}$) were used in sterile distilled water for each concentration.

٤. With the use of spreader the bacterial strains were streaked from the final dilution on the supplemented nutrient agar and incubated for ٢٤ hr. ٣٧ C°.

After the period of incubation, the viable count of bacteria was estimated by multiply the number of colonies/plate by the dilution factor to obtain the viable count/ml in original suspension .

٢.٣.١١ Combination effect of some antibiotics with N -Acetyl L -cysteine (NAC) on the growth of *Enterobacter cloacae* isolates :-

Nutrient agar medium was prepared, sterilized by autoclave at ١٢١ C° for١٥ minutes, then it was cooled to ٥٠ C°. Afterwards, the NAC solution at the concentration ٠.١ mg/ml (sterilized by filtration) was added to the

medium, the same medium was also prepared but without NAC which represent the control medium. The media were poured in sterilized Petri dishes, and left to solidify then they were incubated at ٣٧ C° for ٢٤ hours to ensure that there is no contamination. By using sterile swabs, these media were inoculated with the bacterial isolates by streaking, then the antibiotic discs were placed on the surface of the medium at evenly spaced intervals with flamed forceps or disc applicator.

Then these plates were incubated for ٢٤ hr. ٣٧ C°, antibiotic inhibition zones were measured using caliper then they were compared with the zones of control media .

٢.٣.١٢ Extraction of Plasmid DNA:-

٢.٣.١٢.١ Extraction of Plasmid DNA by Salting out method:-

Extraction of plasmid DNA by using the method (salting out) as mentioned by Kado and Liu (١٩٨١) was done which containing the following steps:

١. The bacterial cells were cultured on brain-heart infusion agar and incubated at ٣٧ C° for ١٨ hours. After that a single colony was taken and cultured in ٥٠ ml of brain-heart infusion broth and incubated in a shaker incubator at ٣٧ C° for ١٨ hours.

٢. The cells suspension was sedimented by centrifugation at ١٠٠٠٠ round /minutes for ١٠ minutes.

٣. The cells precipitate was resuspended in ٥ ml of TES buffer.

٤. A volume of ٦٠٠ microliter of ٢٥٪ SDS in saline EDTA (pH ٨) was added and mixed by inversion then it was incubated in a water bath at ٥٥ C° for ٥ minutes.

٥. Two ml of ٥ M NaCl buffer was added and mixed by inversion and left at room temperature for ٣٠ minutes.

٦. Five ml of phenol - chloroform - isoamyl alcohol solution was added, mixed by inversion and left at room temperature for ٣٠ minutes.

٧. The mixture was centrifuged at ٤٥٠٠ RPM for ١٥ minutes.

٨. The supernatant was transferred to a sterile tube and ٠.٦ ml of the

isopropanol alcohol was added for each ١ ml of the supernatant, mixed by inversion for ٣ minutes then the DNA was picked by sealed Pasteur pipette.

٩. The DNA was washed by ٥ ml of ٧٠ % ethanol and preserved in one ml of TE buffer at -٢٠ C°

٢.٣.١٢.٢ Agarose Gel Electrophoresis:-

The method of Sambrook, *et.al*.(١٩٨٩) was used which containing the following steps:

١. ٠.٧ gm of the agarose was dissolved in١٠٠ ml of TBE buffer by using water bath at ١٠٠ C° after that it was cooled to ٥٠ C° and ١٠

microliter of ethidium bromide solution was added to it.

٢. Suitable amount of the agarose was poured in the tray and left to

solidify.

٣. The comb was risen from the agarose and the tray was transferred to

the electrophoresis chamber and covered with TBE buffer.

٤. Ten microliters of the DNA was mixed with ٣ microleters of the

Loading buffer and placed in the agarose wells.

٥. The electrophoresis process was carried using low voltage (about

٦ volt/cm and by passing ٢٠ milliamper) for ١-٢ hour.

٦. The bands of the plasmid DNA were detected by UV-transllminator in a wave length of ٣٠٢ nanometer.

Chapter Three: Results and Discussion

٣.١ Bacterial Isolation:-

In this study, ١٥٠ urine samples were obtained from patients suffering from urinary tract infections (UTIs) who attending to Surgical Teaching Hospital in Hilla-department of urology the period extending from October ٢٠٠٤ to June ٢٠٠٥ were included . Those patients were distributed as (seventy five male and seventy five female) with the age range between (٧ – ٨٠) years old . In addition to that , ٣٠ healthy individuals were also under this study as control group.

Urine samples were then subjected for culturing on selective media to isolate *Enterobacter* bacteria. A total of (١٥٠) urine samples, only (١٢٦) samples showed positive cultures that mean the ratio is (٨٤٪), whereas no growth was seen in the other (٢٤) samples which had the ratio(١٦٪),the negative culture indicate the presence of micro-organisms that might be cultured with difficulty such as Chlamydia or Mycoplasma or other agents.

Among ١٢٦ isolates ,only nine isolates were found to be related to *Enterobacter* spp., seven of them was belonging to *Enterobacter*

cloacae (the objective of this study), and two isolates for *Enterobacter aerogenes* (Table ٣.١) .

Table ٣.١: The Main Microorganisms Isolated From Urine Samples.

Type of Microorganism		Number	Percentage
Gram positive bacteria	*Staph.saprophyticus*	٨	٥.٣٪
	Enterococcus spp.	٣	٢٪
	Staph.aureus	٢	١.٣٪
	Corynebacterium urealyticum	١	٠.٧٪
Gram Negative bacteria	*E.coli*	٧٣	٤٨.٧٪
	K. pneumoniae	١٢	٨٪
	* *E. cloacae*	٧	٤.٧٪
	Proteus spp.	٦	٤٪
	Acinetobacter spp.	٥	٣.٣٪
	P. aeroginosa	٣	٢٪
	* *E. aerogenes*	٢	١.٣٪

yeast	*Candida* spp.	٤	٢.٧٪
Total		١٢٦	٨٤٪

٣.١.١ *Enterobacter* spp. :-

In this study, only (nine) isolates of *Enterobacter* spp. had been isolated that mean (٦٪), seven of them belongs to *E.cloacae* (٤.٧٪), while the reminder two isolates were *E.aerogenes* (١.٣٪) , in addition, no isolates of *E. cloacae* had been isolated among the healthy controls, this explains that *E. cloacae* represent the main causative agent of UTI for the cases under study (Table ٣.١).This result of *E.cloacae* isolates is similar to those results obtained by (Jones ,*et. al*., ١٩٩٩) who have isolated *E.cloacae* with the percentage of (٣.٨٪) from patients with UTIs in North America .

