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College of Medicine

Bacteriological Study on Extended-Spectrum Beta-Lactamases Produced by *Enterobacteriaceae* Isolated from Children with Bacteremia in Hilla City

Athesis

Submitted to the Council of the College of the Medicine; University of Babylon In Partial Fulfillment of the Requirements For the Degree of Master of Science In Medical Microbiology

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بسم الله الرحمن الرحيم

((قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا

إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ))

صدق الله العلي العظيم

سورة البقرة

الآية (٣٢)

Supervision Certificate

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

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We, the examiner committee, certify that we have read the thesis entitled **(Bacteriological Study on Extended-Spectrum Beta-Lactamases Produced by *Enterobacteriaceae* Isolated from Children with Bacteremia in Hilla City)** and examined the student **(Forqan Mohammed Hussein Al-Asady)** in its contents, and that in our opinion it is accepted as a thesis for the degree of Master of Science in Medical Microbiology with **excellent** estimation.

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Dedication

I dedicate this work to:

- ***The memory of My mother.***
- ***All members of my family.***
- ***Flower of My life: Hussein.***
- ***My wife.***

Forqan

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Forqan

جامعة بابل
كلية الطب

دراسة بكتريولوجية عن البكتريا المعوية المنتجة لإنزيمات البيتالاكتاميز واسعة الطيف والمعزولة من الأطفال المصابين بتجرثم الدم في مدينة الحلة

رسالة

مقدمة الى مجلس كلية الطب/جامعة بابل

جزء من متطلبات نيل درجة الماجستير

في علم الأحياء المجهرية الطبية

من قبل

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بكالوريوس صيدلة/ جامعة بغداد (2003)

المشرف
الأستاذ الدكتور
حبيب صاحب نهر

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شوال ١٤٣٠هـ

تشرين الأول ٢٠٠٩م

الخلاصة:

تضمنت هذه الدراسة, جمع 313 عينة دم من أطفال مصابين بارتفاع درجة الحرارة والداخلين إلى مستشفى الولادة والأطفال في الحلة للفترة من تشرين الأول/2008 إلى شباط/2009. أظهرت النتائج بان 43 عينة (13.7%) فقط من مجموع العينات المدروسة, أعطت نتائج ايجابية لنمو43 عزلة بكتيرية. من هذه ال 43 عزلة, 34 عزلة بكتيرية (10.9%) شخصت كأنواع تابعة للعائلة المعوية *Enterobacteriaceae*.

أظهرت الدراسة إن العزلات التابعة للأنواع *Klebsiella* spp. وال *E. coli* هي أكثر الأنواع التي تم عزلها من عينات دم المرضى (50%) و (32.4%) على التوالي, مقارنة بالأنواع البكتيرية المعزولة الأخرى, (11.7%).*Enterobacter* spp و *Proteus mirabilis* (5.9%). أوضحت الدراسة بان الأطفال حديثي الولادة والأطفال الرضع هم اكثرعرضة للإصابة بتجرثم الدم, بالمقارنة بأعمار الأطفال الأخرى. كما انه لا توجد هنالك فروق معنوية بين إصابة الذكور (47.1%) و الإناث (52.9%) بين الأطفال المرضى.

أظهرت النتائج في مسح أولي للعزلات إن جميع العزلات (34 عزلة) كانت 100% مقاومة لمضادات البيتالاكتام (ß-lactam) (امبيسيلين و اموكسيسيلين). ووجد فيما بعد, بان 15 عزلة (44%) من مجموع العزلات المقاومة للبيتالاكتام كانت لها القابلية على إنتاج إنزيمات البيتالاكتاميز باستخدام طريقة اليود القياسية السريعة (rapid iodometric method), وان غالبية العزلات المنتجة لإنزيمات البيتالاكتاميز كانت تابعة للأنواع (*K. pneumoniae pneumoniae* subsp. و *E. coli*).

تم دراسة تأثير مجموعة من المضادات الحيوية على العزلات المنتجة لإنزيمات البيتالاكتاميز(β-lactamases) (15عزلة) باستخدام طريقة انتشار القرص (disk diffusion) ووجد بان غالبية العزلات كانت تمتلك مقاومة عالية للمضادات الحيوية وخاصة مجموعة البيتالاكتام, يستثنى من ذلك مجموعة ال carbapenems مثل ال meropenem و imipenem, حيث أظهرت جميع العزلات 100% حساسية تجاه هذه

المضادات. وكنتيجة لذلك, تعتبر ال Carbapenems من الأدوية الفعالة جدا في علاج العدوى الناتجة عن البكتريا المنتجة لإنزيمات البيتالاكتاميز واسعة الطيف.

أظهرت نتائج التحري عن إنزيمات البيتالاكتاميز واسعة الطيف, إن طريقة تحديد التركيز المثبط الأدنى (MIC) مع و بدون استخدام ال clavulanate من بين اكثرالطرق دقة وهذه الدقة تجسدت في الحصول على 12 عزلة بكتيرية (80%) منتجة لإنزيمات البيتالاكتاميز واسعة الطيف من مجموع 15 عزلة بالمقارنة ب 10 عزلات (66.6%) منتجة باستخدام طريقة Inhibitor potentiated disk diffusion method و 7 عزلات (46.6%) منتجة باستخدام Disk approximation, وهكذا تعتبر الطريقة الأخيرة من اقل الطرق دقة في الكشف عن إنزيمات البيتالاكتاميز واسعة الطيف.

أوضحت نتائج تحديد التركيز المثبط الأدنى Minimum inhibitory concentrations (MICs) للعزلات 12 المنتجة للبيتالاكتاميز واسعة الطيف تجاه أربعة من مضادات البيتالاكتام, إن جميع العزلات (8 *Klebsiella pneumoniae* subsp. *pneumoniae* و *E. coli* 4) كانت عالية المقاومة للامبيسيلين. وكما أظهرت مقاومة عالية للسفتازديم (ceftazidime) وبدرجة اقل للسيفوتاكزيم (cefotaxime), ولكنها كانت حساسة بشكل كبير للاميبنيم (imipenem) . وتشير قيم MICs العليا للعزلات المنتجة لإنزيمات البيتالاكتاميز واسعة الطيف في هذه الدراسة إلى أن إنزيمات البيتالاكتاميز واسعة الطيف قد تكون متوطنة في منطقة الدراسة.

كشفت نتائج طريقة ال polymerase chain reaction (PCR) بان من بين العزلات 12 المنتجة لإنزيمات البيتالاكتاميز واسعة الطيف وعزلتين غير منتجة لهذه الإنزيمات, عزلة واحدة وهي (*Klebsiella pneumoniae* subsp.*pneumoniae*) تمتلك إنزيم من نوع TEM, 4 عزلات (2 *E. coli*, 1 *K. oxytoca*, *1 K.* *pneumoniae* subsp. *pneumoniae*) تمتلك انزيم من نوع SHV, 7 عزلات (6 *Klebsiella pneumoniae* subsp.*pneumoniae* و *E. coli* 1) أظهرت القابلية

على امتلاك كلا النوعين من الإنزيمات TEM و SHV, وهنالك عزلتين فقط كلاهما *E. coli* أظهرت عدم امتلاكها لأي من إنزيمات TEM أو SHV.

أظهرت النتائج التي تم الحصول عليها بطريقة ال PCR, بان كلا الانزيمين TEM و SHV هي واسعة الانتشار بين الأنواع العائدة لعائلة *Enterobacteriaceae*. بالإضافة إلى ذلك, تعتبر طريقة ال PCR من الطرق الجينية العالية الدقة في تحديد أنواع إنزيمات البيتالاكتاميز واسعة الطيف (ESBLs).

Abstract:

The present study included 313 blood samples collected from febrile children admitted to Pediatric and Maternity Hospital in Hilla city during the period from October 2008 to February 2009. Only 43 samples (13.7 %) gave positive results for 43 bacterial isolates. Of these, 34 bacterial isolates (10.9%) which were identified as *Enterobacteriaceae*.

The study revealed that *Klebsiella* spp. (50%) and *Escherichia. coli* (32.4%) were the most common isolated bacteria as compared with other members of *Enterobacteriaceae*. The study showed that neonates and infants were at increased risk for blood stream infections by *Enterobacteriaceae*, in comparison to other pediatric age groups. The susceptibility of males (47.1%) and females (52.9%) to blood stream infection was nearly the same (no significant difference).

Results showed that all 34 isolates were resistant to ß-lactam antibiotics (ampicillin and amoxicillin) in the initial screening test. Subsequently, out of these 34 isolates, only 15 isolates (44%) (Mostly *K. pneumoniae* subsp. *pneumoniae* and *E. coli*) were found to be ß-lactamase-producers by rapid iodometric method.

Antibiotic susceptibility pattern of these 15 ß-lactamase-producers by disk diffusion method revealed that the majority of these isolates were highly resistant to the antibiotics tested especially ß-lactam antibiotics, except for carbapenems (meropenem and

imipenem), to which all the isolates were sensitive. As a result, carbapenems (meropenem and imipenem) were considered the drugs of choice for treatment of infections caused by these isolates.

Among the three methods used for ESBL detection, determination of minimum inhibitory concentration (MIC) with and without clavulanate was the most accurate method since 12 isolates (80%) were identified as ESBL-producers in comparison to 10 isolates (66.6%) by inhibitor potentiated disk diffusion test and 7 isolates (46.6%) detected by disk approximation test. Consequently, disk approximation method may be associated with the lowest recovery in the detection of ESBL enzymes.

MICs of ESBL-producers against 4 β-lactam antibiotics showed that all 12 ESBL-producers were highly resistant to ampicillin (MIC >128 mg/ml). They were also resistant to ceftazidime and to a less extent to cefotaxime; but, they showed a higher sensitivity to imipenem. The high MIC results (from 64 to >128 μg/ml) of ESBL-producing isolates in the present study suggested that ESBL enzymes are endemic in the area of the study.

Polymerase chain reaction (PCR) results revealed that among the 12 ESBL-producers and 2 non ESBL-producers, 1 isolate (*K. pneumoniae* subsp. *pneumoniae*) had TEM enzyme type, 4 isolates (1 *K. pneumoniae* subsp. *pneumoniae*, 2 *E. coli* and 1 *K. oxytoca*) had SHV enzyme type, 7 isolates (6 *K. pneumoniae* subsp. *pneumoniae* and 1 *E. coli*) had both TEM and SHV enzyme type, and 2 isolates (both *E. coli*) do not produce neither TEM nor SHV enzymes.

Results of PCR showed that both TEM and SHV enzymes were highly prevalent among species of *Enterobacteriaceae* that responsible for blood stream infections in children. In addition to that, PCR test was considered as an accurate genotypic method used for detection of ESBL enzyme type.

Conclusions:

1- Gram-negative bacilli (mainly *Enterobacteriaceae*) were the most common pathogens recovered from blood of febrile children.