The isolation of *E.cloacae* varies from one study to another and this is affected by several factors such as changing patients population , time and number of samples collection and the extensive use and mis-use of the antibiotics, in addition to that, the

environmental conditions that affect on the dissemination of this bacteria in the community and hospital (Kartali *,et.al*., ٢٠٠٢).

The present work is concerned with *Enterobacter cloacae* in order to find resistant bacteria such as *E.cloacae* because there is little studies carried out on this bacteria, so our study is aimed to show the prevalence of this bacteria among other bacteria associated with UTI.

Helander and Helen (٢٠٠٥) have pointed that the ratio of *E.cloacae* isolation was (٢.١ %) from patients with UTIs in Sweden .

While Hryniewicz *,et .al.,*(٢٠٠١) showed that the isolation percentage

of *E.cloacae* in Poland was few (١.٧٪) if compared with other causative agents isolated from the UTIs cases .

On the other hand , in a study carried out in Taiwan ,the percentage of isolation of *E.cloacae* from patients with UTIs was very low (٣٪) (Lau*, et.al*., ٢٠٠٤).

Moreover, Ishii, *et.al*. (١٩٩٦) had isolated *E.cloacae* as a causative of (١٤.٢ %) of the cases studied , these isolates were multi drug resistant strains, and only from complicated UTIs .

In addition to *E.cloacae,* two isolates of *E.aerogenes* had also been isolated. *E.aerogenes* can cause UTI ,and it represents with *E.cloacae* most clinically significant species of the genus *Enterobacter.* Surveillance studies in ١٩٩٧ found that ٥ % of UTIs in Europe were caused by *Enterobacter* spp. In recent years ,infections of hospital patients with these two species has been reported more often than formerly , most of these infections are of the urinary tract (Fluit ,*et. al.*,٢٠٠٠).

Enterobacter spp. appear well adapted for survival and proliferation and have the ability for transmissibility in solutions and on surfaces of medical devices , the options for control of these organisms are quite limited , *Enterobacter* spp. have spilled over into the community , occasionally infecting other wise well individuals causing several infections including UTIs (Sanders and Sanders,١٩٩٧), this may be one of the reasons for this relatively high prevalence of *E.cloacae* isolated from UTIs patients in this study . In addition, the number of isolates of *E.cloacae* was higher than isolates of *E. aerogenes* which was isolated only from two cases (١.٣٪).

E.cloacae with *E. aerogenes* represent by far most frequently encountered human pathogens among the genus (Haddy ,*et. al* .,١٩٩١).

However, in this work another types of bacteria were isolated as shown in the (Table ٣.١), these bacteria were included : *Escherichia coli*, the predominant,(٧٣)isolates, followed by: *Klebsiella pneumoniae* (١٢) , *Proteus* spp.(٦), *Acinetobacter* spp.(٥), *Pseudomonas aeroginosa* (٣), *Staphyllococcus saprophyticus*(٨),*Enterococcus* spp.(٣),*Staphyllococcus aureus* (٢) and one isolate of *Corynebacterium urealyticum*. In addition to bacteria, yeasts were isolated as four (٤)isolates of *Candida* spp.The biochemical tests used for identification these Gram-positive and Gram-negative bacteria were performed according to MacFaddin, (٢٠٠٠). (Table ٣.٣)and (Table ٣.٤).

These results are correlated with the results of the previous studies which performed in Hilla by AL-Saigh (٢٠٠٥), and Al- Amedi (٢٠٠٣), they found the same bacteria in UTIs patients although the objectives of these studies were different.

In addition ,these results are accepted with those obtained by Thirion and Williamson, *et.al*.(٢٠٠٤), Ronald (٢٠٠٣) and Orenstein and Wong (١٩٩٩) all of them have pointed that these microorganisms are the most common causative agents of UTIs.

However, *E.coli* remains the predominant uropathogen that causes wide range of UTIs (Lepelletier, *et.al.,*١٩٩٩). The recent studies showed that ٤٧٪ of patients with UTIs have *E.coli* in their cultures (Gordon and Jones, ٢٠٠٣).

On the other hand, twelve isolates of *K.pneumoniae* have been isolated in this study, It is one of the causes of urinary tract infection (UTI), especially among patients with prolonged catheterization, and in immunocompromised individuals (Struve ,*et.al.*,٢٠٠٣).

Furthermore, eight isolates of *Staph. saprophyticus* had also been isolated. This coagulase negative Staphylococci are predominant among female patients and may be involved in recurrent infection and in stone formation ,it is responsible for ١٠-١٥٪ of community acquired UTIs (Todar ,٢٠٠١).

In addition ,six isolates of *proteus* spp. had also been isolated, *Proteus* spp. can cause hospital acquired UTI ,previous infection with this organisms is often associated with the formation of urinary stones, which further predispose the patient to infection (O'Hara,*et.al.,*٢٠٠٠).

Five isolates of *Acinetobacter* spp. had been isolated . This bacteria can cause UTIs and it affected severally ill patients (Koelman, *et.al.*,١٩٩٧). Al-Shukri (٢٠٠٣) has pointed that *Acinetobacter* spp. can be isolated from urine samples,the isolation rate was ٣.٤٪ that obtaining from patients with UTIs, this study had been carried out in Hilla province ,too.

Three isolates of *P.aeroginosa* had also been isolated, this bacteria has one of the broadest ranges of infectivity among all pathogenic microorganisms as opportunistic pathogen ; it can cause several

infections like UTIs (Bodey, ١٩٨٣).

Enterococcus spp. had also been isolated (٣ isolates). It account for less than ٥٪ of UTIs , *Enterococci* infrequently cause of uncomplicated cystitis and pyelonephritis (Huebner, *et.al*.,١٩٩٩).Moreover, two isolates of *Staph. aureus* had been isolated ,the presence of this bacterium in urine often indicate pyelonephritis acquired via hematogeneous spread or the ascending route (Measley and levison, ١٩٩١).

Furthermore, one isolate of *Corynebacterium urealyticum* had been isolated, this bacteria is the commonest species of the genus *Corynebacterium* that cause UTI in human, the frequency of this bacteria is very low that was isolated in one case from a total of ٣١٠ patients with recurrent UTI (٠.٣٢٪) (Suarez ,*et.al*.,٢٠٠٢).

In addition to bacterial isolates, yeast had also been isolated which was represented by *Candida* spp. and it is known that this yeast was proved to be one of the causative agent of UTIs in human particularly in women patients (Fiedel ,*et.al*.,١٩٩٩).

٣.١.٢ The Characteristics of *Enterobacter cloacae* :-

E.cloacae is gram-negative , rod-shaped bacterium , motile, encapsulated with small-polysaccharide capsule , facultativelly anaerobic bacteria , that is catalase positive and oxidase negative ,this result is identical with those obtained by(Collee,*et.al*.,١٩٩٦).

On the MacConkey agar all *Enterobacter* spp. have pink colonies (weak lactose fermentation),not as mucoid as *Klebsiella* and on EMB agar it is centrally dark pink to purple color colonies, these results identical with those obtained by Baron ,*et. al*.(١٩٩٤).

Therefore, it was impossible to distinguish *E.cloacae* on these media, because *Klebsiella* spp. very resemble *Enterobacter* spp. in the morphlogical characteristics, however *Enterobacter* spp. can be differentiated from *Klebsiella* spp. through motility and ornithine decarboxylase which are positive for all *Enterobacter* spp., in addition *Enterobacter* colonies may be some what mucoid but the amount of the extracellular material formed is usually not great as with *Klebsiella*, these results are resemble those of Montgomerie (١٩٧٩).

Additionally, all *E. cloacae* isolates were resistant to cephalothin and that similar to the results obtained by (Ehrhardt, *et.al.*,١٩٩٣) who found that most *Enterobacter* spp. are resistant to cephalothin whereas *Klebsiella* spp. are often susceptible to this agent, therefore, the susceptibility to cephalothin is considered as the

diagnostic feature for the differentiation between *Enterobacter* spp. and *Klebsiella* spp.

E.cloacae can be differentiated from other species of the genus *Enterobacter* through chemical tests including lysine decarboxylase ,methyl red ,esculin hydrolysis and gelatin liquefaction , all of them collectively are negative only for the species *E.cloacae* (MacFaddin , ٢٠٠٠), so that all of these four differentiated tests have been performed in this study and the results are summarized in (Table ٣.٢) .

This table is carried out according to MacFaddin, (٢٠٠٠),Baron, *et.al*., (١٩٩٤) and Collee, *et.al*.,(١٩٩٦).

Table ٣.٢: Diagnostic Features of *Enterobacter cloacae*

Test	Result
Growth on MacConkey agar	+
Lactose fermentation	+
Catalase	+
Oxidase	-
Growth on EMB	Centrally dark pink to purple colonies
Gram stain	-(red)
Shape of cell	Rod
Growth on TSI	A/A with gas
H_2S production	-
Indole test	-
Urease	Variable
Citrate	+
lysine decarboxylase	-
Ornithine decarboxylase	+
Esculin hydrolysis	-
Gelatin hydrolysis	-
MR	-
VP	+
Manitol fermentation	+
Inositol	-
Glucose	+
Sucrose	+
Motility	+
Cephalothin	Resistant

Table ٣.٣: Biochemical Tests used for identification

Gram-positive bacteria

Test	*S.aureus*	*S.saprophy*	*Corynebacteri*	*Enterococc*
Gram stain	G + ve cocci (clusters)	G + ve cocci (clusters)	G + ve Rods (chinees letter)	G + ve long
Albert stain	–	–	+	–
Esculin test	–	–	–	+
Oxidase	–	–	–	–
Catalase	+	+	+	–
Coagulase	+	–	–	–
Haemolysis	+	–	–	–
Urease	–	+	+	–
Manitol ferment	+	+	–	+
Novobiocin resistace	–	+	–	–
Motility	–	–	–	–

Table ٣.٤: Biochemical Tests used for identification

Test	*E. coli*	*K.pneumoniae*	*Proteus*	*P.aeroginosa*	*Acinetobacter*
Gram stain	G- ve, Short rods	G- ve, short rods	G-ve rods	G- ve rods	G- ve coccobacilli or diplococci
Lactose ferment	+	+	–	–	–
Haemolysis	–	–	–**	+	–
EMB	Metalic	Centrally dark	Pale	Pale	Pale
Oxidase	–	–	–	+	–
Catalase	+	+	+	+	+
MR	+	–	–*	–	–***
VP	–	+	–	–	–
Indole	+	–	V	–	+
TSI	A/A/G	A/A/G	A/A or ALK/A	ALK/ALK	ALK/ALLK
H_2S	–	–	+	–	–
Motility	+	–	+	+	–
Citrate	–	+	+	+	–***
Urease	–	+	+	–	–***
Swarming	–	–	+	–	–

Gram-negative bacteria

V = Variable

* Positive in ٣٣.٣ %

** Positive in ٦٦.٧ %

*** Positive in ٤٠ %

٣.٢ Investigation of some factors associated with the pathogenecity of *E. cloacae* :-

Very little is known about the virulence factors of *E.cloacae*(Bonnet, *et. al.,* ٢٠٠٤), however recently the somatic and capsular antigens of the genus *Enterobacter* and other virulence factors under intensive investigation (Isenberg and D'Amato ,*et.al.*,٢٠٠٠).

٣.٢.١ Capsule:-

Capsule detection by using capsule staining technique is carried out for *E.cloacae* isolates and it was found that all *E.cloacae* isolates had a capsule surround the bacterial cell.

These results are identical with those obtained by Andersen, *et.al.,*(١٩٩٩) who stated that strains of *E.cloacae* had a polysaccharide capsule as one of its virulence factors .

Lassiter, (١٩٩٢) decided that *E.cloacae* capsular polysaccharide prevents activation of the alternative complement system protecting the bacteria from opsonization , phagocytosis and bacteriolysis .

Although the host will normally make antibodies directed against the bacterial capsule ,some bacteria are able to subvert this

response by having capsules that resemble host polysaccharide (Brogden ,*et.al*., ٢٠٠٠).

٣.٢.٢ Detection of Haemolysin, Siderophores and Extracellular Protease Production:-

Microorganisms evolve a number of mechanisms for the acquisition of iron from their environments . One of them is the production of haemolysins , which act to release iron complex to intracellular heme and haemoglobin . Another mechanism for the iron acquisition is to produce siderophores which chelate iron with a very high affinity and which compete effectively with transferrin and lactoferrin to mobilize iron for microbial use (Neilands,١٩٩٥).

To investigate the ability of *E.cloacae* to produce haemolysin on human blood agar .The results are show that only one out of seven isolates (١٤.٢٪) are able produce haemolysin extracellularly and the type of haemolysin is alpha (Table ٣.٥) ,the remainder six *E.cloacae* isolates did not produce haemolysin.

Table ٣.٥ : Detection of Production of Haemolysin, Siderophore and Extracellular Protease by *Enterobacter cloacae* isolates.