2- Neonates and infants were at increased risk for bloodstream infection by members of *Enterobacteriaceae* more than other pediatric age groups.

3- *Klebsiella* spp. (mainly *Klebsiella pneumoniae* subsp. *pneumonia*) and *E. coli* were the most common species recovered from blood of those children

4- About half of tested isolates were ß-lactamase-producers, and most of them possessed a higher antibiotic resistance especially for ß-lactam antibiotics. Eighty percent of them were ESBL-producers.

5- Determination of MIC with and without clavulanate was the most accurate phenotypic method for detection of ESBL-producing isolates and the disk approximation method was the least accurate method in detecting such enzymes.

6- Carbapenems (imipenem and meropenem) were the drug of choice for the treatment of infections caused by ESBL-producing isolates.

7- PCR technique was an accurate genotypic method used for detection of ESBL enzyme type.

8- The majority of ESBL-producing isolates have TEM and/or SHV enzyme type.

Recommendations:

1- It is important to make blood culture and subsequently, antibiotic susceptibility pattern for any febrile child not responds to antibiotic treatment especially third generation cephalosporins.

2- Avoiding the use of third generation cephalosporins against pathogenic bacteria that appear resistant to these antibiotics (i.e. ESBL-producers).

3- Tests used for detection of ESBL-producing isolates should be carried out, if possible, in all hospital laboratories in Hilla.

4- Studying the prevalence of ESBL enzymes in other species of Gram-negative and even Gram-positive bacteria.

5- It is necessary to study the presence and the prevalence of other types of ESBL enzymes such as CTX-M, OXA and others.

6- It is important to use PCR technique to provide an accurate detection of ESBL enzymes.

7- In addition to PCR, it is important to use other molecular methods, if possible, such as isoelectric focusing and DNA sequencer.

1-1- Introduction:

Bacteremia is defined as the presence of bacteria in circulating blood whether continuously or transiently. It may be asymptomatic and resolve without treatment, or it may cause serious and immediate consequences including shock, organ failure and death (Huber and Terezhalmy, 2005). Gram-negative bacteraemia is regarded as syndrome secondary to infection usually originates from the gastrointestinal tract or the skin in patients with decubitus ulcers (Vincent *et al*., 2004).

The β-lactam family of antibiotics includes many of the most heavily used antibacterial in clinical medicine. They are important, both historically and currently, because of their effectiveness and generally low toxicity. Production of β-lactamase enzymes that break down the structural β-lactam ring of penicillin-like drugs considered as a predominant mechanism of bacterial resistance to β-lactam antibiotics especially in bacteria causing clinically significant infections of which there are several classes including plasmid-encoded and chromosomally-encoded enzymes (Livermore and Brown, 2001).

The extended-spectrum β-lactams became widely used in the treatment of serious infections due to Gram-negative bacteria. Resistance to the extended-spectrum β-lactam among members of *Enterobacteriaceae* has become a growing problem worldwide

(Jacoby and Han, 1996). Such resistance is often associated with transferrable plasmid-encoded Extended-Spectrum β-lactamases (ESBLs). Most ESBLs from *E. coli* and *K. pneumoniae* derived from TEM or SHV-type β-lactamase by one or more amino acid substitutions which confer resistance to exteded-spectrum ß-lactam antibiotics (Jacoby, 1997).

ESBLs arise by mutations in genes responsible for these plasmid-mediated β-lactamases that alter the configuration of the active site so as to increase the affinity and hydrolytic activity of the enzyme for oxyimino-compounds (Jacoby, 1994).

ESBLs in Gram-negative bacillary pathogens are growing and important problem in hospital practice (Rice *et al*., 1991). Successful detection of the ESBL mechanism is important for timely selection of the appropriate antimicrobial treatment as well as to establish hygienic precaution to prevent further spread of the strains involved.

The easiest and most common molecular method used to detect the presence of a β-lactamase enzyme belonging to a family of enzymes is the polymerase chain reaction (PCR) with oligonucleotide primers that are specific for a β-lactamase gene (Woodford *et al*., 2006). PCR method was used for determination of bla_{TEM} and bla_{SHV} genes that coded for TEM and SHV β-lactamase enzyme, respectively (De Gheldre *et al*., 2003).

The Present Study Aims for:

1- Studying the prevalence of β-lactam resistance in clinical isolates of bacteria belonging to the family *Enterobacteriaceae*.

2- Determining of antibiotic susceptibility patterns for ß-lactamase-producing isolates.

3- Detecting of ESBL-producing isolates and determination of the best methods for the detection of these enzymes.

4- Detecting of bla_{TEM} and bla_{SHV} genes by using molecular methods (PCR).

1-2- Literatures Review:

1-2-1- *Enterobacteriaceae*:

Enterobacteriaceae includes a large, heterogenous group of gram-negative rods whose natural habitat is the intestinal tract of human and animals. Members of *Enterobacteriaceae* are aerobes or facultative anaerobes, ferment a wide range of carbohydrates, possess a complex antigenic structure, and produce a variety of toxins and other virulence factors (Brooks *et al.*, 2007).

Genera of the family *Enterobacteriaceae*, that commonly colonize human or associated with human infections, include: *Escherichia, Shigella, Salmonella, Yersinia, Klebsiella, Raoultella, Proteus, Enterobacter, Citrobacter, Serratia, Edwardsiella, Hafnia, Morganella,* and *Providencia* (Forbes *et al.*, 2007).