Virulence Factor	Isolation No.							Ratio
	١	٢	٣	٤	٥	٦	٧	
Haemolysin	-	-	*+	-	-	-	-	١٤.٣٪
Siderophore	+	+	-	+	+	+	+	٨٥.٧٪
Extracellular protease	-	-	-	-	-	-	-	٠ %

* : **alpha hemolysis**

Stenhouse, (١٩٩٢) mentioned that *Enterobacter* spp. did not produce haemolysin on blood agar, but Simi, *et.al.* (٢٠٠٣) had pointed that clinical isolates of *E.cloacae* can produce low molecular weight haemolysin and this haemolysin may be putative virulence factor in *E.cloacae* infections.

In addition (Prada and Beutin , ١٩٩١) showed that *E.cloacae* can produce alpha haemolysin and that was the first report of α-haemolysin production in *E.cloacae* , this α-haemolysin was tested with specific monoclonal antibody and by DNA- hybridization with an α-haemolysin specific gene probe.

This support our result in that one *E.cloacae* isolate had produced alpha-haemolysis on human blood agar, which lead to greenish discoloration around the colony (incomplete haemolysis)in the medium. The function of haemolysin is to provide the microorganism with iron and it will make the bacteria unable to reproduce any factor for obtaining the iron from environment (Valvano ,*et. al.,* ١٩٨٦).

E.cloacae is also tested for its ability to synthesize siderophore on M٩ media containing dipyridyl ,the results are recorded according to the ability of the isolates to grow or not .

It was showing that six out of seven isolates of *E.cloacae* can produce siderophore under aerobic conditions (Table ٣.٥).

This result is identical with those obtained by (Keller,*et.al.,* ١٩٩٨) who showed that most strains of *E.cloacae* including those of urine

samples are able to produce siderophore, they also reported that the type of this siderophore was aerobactin which influences either

the extent of bacterial translocation from the intestinal tract and the extent of bacterial multiplication in tissues following translocation ,or both. Thus, aerobactin (siderophore) secretion could be an important step in the stage of the opportunistic infection cycle.

In addition, Vartivarian (١٩٩٩) observed that most of *E.cloacae* had produced siderophores as part of their virulence. *E.cloacae* can produce two types of siderophores ,identified as enterochelin and aerobactin ,the aerobactin was excreted in larger amounts than enterochelin, both siderophores appeared to be excreted immediately after their synthesis (Van Tiel- Menkveld ,*et.al.*,١٩٨٢).

The siderophore-mediated iron acquisition system is necessary for bacterial survival in the host .Since most pathogens produce siderophores to secure iron from the host, the lack of the siderophore synthesis may reduce the pathogenecity (Mathew, *et.al.*,٢٠٠١).

In regarding to protease which is class of enzymes involved in essential biological processes, it assists the hydrolysis of large polypeptides cell (Beynom and Bond, ١٩٨٩).The ability of *E.cloacae* to produce exracellular protease by using M٩ (supported by ٠.٢٪ glucose and ١٪ gelatin) was investigated and it was found non of these isolates have the ability to produce extracellular protease

after ٢٤ hours of incubation and there is no transparent area around the colony after the addition of ٣ml (٥٪) of trichloroaceticacid (TCA) (Table ٣.٥).

No previous studies mentioned about the ability of *E.cloacae* to produce extracellular proteases .

However, negative gelatinase (which is type of protease) is one of the diagnostic features of *E.cloacae* to differentiate it from *E.aerogenes* (MacFaddin, *et.al.,* ٢٠٠٠).

٣.٢.٣ Detection of Colonization Factor Antigens (CFA):-

The isolates of *E.cloacae* are tested for their ability to produce colonization factor antigens type ١,٢ or ٣ ,the results showed that only five isolates have the ability to produce CFA/١, while there is no any isolates have the ability to produce CFA/٢ . CFA/١ and CFA/٢ performed in the presence of D-mannose.

Concerning to the production of CFA/٣ in *E.cloacae*, only two isolates have the ability to produce CFA/٣ in the presence of tannic acid (Table ٣.٦)

Table ٣.٦ : The Ability of *Enterobacter cloacae* to Produce

Colonization Factor Antigens (CFA)

Virulence factor	Isolation No.							Ratio
	١	٢	٣	٤	٥	٦	٧	
CFA/١	+	+	-	+	+	-	+	٧١.٤٪
CFA/٢	-	-	-	-	-	-	-	٠٪
CFA/٣	-	+	-	-	-	-	+	٢٨.٥٪

These results are correlated with those obtained with Hornick ,*et.al.* (١٩٩١) who found that the frequency of phenotypic expression of type ١ fimbrial adhesion was (٦٦.٦٪)among *E.cloacae* strains and for type ٣ fimbrial adhesin was (٢١٪) while non of these isolates produced type ٢ fimbriae or(P fimbriae), they also found that even some strains of *E.cloacae* did not express type ٣ fimbriae on their surface, did possess sequences similar to the gene cluster for expression of this type of fimbriae , in addition to these fimbrial types, *E.cloacae* also may have produced other distinct adhesions that need for specific method for detection.

In other study carried out by Adegbola and Old, (١٩٨٣) they found mannose-sensitive hemagglutinin (MSHA) or type (١) fimbriae in most of the *E.cloacae* strains studied.

The close antigenic relationship between type ١ and ٢ fimbrial types may indicate that the type ٢ fimbriae are non adhesive variants of type ١ fimbriae (Maurer and Orndroff, ١٩٨٥).

Furthermore, Gerlach and Clegg (١٩٨٨) pointed that seven isolates out of ten of *E.cloacae* produced MR/K HA or (type ٣) fimbriae .

The type ٣ fimbriae are responsible for colonization the surfaces of indwelling devices (urinary catheters) (Mobley, *et.al*., ١٩٨٨), however, *E.cloacae* among those organisms that commonly contaminating these devices and developing biofilms (Stickler,١٩٩٦).

These factors (CFA١,٢ and ٣) are considered primary factors which

cause adhesion of bacteria to the target cell of the host,and their presence indicates that the bacteria contain cell surface fimbrial antigens.

٣.٢.٤ Bacteriocin production:-

Bacteriocin is antimicrobial protein produced by bacteria that kill or inhibit the growth of other bacteria related to the same group or species (Cleveland ,*et .al*., ٢٠٠١)

Bacteriocin production is investigated by using all isolates of *E.cloacae* in the production process , the same isolates also used as sensitive strains in addition to other bacterial types which is *E.coli* and *K.pneumoniae*. It was found that there is no isolate of *E.cloacae* was able to produce bacteriocin.

The reasons why these isolates have no the ability to produce bacteriocin although many previous studies have pointed that *Enterobacter* can produce a type of bactriocin called cloacin, may be related to the siderophore production which is synthesized by six isolates of *Enterobacter* under study,and this compound can inhibit the activity of bacteriocin as observed by the studies of VanTiel-Menkveld *,et.al*. (١٩٨٢).

However, one isolate as mentioned in item ٣-٢-٢ has no ability to

produce the siderophore, at the same time this isolate may have the

ability to produce bacteriocin, but this bacteriocin is not detected and that may be related to that the amount of bacteriocin produced by this isolate is very little and can not be detected in culture media, or the bacteria produced it but there are no sensitive bacteria (other isolates) are present.

The killing activity of the bacteriocin cloacin (which produced by some strains of *E.cloacae*) was inhibited by siderophore because the receptor for the uptake of this siderophore (which is found on the outer membrane) also functions as receptor for the bacteriocin cloacin, the siderophore is able to protect susceptible cells against the bacteriocin cloacin , this protection is a result of competition

between both compounds for the same receptor sites, therefore cloacin -susceptible cells of *E.cloacae* produce large amounts of the siderophore, which competitively inhibit the killing activity of cloacin DF١٣ (Bouchet, *et.al.* ١٩٩٤).

Marolda , *et.al.* (١٩٩١) has found that there is polymorphism in the siderphore-cloacin receptor in strains of *E.cloacae* ,(this receptor protein exhibit apparent molecular mass of ٨٥ KDa), siderphore producer *E.cloacae* possess a low susceptibility to cloacin and express this outer membrane protein which immunolgically cross- reacts with the receptor.

E.cloacae bacteriocin (cloacin DF١٣) secreted as a protein complex comprising a ٥٦- KDa activity protein and a ١٠- KDa immunity protein (Thomas and Valvano, ١٩٩٣).

Also Traub, *et. al.,* (١٩٨٢) pointed that the host ranges of *E.cloacae*

bacteriocin were essentially species specific , and was inactive against *K.pneumoniae*, while Riley, *et.al.,*(٢٠٠٣) showed that the activity of *E.cloacae* bacteriocin was affected not only *Enterobacter* spp. but also other closely related bacteria such as *K. pneumoniae.*

٣.٣ Effect of some Antibiotics on *Enterobater cloacae* :-

Some antibiotics are used to show their effect on the *E.cloacae* isolates, it has been found that there is clear variation in the resistance and most isolates showed resistance to one or more of these antibiotics.

It has been found that all *E.cloacae* isolates were resistant (١٠٠٪) to Amoxicillin, Tetracycline and Doxycyclin. whereas some isolates showed resistance in lesser degrees to Trimethoprim-Sulfamethoxazole (٨٥.٧٪), to Cefexime (٢١.٤٪) while to Cefotaxime and Gentamicin the resistance for both of them was (٥٧.١٪).

On the other hand, most of these isolates were resistant to Ciprofloxacin at the ratio of (١٤.٣٪), while these isolates were resistant to Nalidixic acid and to Amikacin with low higher degree (٢٨.٦٪) for each of them and to Nitrofurantoin (٤٢.٨٪) (Table ٣.٧).

Table ٣.٧: Effect of Antibiotics on the Growth of *Enterobacter cloacae*

Antibiotic	Isolation No.						
	١	٢	٣	٤	٥	٦	٧
Amoxicillin	+	+	+	+	+	+	+
Cefalothin	+	+	+	+	+	+	+
Tetracycline	+	+	+	+	+	+	+
Doxycyclin	+	+	+	+	+	+	+
TMP-SMX	-	+	+	+	+	+	+
Cefixime	-	+	+	-	+	+	+
Cefotaxime	+	-	+	+	+	-	-
Gentamycin	-	-	+	+	+	+	-
Nitrofurantoin	+	-	-	-	-	+	+
Amikacin	-	-	+	-	+	-	-
Nalidixic acid	-	-	+	-	+	-	-
Ciprofloxacin	-	-	-	-	+	-	-

Resistance (+) , Sensitive (-)

All isolates of *E.cloacae* are resistant to Amoxicillin, this result is identical with those obtained by (Conceicao, *et.al*., ٢٠٠٤) who have pointed that *E.cloacae* produce chromosomally encoded beta-lactamases that mediate resistance to Amoxicillin and cephalosporins .Amoxicillin which is generally effective for treatment of infections involving the urinary tract, is not active against penicillinase and beta-lactamase producing organisims (Warren ,*et.al.,*١٩٩٩).

In addition, some *E.cloacae* isolates have shown resistance to the Cefotaxime ,the resistance rate was (٥٧.١٪) this result is in agreement with those obtained by Redondo, *et.al*. (٢٠٠٤) they have pointed that the resistance rate of *E.cloacae* was (٥٣٪) in the survey study from ١٩٩٥-١٩٩٧,while in the same study resistance rate of *E.cloacae* to Cefotaxime was (٦١٪) in ٢٠٠١.In other study performed by Jiang ,*et.al*.,(٢٠٠٥) the (٤٦.٥٪) isolates of *E.cloacae* were found to be resistant to Cefotaxime and that differ from the result obtained by this study. In addition to beta-lactamases other mechanism is the production of novel cefotaxamase enzyme in which the gene encoding for this enzyme is present on plasmid detected in *E.cloacae* (Bonnet ,*et.al*.,٢٠٠١).

The resistance of *E.cloacae* isolates to Cefixime which is also belongs to third generation cephalosporins was (٧١.٤٪) ,this result is correlated with those of Inoue and Mitsuhashi, (١٩٩٤) who have pointed that *E.cloacae* was resistant to Cefixime , while Smith and Eng, (١٩٨٨) showed that the susceptibility rate of *E.cloacae* to Cefixime was ٩١٪. Mutations in structural genes of chromosomal beta-lactamase in *E.cloacae* isolates is the reason for resistance to oxyimino- cephalosporins including cefixime and cefotaxime (Nukaga, *et.al*.,١٩٩٥).

Although some *E.cloacae* isolates often appear susceptible to broad spectrum cephalosporins in vitro, antibiotic pressure can facilitate the emergence of derepressed mutant which produce these enzymes at high levels constitutively (Kaye, *et.al*., ٢٠٠١).

Generally, *E.cloacae* resistance to beta-lactam antibiotics is mediated by chromosomally beta-lactamases and also by reduced permeability of these antibiotics inside the cell by alterations in the outer membrane proteins (porins)(Michalkova -papajova , ٢٠٠١).

All *E.cloacae* isolates are completely resistance (١٠٠٪) to Cephalothin which is first generation cephalosporin, this result is identical with those obtained by Muytjens and Repe (١٩٨٦) who have pointed that *E.cloacae* have resistance to Cephalothin. Resistance of *E.cloacae* to Cephalothin represent intrinsic resistance and differentiate it from *Klebsiella* spp. which is susceptible to it

(Murray,*et.al*.,٢٠٠٣), therefore, the use of this antibiotic in this study not as treatment antibiotic but as diagnostic antibiogram of *E.cloacae*.