Most genera and species in the family *Enterobacteriaceae* do not form spores, are motile by peritrichous flagella or non motile, grow on peptone or meat extract media without the addition of sodium chloride or other supplements, and are catalase positive and oxidase negative (Baron *et al.*,2003).

Clinically, most of the genera mentioned above are opportunistic pathogens, causing diverse of infections, in addition to the nosocomial infection being resulted by most of these genera. However, the clinically most important species are briefly described below:

1-2-1-1- *Escherichia coli:*

It is variable in motility and commonly found in the lower intestine of warm-blooded animals. It was discovered by Theodor Escherich in 1885 (Feng *et al.,* 2002).

E. coli and related bacteria has been reported to transfer DNA via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population. This process led to the spread of the gene encoding Shiga toxin from *Shigella* to *E. coli* O157:H7, being carried by a bacteriophage (Brussow *et al.,* 2004). Dairy and beef cattle are primary reservoirs of *E. coli* O157:H7 which can cause serious food poisoning in humans, and are occasionally responsible for costly product recalls (Vogt and Dippold, 2005).

E.coli is not always confined to the intestine, and their ability to survive for short period outside the body makes them an ideal indicator organism to test environmental samples for fecal contamination (Heaton and Jones, 2007).

Virulent strains of *E. coli* can cause cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI), traveler's diarrhea, and other clinical infections such as neonatal meningitis and pneumonia. Enterohemorrhagic *E coli* (EHEC) causes hemorrhagic colitis or hemolytic-uremic syndrome (HUS) (Harrington *et al*., 2006).

1-2-1-2- *Klebsiella* species:

Klebsiella species are described as non-motile, oxidase-negative rod shaped organisms with a prominent polysaccharide-based capsule, and are generally positive in the Voges-Proskauer test (Ryan and Ray, 2004). Frequent human pathogens, *Klebsiella* organisms can lead to a wide range of diseases, notably pneumonia, urinary tract infections (UTI), septicemia, Ankylosing spondylitis, and soft tissue infections (Podschun and Ullmann, 1998).

Klebsiella species are usually identified and differentiated according to their biochemical reactions. Capsule is essential to the virulence of *Klebsiella* (Highsmith and Jarvis, 1985). *Klebsiella* is carried in the nasopharynx and the bowel; however, feces are probably the most significant source of patient infections (Montgomerie, 1979).

1-2-1-3- *Enterobacter* species:

Enterobacter species are motile, rod-shaped cells, some of which are encapsulated. They also possess peritrichous flagella. As facultative anaerobes, some *Enterobacter* bacteria ferment both glucose and lactose as a carbon source. Gas is produced from the metabolic processes, but they do not produce hydrogen sulfide (Roberts *et al*., 1999).

Enterobacter bacteria are nosocomial opportunistic pathogens that are causing more infections including up to 5% of hospital-

acquired septicemias, 5% of nosocomial pneumonias, 4% of nosocomial urinary tract infections, and 10% of postsurgical peritonitis cases (Hoffmann *et al.*, 2003).

Nosocomial *Enterobacter* colonization and infection are frequently associated with contaminated medical devices and instrumentation; however, *Enterobacter* species are commonly consumed in food, and an endogenous source should also be considered (Janda *et al.*, 1998).

1-2-1-4- *Proteus* species:

Proteus includes opportunistic pathogens responsible for many human urinary tract infections (Guentzel, 1996). *Proteus mirabilis* is an important pathogen of urinary tract, especially in patients with indwelling urinary catheters (Mobley and Warren, 1987). Urinary tract infections with *Proteus mirabilis* usually start with colonisation of the bladder, causing bacteriuria and cystitis (Bahrani *et al.*, 1991).

Several potential virulence factors have been reported for *P. mirabilis*, these include urease production (Mobley and Hausinger, 1989), cell invasiveness, swarming motility by flagella (Allison *et al.*, 1992), cleavage of IgG and IgA by a proteolytic enzyme, and outer-membrane proteins (Moayeri *et al.*, 1991). The inducible urease is responsible for the formation of bladder and kidney stones at later stages of infection as a result of urea hydrolysis (Mobley and Hausinger, 1989). Furthermore, the haemolysin secreted by *Proteus*

mirabilis was a cytotoxic for urinary tract epithelial cells (Mobley *et al.*, 1991).

1-2-1-5- *Raoultella* species:

The name *Raoultella* is proposed as a genous name for species of cluster II which contained *Klebsiella ornithinolytica, Klebsiella planticola* and *Klebsiella terrigena*, organisms characterized by utilization of L-sorbose as carbon source. (Drancourt *et al.*, 2001).

However, in the United States, a survey of 436 *Klebsiella* strains isolated from newborns found that only one isolate which could be identified as *Raoultella planticola*, indicating that its prevalence may differ geographically (Westbrook *et al.*, 2000).

Other members of *Enterobacteriaceae* include: *Salmonella, Shigella, Yersienia* (pathogenic), *Serratia, Citrobacter, Morganella*, and *Providencia* (apportunistic) (Forbes *et al*., 2007).

1-2-2- Bacteremia Caused by *Enterobacteriaceae*:

Blood is normally sterile so, culturing of such blood does not normally lead to isolation of the bacteria. Bacteremia is most commonly diagnosed by blood culture, in which a sample of blood is allowed to incubate with a medium that promotes bacterial growth. If bacteria are present in the blood stream at time of sample collection, the bacteria will multiply in the medium and can thereby detected (Thomson *et al*., 2004). Any bacteria that incidentally find their way

to the culture medium will also multiply, for this reason blood cultures must be drown with great attention in sterile process (Brooks *et al*., 2007).