Aminoglycosides have also been used which was including gentamycin and amikacin, the resistance rate of *E.cloacae* to gentamycin

was (٥٧.١٪) while it's resistance to Amikacin was (٢٨.٦٪) , these results are correlated with those of Carapeti, *et.al*.,(١٩٩٦) who have showed that *E.cloacae* strains had partial resistance to Gentamycin (٥٨٪) while it was completely sensitive to Amikacin (١٠٠٪) while Park, *et.al*.(٢٠٠٣) had stated that the resistance rate of *E.cloacae* to Gentamycin was (٣٣.٣٪) while to Amikacin it was (٥٤٪) and that differ from the results in this study .

The mechanism of *E.cloacae* resistance to aminoglcosides is mediated by the production of more than one type of aminoglycosidases located on the R plasmid (Maes and Vanhoof, ١٩٩٢).

Other mechanism is post transcriptional modification of ١٦ S rRNA which can confer high level resistance to all aminoglycosides except Streptomycin in gram negative human pathogens including *E.cloacae* (Galimand ,*et.al*.,٢٠٠٥).

In addition , some quinolones antibiotics have also been used such as Nalidixic acid and Ciprofloxacin and the results showed that these antibiotics had good activity against *E.cloacae* isolates , the resistance rate of *E.cloacae* to Nalidixic acid was (٢٨.٦٪), this result is correlated with those obtained by Robert, *et. al*., (٢٠٠١) who had showed that the susceptibility rate of *E.cloacae* to Nalidixic acid was (٨٢٪), while Liverelli, *et.al*., (١٩٩٦) had pointed that *E.cloacae* isolates was completely sensitive to Nalidixic acid . However, Nalidixic acid is now rarely used in practice because of the risk of seizures and phototoxicity (Schaeffer ,٢٠٠٢).

On the other hand, the resistance rate of *E.cloacae* to Ciprofloxacin was (١٤.٢٪), this result is identical with those of Davin - Regli *, et. al.,*

(١٩٩٩) who mentioned that the resistance rate of *E.cloacae* to Ciprofloxacin was (١٣٪). This result is also identical with that of Kunapuli, *et.al*, (٢٠٠٣) who found that the susceptibility rate for Ciprofloxacin was more than ٨٥٪ for *E. cloacae .*

Alteration of the drug target and the ability of the drug to enter and/or accumulate in the cell are the mechanism that most often involved in resistance of *E.cloacae* to quinolones. In adition to that, quinolone resistance gene which is plasmid-mediated is the other method for the resistance to those antibiotics in *E.cloacae* (Jacoby *,et.al.,*٢٠٠٦).

Resistance rate of *E.cloacae* to Trimethoprim-Sulfamethoxazole was (٨٥.٧٪) and this is identical with those of Bell, *et.al.*, (٢٠٠٣) who have pointed that the percentage of *E.cloacae* resistance strains in Singapore was (٨٣٪) while in the same survey the resistance rate of *E.cloacae* to it was (١٠٠٪) in Hong Kong and (٢٨٪) in Japan .The mechanism of *E.cloacae* resistance to Trimethoprim-Sulfamethoxazole is either by changing the target site of this antibiotic or changing its permeability through the outer membrane (Cohen, *et.al.*, ١٩٩٣).

Resistance rate of *E.cloacae* to Nitrofurantoin was (٤٢.٨٪) and that do not in agreement with some results such as those obtained by Astal(٢٠٠٥) who showed that the *E.cloacae* were more susceptible to Nitrofurantion because only (٨.٣٪) of *E . cloacae* isolates were resistant to it and therefore may still be useful for treatment of cystitis and uncomplicated UTIs caused by this organism and that differs from our result . This result also differs from those of Kenneth, *et .al.*, (٢٠٠٣) who found that the susceptibility rates of *E. cloacae* to Nitrofurantoin was ١٣٪, while some authors decided that *E.cloacae* can be reported as resistant to Nitrofurantion without prior testing (Srga and Srga, *et.al.,* ٢٠٠٥).

The mechanism of *E.cloacae* resistance to Nitrofurantoin is due to the presence of efflux pumps which is energy dependent mechanism, these pumps is multi drug systems mediated by

transport proteins which confer resistance to toxic compounds (Moreira , ٢٠٠٢).

Furthermore, all *E.cloacae* isolates were resistant to Tetracycline (١٠٠٪) and to Doxycyclin (١٠٠٪),these results are identical with those obtained by Zhoue ,*et.al.(*٢٠٠٣) who showed that *E.cloacae* have high resistance to these two antibiotics.

Resistance of *E.cloacae* to Tetracycline results from the absence or diminished level of outer membrane protein that involved in the permeation of Tetracycline as well as other antibiotics inside the cell (Kaneko, *et.al.,*١٩٨٤).

Doxycyclin resistance transports with Tetracycline resistance on the same plasmid (Murray, *et.al*., ١٩٩٩).

In general , these results also show that there is two isolates of

E.cloacae (isolation No.٣ and ٥) were multi drug-resistant (Table ٣.٧), these results are in agreement with those of Ndugulile, *et.al.*, (٢٠٠٥)who found that some *E.cloacae* isolates including those isolated from urine samples had multiple resistance to antibiotics including beta-lactams, aminoglycosides, fluroquinolones and Trimethoprim-Sulfamethoxazole, other study showed that multi-resistant *E.cloacae* isolates were responsible for UTIs for several years in certain hospital (Kaminska,

et. al.,٢٠٠٢).

One possible mechanism to this resistance is the co-transmission of ESBLs and resistance to other antimicrobials within the same plasmids (Pitout ,*et.al.*,١٩٩٨).This result also identical with those of (Komatsu, *et.al*, ١٩٩٠) who found that the *E.cloacae* isolates were multiple-antibiotics resistant to quinolones, beta-lactams and Tetracycline, the reason for this resistance was the presence of certain genes on the *E.cloacae* chromosome which made and increase this multiple resistance.

٣.٤ The effect of N - Acetyl L - cysteine (NAC) and its combination with some antibiotics on the *Enterobacter cloacae* isolates :-

N-Acetyl L-Cysteine (NAC) is considered to be a non antibiotic drug but to have antibacterial (bacteriostatic) properties .It has been used in medicine for the treatment of chronic bronchitis, cancer, paracetamol intoxication and cystic fibrosis (Stey, *et.al.*,٢٠٠٠).

This molecule is a thiol-containing antioxidant that disrupts disulfide bonds in mucus, the positive effects of NAC treatment

have primarily been attributed to the mucus-dissolving properties of this agent and it competitively inhibits amino acid (cysteine) utilization (Ventura, *et.al*., ١٩٩٩).