Growth in media is indicated by the presence of turbidity, gas bubbles, and haemolysis of the blood in culture bottle or even by observation of bacterial colonies suspended in the bottle (Washington, 1975). Gram-stained smears should be made from any broth that shows visible signs of growth, cultured characteristics, biochemical, serological, and genetic analysis are widely used to confirm the diagnosis of bacterial isolates (Collee *et al*., 1996).

Bloodstream infections by Gram-negative bacilli are associated with high morbidity and mortality rates that seem to be correlated with the severity of the underlying diseases and with the use of inadequate empirical antimicrobial drug therapy (Pong and Bradley, 2004).

Schiappa *et al*. (1996) published a case-control study of 31 cases of bacteremia caused by ESBL-producing *E. coli* or *K. pneumoniae* strains. A study performed in a pediatric oncology ward (Ariffin *et al*., 2000), the sepsis-related mortality rate was higher among 16 patients infected with ceftazidime-resistant *K. pneumoniae* strains (50.0%) than 15 patients infected with ceftazidime-susceptible *K. pneumoniae* strains (13.3%).

Blood stream infections (BSI) risk factors and the impact of ESBL-related bacteremia on treatment outcome have been

investigated solely in infections due to ESBL-positive isolates of *E. coli* and *K. pneumoniae* (Endimiani *et al*., 2004). The enhanced complexity of the patient, with a consequent increase in the duration of the hospital stay and greater need for intravascular devices, has increased the risks for acquiring bloodstream infections (Biedenbach *et al*., 2004). Initially more prevalent in *K. pneumoniae* and *E. coli*, ESBLs are now also emerging resistance determinants in other enterobacterial species, including *P. mirabilis* (Stürenburg and Mack, 2003).

Concerning BSI, several studies have analyzed risk factors, clinical outcome, and treatment outcome of BSI caused by ESBL-positive isolates of *E. coli* and *K. pneumoniae* (Kim *et al*., 2002), while analogous aspects have not been investigated for BSI caused by ESBL-producing *Proteus mirabilis* isolates. Although *P. mirabilis* is a less common cause of BSI (1 to 2% of a total cases, ranking forth among Gram-negative bacilli following *E. coli*, *K. pneumoniae*, and *Enterobacter* spp.) (Sader *et al*., 2003). The increasing diffusion of ESBL in strains of this species is now making this issue more interesting.

Proteus mirabilis is one of the most common gram-negative pathogens encountered in clinical specimens and can cause a variety of community- or hospital-acquired illnesses, including urinary tract, wounds, and blood stream infections (BSI) (O'hara *et al*., 2000). Coresistance to aminoglycosides, fluoroquinolones, and trimethoprim-

sulfamethoxazole has been frequently reported among ESBL-producing *P.mirabilis* (ESBL-P-PM) strains (Winokur *et al*., 2001).

In recent years, it has also become apparent that *Enterobacter cloacae* can acquire and express genes encoding extended-spectrum β-lactamases (ESBLs) (Canto´n *et al*., 2002). *Enterobacter* spp. can cause difficult to treat nosocomial infections in critically ill patients owing to mutations. The expression of a chromosomal gene encoding the AmpC ß-lactamase (Jacoby and Munoz-Price, 2005) can be increased during therapy with third-generation cephalosporins such as cefotaxime, ceftazidime and ceftriaxone (Livermore and Brown, 2001).

Bloodstream infections also involve other members of *Enterobacteriaceae* such as *Salmonella, Serratia and Citrobacter* (Pong and Bradley, 2004).

1-2-3- ß-Lactam Antibiotics:

The history of ß-lactam antibiotics began in 1929 when Alexander Fleming description of antibacterial activity of a substance produced by *Penicillium* mould and gave it the name penicillin. However, the phenomenon of antibiotic was already well known at this time (Albert and Sussman, 1998).

Antibiotics are organic substances produced by microorganisms and capable at low concentration of inhibiting the growth of, or destroying another microorganism. These antibiotics have been

isolated from numerous sources but principally from bacteria and fungi (Ibezim, 2005).

The introduction of penicillin into clinical practice has provided the stimulus for the discovery of many other antibiotics. The major expansion of the ß-lactam field only occurred in the early 1960s with the development of the semi-synthetic penicillins, quickly followed by the development of the semi-synthetic cephalosporins and other ß-lactam antibiotics (Rolinson, 1998).

The ß-lactam groups differ from each other by additional rings (thiazolidine ring of pencillins, cephem nucleus of cephalosporins, double ring structure of carbapenems and none for monobactams). The various antibiotics in each group differ by the nature of one or two side chains (Poole, 2004)

Ceftobiprole has been described as "fifth generation" cephalosporin (Widmer, 2008). ß-lactams are the widely used antibiotics because of their efficacy, broad spectra, and low toxicity. The clinically useful ß-lactams belong either to pencillin (penam) or cephalosporin (cephem) group. The ß-lactams also include the carbapenems e.g. imipenem, the monobactams e.g. aztreonam, and the ß-lactamase inhibitor e.g. clavulanate **(Table 1-1)**. Monobactam have a monocyclic ß-lactam ring and are resistant to broad spectrum ß-lactamase. They are active against Gram-negative rods but not against Gram-positive bacteria or anaerobes. The first such drug to become available was aztreonam. Imipenem the first drug of the carbapenems,

had good activity against many Gram-negative rods, Gram-positive organism and anaerobes. It is very stable in the presence of bacterial broad spectrum, extended spectrum and AmpC ß-lactamases (Mandell and Petri, 1996).

The production of ß-lactamase is the most frequently encountered mechanism of bacterial resistance to ß-lactam antibiotics. Some variants are able to inactivate newer cephalosporins, are called extended spectrum ß-lactamases (ESBLs) and are predominantly plasmid mediated (Queenan and Bush, 2007).

Efficacy of ß-lactamases in hydrolyzing the ß-lactam ring, which is necessary for activity of antibiotic, varies widely and the enzymes could be conceived as a family with a spectrum of activity. Therefore, those enzymes with strong affinity for penicillins (6-aminopenicillanic acid derivatives) are called penicillinases. Penicillinase was the first β-lactamase to be identified, it was first isolated by Abraham and Chain in 1940 from *E. coli* even before penicillin entered clinical use (Abraham and Chain, 1940).

Those at cephalosporin (7-aminocephalosporanic acid derivatives) end of spectrum are cephalosporinases. There are also broad spectrum ß-lactamases that are active on both penicillins and cephalosporins.

Table (1-1): Groups and Examples of ß-Lactam Antimicrobial Agents (modified from Samaha-Kfoury and Araj, 2003).

ß-Lactam Groups	Examples of Antimicrobial Agents
Penicillins	• **Penicillin G, penicillin V**
	• **Penicillinase resistant penicillins:** methicillin, nafcillin, oxacillin, cloxacillin.
	• **Aminopenicillins:** ampicillin, amoxicillin.
	• **Carboxypenicillins:** carbenicillin, ticarcillin.
	• **Ureidopenicillins:** mezlocillin, piperacillin.
Cephalosporins	• **First generation:** cefazolin, cephalothin, cephalexin.
	• **Second generation:** cefaclor, cefamandole, Cefuroxime.
	• **Third generation:** cefotaxime, ceftriaxone, Cefoperazone, ceftazidime, ceftizoxime.
	• **Fourth generation:** cefepime, cefpirome.
	• **Fifth generation:** ceftobiprole.
	• **Cephamycin:** cefoxitin, cefot cefmetazole
ß-Lactamase inhibitor	Ampicillin + sulbactam, Amoxicillin + clavulanic acid, Piperacillin + tazobactam.
Carbapenems	Imipenem, meropenem, ertapenem.
Monobactams	Aztreonam.

1-2-4- Bacterial Resistance to ß-Lactam Antibiotic:

Resistance to ß-lactam antibiotics in bacteria could be due to four mechanisms (Piddock *et al.,* 1997).

1-2-4-1- Destruction the Active Drug by the ß-Lactamase:

The most common form of resistance to ß-lactam agents is caused by enzymes that render molecules inactive by opening the ß-lactam ring. In Gram-positive, ß-lactamase is excreted into the medium and therefore, destroys the antibiotics extracellularly (Albert and Sussman, 1998). In contrast, the ß-lactamase of Gram-negative bacteria is located in the periplasmic space, where they attack the antibiotic before it can reach its receptor site (Wiedemann *et al.,* 1989).

1-2-4-2- Decreased Affinity of the Target Penicillin Binding Proteins (PBPs):

Resistance to ß-lactam antibiotics can also attributed to alteration in the PBPs in the bacterial cell membrane which accounts for both the low-level and the high-level rsistance exhibited by *Streptococcus pneumoniae* to penicillin G and for the resistance of *Staphylococcus aureus* to methicillin and other ß-lactamase-resistance penicillins. Hence, resistance to penicillin and cephalosporin may be a function of the loss or alteration of PBPs (Piddock *et al.,* 1997).

1-2-4-3- Decreased Permeability of Drug into the Cell:

Porins are major water-filled outer membrane proteins that allow diffusion of small polar molecules across the G-proteins. Proteins that line these channels are called outer membrane proteins. Polar antibiotic drugs, including ß-lactams, floroquinolones, and aminoglycosides, enter the periplasmic space through porin channels. Specificity of the outer membrane protein for the antibiotic drug is important.

Bacteria that produce either down regulation of porin expression, or modification of porin protein, can lead to antibiotic resistance for example reduced permeability of the outer membrane of Gram-negative bacteria can lead to resistance to third generation cephalosporins in *Enterobacter cloacae*, *Serratia marcescens* and in other members of *Enterobacteriaceae* (Livermore, 1995).

1-2-4-4- Efflux Pumps:

Several classes of pumps that remove one or more antibiotics from bacterial cell have been described in Gram-positive and/or Gram-negative bacteria. They may be quite selective or they may have broad substrate specificity. The majorities of these pumps are located in the cytoplasmic membrane and use proton motive force to drive drug efflux (Li *et al.,* 1994). An example of this mechanism of resistance is *Pseudomonas aeruginosa* (Aires *et al*., 2002).

1-2-5- Occurance of ß-Lactamase Resistance:

The ß-lactamases are the major reason of bacterial resistance to penicillins, cephalosporins and related antibiotics. The emergence of resistance to ß-lactam antibiotics began even before the first ß-lactam, penicillin was developed. The enzyme was established as the mechanism of resistance in penicillin-resistant strains of *Staphylococcus aureus* (Li, *et al.*, 1994).