In this study the effect of NAC at different concentrations (٠.٠٥-١.٠ mg/ml) on *E.cloacae* isolates has been investigated by using colorimetric method as shown in (Figure ٣.١).

It has been observed that the effect of NAC on the *E.cloacae* was only on the growth of this bacterium but not on the capsular polysaccharides where the size of the colonies remains similar to the size of colonies pretreated with this agent, while the number of these colonies were affected, the growth absorbance of *E.cloacae* without addition of NAC is ٢٠.١; the growth rate decreased very slowly when NAC is added at different concentrations, at the concentration ٠.٠٥ mg/ml the absorbance started to decrease gradually until it became ١١.٨ at the concentration ١.٠ mg/ml.

The mechanism of action of NAC on the growth of bacteria in general is unknown, however in the case of *E.cloacae* this may be due to that it considered as resistant organism and have several mechanisms to resist multiple kinds of antibiotics. However, the structure of extracellular polysaccharides (EPS) of *E.cloacae* may have specific components and that may interpret the results above about that the NAC had no effect on *E.cloacae* EPS therefore the effect was only on the growth but not on the polysaccharides.

The same results are shown by Olofsson,*et.al*.,(٢٠٠٣)who have pointed

that the NAC had the effect on the growth but not on the EPS of the

E.cloacae, but in general the effect of the NAC on the gram-negative strains were less than the effect of it on the gram-positive strains that tested in their study. In other study performed by Parry and Neu (١٩٧٧) they have pointed that the growth of *E.cloacae* was affected by the presence of the NAC but it considered highly resistant to its effect if compared with the very sensitive *Pseudomonas* strains that tested.

Despite its importance as a medicine, the effect of NAC on bacteria has been poorly studied and not investigated in depth(Perez-Giraldo *et.al.*, ١٩٩٧).

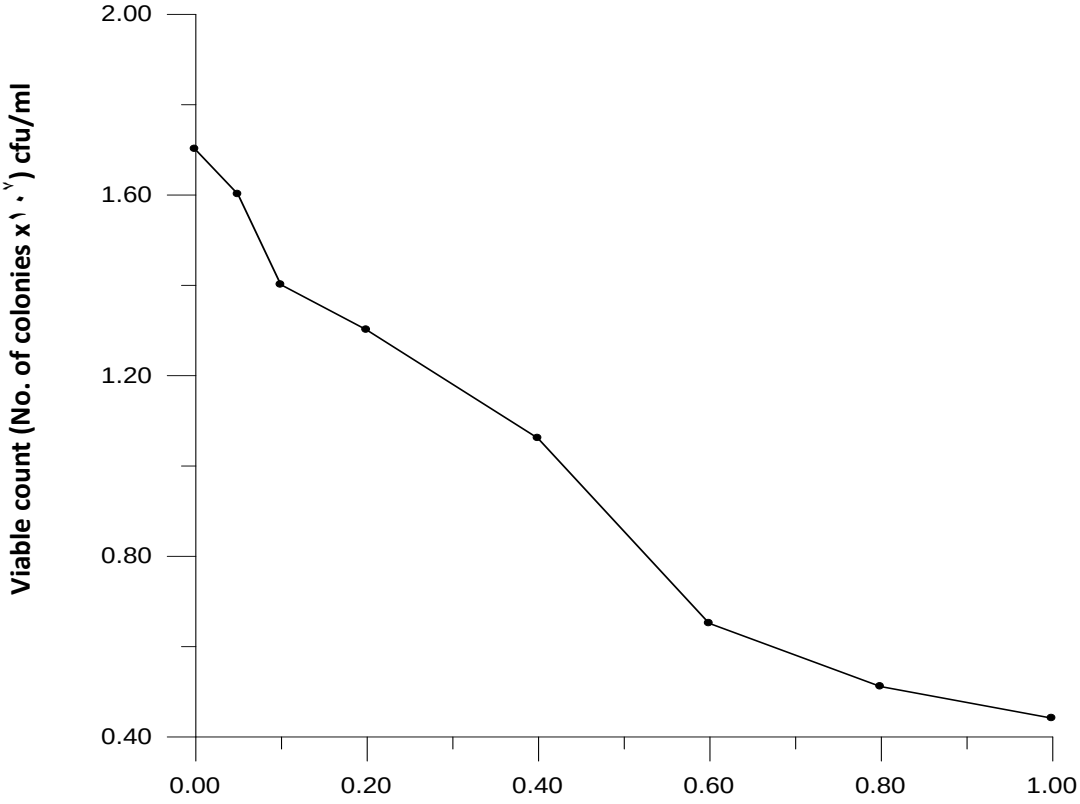


Figure (٣.١): The Effect of NAC on the Growth of *Enterobacter cloacae*

The effect of the combination of (NAC) with some antibiotics on *E.cloacae* isolates was also investigated by the interaction of (NAC) with these antibiotics, the concentration of (NAC) was (٠.١ mg/ml).

It has been observed that the combination of (NAC) at this concentration with some antibiotics such as Amoxicillin, Tetracycline and Trimethoprim-Sulfamethoxazole have no effect on the action of these antibiotics against *E.cloacae* growth where the isolates of *E.cloacae* still resistant to these antibiotics even with the presence of the (NAC).

The same results are shown by Parry and Neu (١٩٧٧), who have claimed that the strains of *E.cloacae* which isolated from all clinical specimens were not susceptible(resistant) to this combination and more resistant than other tested microorganisms such as *P.aeruginosa* and *Staphylococcus aureus*. However this study represent the only performed study about this subject and there is no other or newly studies that supporting our results.

In general there is very few studies about the effect of NAC on the bacteria, and there is no sufficient studies about the effect of NAC on the growth and EPS of *E.cloacae.*

There are no previous studies about the effect of (NAC) on *E.cloacae,* this study is considered as the first study that investigated the effect of (NAC) on the growth of *E. cloacae* isolates and the effect of the combination of NAC with antibiotics on these isolates .

٣.٥ Screening for Plasmid content :-

An attempt was carried out to study the plasmid content of *E.cloacae* isolates. Plasmid content were detected in only two isolates of *E.cloacae,* isolates number (٣ and ٥) by using the Salting out method for the extraction of plasmids present in these isolates (Kado and Liu,١٩٨١) as an attempt to show whether this bacteria have the extra chromosomal element (plasmid) or not.

It was seen that these two isolates contain one plasmid at each, these plasmids were identical in their size according to their migration on the agarose of gel-electrophoresis system (Figure ٣.٢).

The presence of such plasmids may confer the bacteria additional factors such as the ability to resist more than one antibiotic (not detected in this study).