Throughout the 1960s and 1970s, a relentless raise in reports of resistance to ß-lactam as consequence of selection of bacteria that produce ß-lactamase (Heritage *et al.*, 1999), while in early 1980s, a level of 30%-40% in both hospitals and community in the United Kingdom and in many other countries was elevated (Verbist and Piot, 1986).

Over the last 20 years, many new ß-lactam antibiotics have been developed that were specifically designed to be resistance to the hydrolytic action of ß-lactamase. However, bacteria in turn, developed a new ß-lactamases with broad spectrum activity (Bradford, 2001).

1-2-6- Mechanisms of Action of ß-Lactamase:

The most important mechanism of resistance is the production of one or more ß-lactamase enzymes that hydrolyses the ß-lactam bond characteristic of this family of antibiotics by two major mechanisms (Heritage *et al.*, 1999).

The first mechanism in which, ß-lactamases have a serine based mechanism of action. They are divided into three major classes (A, C, and D) on the basis of the amino acid sequences (Jacoby and Munoz-Price, 2005). **Figure (1-1)** illustrates the action of serine ß-lactamases, in which the enzyme first associates noncovalently with the antibiotic to yield the noncovalent Michaelis complex. The ß-lactam ring is then attacked by the free hydroxyl on the side chain of a serine residue at the active site of the enzyme, yielding a covalent acyl ester. Hydrolysis of the ester finally liberates active enzyme and the hydrolyzed inactive drug. This mechanism is followed by ß-lactamases of molecular classes A, C, and D (Waley, 1992).

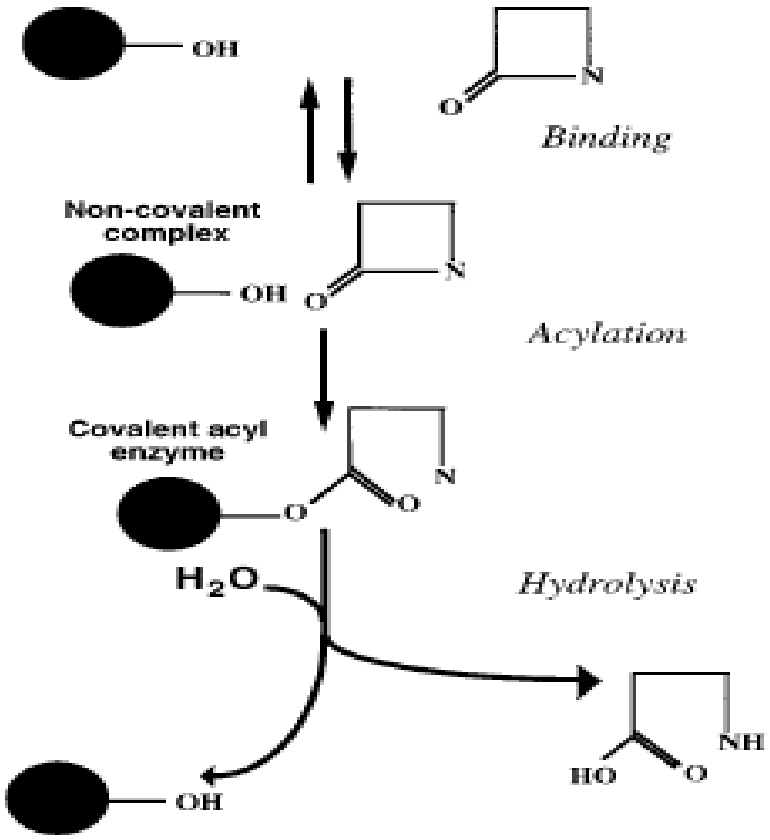

Figure (1-1): Action of Serine β-Lactamases

(From: **Waley,** 1992).

The second mechanism which includes a less commonly encountered group of ß-lactamases, which is the metallo ß-lactamases, or class B ß-lactamases. These enzymes use a divalent transition metal ion, mostly zinc, linked to histidine and/or cystine residue, to react with carbonyl group of the amide bond of penicillins, cephalosporins, and carbapenems, but not monobactams (Page, 2002).

ß-lactamases inactivate the ß-lactam antibiotics via breaking the amide bond in ß-lactam ring bringing about the destruction of antibiotic molecule, turning the antibiotic into an inactive compound. The compound resulting from break down of penicillins called penicilloic acid which has two acidic groups, whereas, there is one acidic group in the original compound. In contrast to the stable penicilloic acid, the compound resulted from break down of cephalosporins (cephalosporonic acid) is unstable which dissociate quickly into two inactivated fragments (Sykes and Matthew, 1976).

1-2-7- Classification of ß-Lactamases:

The ß-lactamases have been designated by the Nomenclature Committee of the International Union of Biochemistry as "enzymes hydrolyzing amides, midines and other C-N bonds… separated on the basis of the substrate: … cyclic amides" (Webb, 1984). These enzymes are the major cause of bacterial resistance to ß-lactam antibiotics and have been the subject of extensive microbiological, biochemical, and genetic investigation.

The ß-lactamases are produced by many Gram-positive and Gram-negative bacteria. They differ in substrate profile, molecular weight, isoelectric point (IP), genetic determination, and susceptibility to inhibition by several inhibitors. Such classification was first proposed by Jack and Richmond in 1970 and was expanded by Richmond and Sykes in 1973.

This system subdivided the enzymes into five classes. Class I enzymes are predominantly cephalosporinases; class II are penicillinases; class III enzymes show broad-spectrum activity and are sensitive to inhibition by cloxacillin but resistant to p-chloromercuribenzoate (pCMB); class IV are also broad-spectrum enzymes but resistant to inhibition by cloxacillin and sensitive to pCMB, finally; class V enzymes are penicillinases able to hydrolyse cloxacillin and resistant to pCMB inhibition (Wiedmann *et al.*, 1989).