However, both isolates (٣ and ٥) were isolated from two patients suffering from UTIs, but their plasmids had the same shape

and size, this may give an indication that the source of these two isolates was one.

These results resemble those obtained by (Darini, *et.al.*, ١٩٩٩) who have pointed that the *E.cloacae* which isolated from three hospital wards including nephrology ward were containing plasmids that identical in their sizes .

These results also resembles the results obtained by Tsivitanidou, *et.al.,* (٢٠٠٣) , they found that all *E.cloacae* isolates were identical in plasmid content ,these isolates were responsible for an outbreak in neonatal intensive care unite (NICU), they concluded that one epidemic strain caused the outbreak .

In addition, the study of Sherly, *et.al*.(٢٠٠٣) found that the mean of plasmids carriage by plasmid bearing strains of *E.cloacae* was (١.٦) plasmids, also they have shown that some *E.cloacae* isolates were harboring large plasmids.

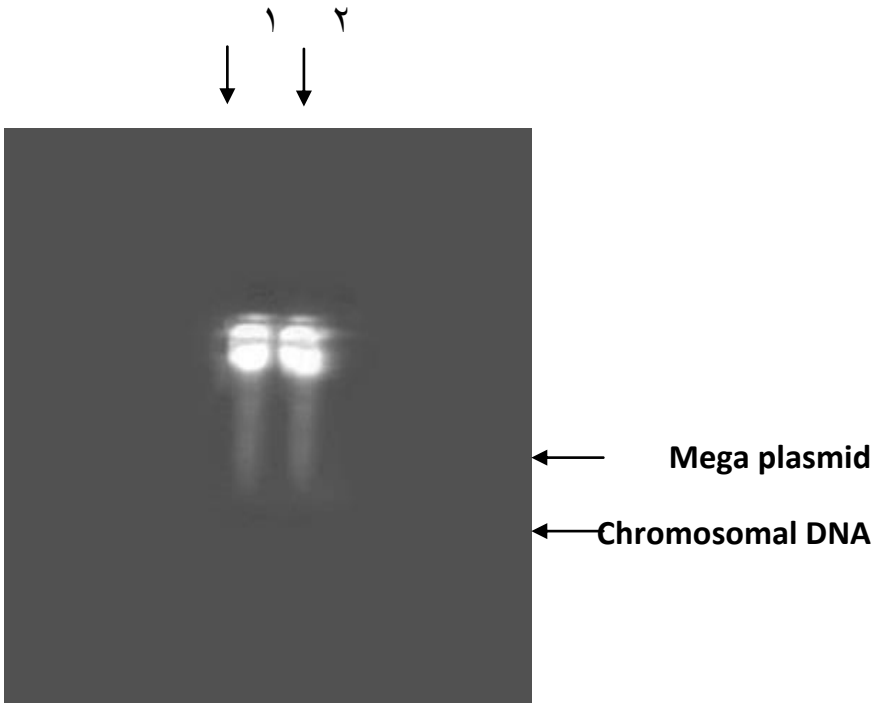
Ni Rian*, et.al*.,(١٩٩٤)have mentioned that many *Enterobacter* strains including *E.cloacae* may possess few , if any plasmids .

The plasmid profile of these two isolates of *E.cloacae* carried out in this work , consisted only one band for each isolate ,in addition to that, only these two isolates have approximately the same multiple -resistance to the antibiotics used against *E.cloacae* isolates ,this confirms the possible clonal relatedness among them

and the same epidemiological source of the infection ,however ,in *E.cloacae* the resistance to beta-lactam antibiotics generally mediated by chromosomally expression enzymes (Frankard ,*et.al.*,٢٠٠٤) and to other antibiotics such as quinolones ,aminoglycosides and Trimethoprim-Sulfamethoxazole is mediated by the plasmids (Alhambra, *et.al.*, ٢٠٠٤).

It is noteworthy that extra-chromosomal elements can be easily acquired or lost depending on the antibiotic pressure ,alternatively plasmid can integrate into the chromosomal DNA , being undetectable by the usual extraction techniques and the plasmid transfer of DNA is extremely common among enterobacteriaceae (Weischer and Kolmos, ١٩٩٣). The extent of discrimination provided by DNA typing of bacteria depends on the degree of genetic divergence from non-lethal mutations, acquisitions, or deletions in DNA over time(Hartstein,*et.al.*,١٩٩٥).

All *Enterobacter* spp . thought to be contributing to the resistance plasmid pools in institutions by their ability to transfer extra chromosomal resistance factors to susceptible bacteria (Drancourt, *et.al.*,٢٠٠٠). Plasmid profile is one of the molecular techniques that use in the molecular epidemiology, although it is easiest to determined but it is not enough if not supporting with other techniques such as fingerprinting and hybridization of DNA.



Figure(٣.٢) Electrophoresis of Plasmid DNA

٣.٦ Conclusions and Recommendations

٣.٦.١ Conclusions:

The results of this study can yield the following conclusions:

١. The prevalence of *E. cloacae* infection was ٧/١٥٠ isolates in patients who suffering from UTIs with the age more than ٧ years and from both sexes, this result nears the results of other studies.

٢.*E.cloacae* isolates varies in their resistance to the different antibiotics

and some isolates have the resistance for two antibiotics or more.

٣. *E.cloacae* isolates was revealed to possess more than one virulence

factor such as capsule, colonization factor antigens and siderophores.

In addition to that, it was found that *E. cloacae* can produce the

haemolysin as the virulence factor.

٤. There is no isolate has the ability to produce the bacteriocin and that may be due to several factors, the important factor among them

may be the production of the siderophore.

٥. N-acetyl - L- cystein (NAC) is markedly effective on the growth of

E.cloacae.

٦. Third - generation cephalosporins showed ratherly good in vitro activity against *E.cloacae* isolates.

٧. Plasmid content was detected in two isolates of *E.cloacae* and it was found that these two isolates have one plasmid at each, with the same shape and size.

٣.٦.٢ Recommendations

According to the results obtained in the present study, it is recommended that further investigation to made following:

١. Study the incidence of *E.cloacae* infections in diseases other than

Urinary tract infections .

٢. Using combination of some antibiotics and newer antibiotics in

treatment the infections that caused by *E.cloacae* because this

bacteria have revealed resistance to more than one of the

common use antibiotics.

٣. Current studies must be performed to detect and describe the

bacteriocin production , which is helpful in treatment of bacterial

infection.

٤. Study the molecular basis of *E.cloacae* resistance to antibiotics and

using other molecular techniques to detect the gene that present in

the molecular content of this bacteria.

٥. Examine the effect of some concentrations of(NAC) that higher than that used in this study on the growth of *E. cloacae* isolates.

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