Sykes and Matthew (1976) proposed two major groups, classes-A and B. Class A includes types of enzyme that chromosomally mediated and subclassified into (a) penicillinases, (b) cephalosporinases, and (c) broad-spectrum ß-lactamases. Class B enzymes are determined by R-plasmids and subclassification leads to (a) isoxazolyl-non-hydrolysing, (b) isoxazolyl-hydrolyzing, and (c) other ß-lactamases.

Ambler (1980) classified ß-lactamases from Gram-positive and Gram-negative bacteria on the basis of amino acid sequence of the

active site of the enzymes. Accordingly, four classes A, C, and D comprises distinct groups of serine enzymes, and class B contains only ß-lactamases which require bivalent metal actions such as Zn++. The classification system proposed by Bush in 1989 which was further updated in 1995, classified ß-lactamases by their substrate preference and susceptibility to inhibition by clavulanate (Bush *et al.,* 1995).

Phenotypic classification face the problem that point mutation can greatly alter substrate specificity and inhibitor susceptibility (Vedel *et al.,* 1992), changing the group to which an enzyme is assigned. On contrast, sequence-based classification of Ambler is more stable because it cannot be destroyed by mutations (Livermore, 1995).

1-2-8- Genetic Factors Controlling the Production of ß-Lactamases:

1-2-8-1- Chromosomally-Mediated ß-Lactamases:

In Gram-positive bacteria, no chromosomal ß-lactamases have been detected in staphylococci, streptococci, enterococci. Chromosomally mediated resistance to ß-lactam antibiotics in members of these genera is due to other mechanisms such as alteration of PBPs (Williamson, 1983). However, many *Bacillus* spp. and some closteridia produce inducible chromosomal ß-lactamase (Appelbaum *et al.,* 1994). In Gram-negative bacteria, the chromosomal ß-lactamases are almost ubiquitous in enterobacteria, except for

Salmonellae (Decre *et al.*, 1992). They vary considerably in amount, mode of action, production, and contribution to resistance (Haeggman *et al*., 2004).

Some species typically have molecular class A enzymes, but a greater number have class C types. Expression may be inducible, high level constitutive or low level constitutive of chromosomal ß-lactamases in *Enterobacteriaceae*, according to the species and the strain (Livermore, 1995). *E. coli* presents the simplest case, as they usually have only insignificant level of inducible molecular class C enzymes, often called AmpC types (Jacoby and Munoz-Price, 2005).

Klebsiella spp. has a class-A (TEM-1, TEM-2, SHV-1) chromosomal ß-lactamases, which differ greatly from class C types. Most *K. pneumoniae* isolates have chromosomally mediated SHV-1 (from sulfhydryl variable) ß-lactamases. All these ß-lactamases of *Klebsiellae* are constitutive and usually produced at low levels, which are sufficient to protect against ampicillin, amoxicillin, carbenicillin, and ticarcillin (Chaves *et al*., 2001).

1-2-8-2- Plasmid-Mediated ß-Lactamases:

In Gram-positive bacteria, plasmid-coded ß-lactamases are predominant in *S. aureus,* which one of the most isolated species not only in community but also in hospital-acquired infections. It was also reported that coagulase-negative staphylococci produce plasmid-coded ß-lactamases in nearly 100% of resistant strains (Jacoby, 1996).

In Gram-negative bacteria, over 200 different plasmid-mediated ß-lactamases have been reported (Bradford, 2001). The first plasmid mediated ß-lactamase, TEM-1, was described in 1965 in *E. coli* isolate from a patient in Athens, Greece, named Temoneira (hence the designation TEM) (Medeiros, 1984). Being plasmid and transposon mediated, has facilitated the spread of TEM-1 to other species of members of the family *Enterobacteriaceae*, *P. aeroginosa*, *Haemophilus influenza*, and *Neisseria gonorrhoeae* (Frere, 1995; Bush, 2001).

Another plasmid mediated ß-lactamase found in *K.pneumoniae* and *E. coli*, SHV-1. The SHV-1 ß-lactamase is chromosomally encoded in the majority of *K. pneumoniae* but is usually plasmid-mediated in *E. coli* (Bradford, 2001). In general, plasmid-mediated ß-lactamases are distinct from the chromosomal types, but a few overlaps exist such as SHV-1 ß-lactamase, which is common as plasmid type and a typical chromosomal origin (Livermore, 1991).

1-2-9- Extended-Spectrum ß-Lactamases (ESBLs):

The commonest plasmid-mediated ß-lactamases encountered in *Enterobacteriaceae* are TEM-1, TEM-2, SHV-1, and OXA-1 enzymes, which have a weak activity against first generation cephalosporins (West, 2000). Over the last 25 years, many new ß-lactam antibiotics have been developed that were specifically designed to be resistant to the hydrolytic action of ß-lactamases. One of these ß-

lactam classes was the oxyimino-cephalosporins, which became widely used for treatment of serious infections due to Gram-negative bacteria in the 1980, which include:

- Oxyimino-cephalosporins (include all the parenterally administered cephalosporins such as cefotaxime, ceftazidime, ceftriaxone.
- Oxyiminomonobactams (like aztreonam).
- Carbapenems (like imipenem).
- 7-α-methoxy B ß-lactams (Cephamycins) include cefoxitin and cefotetan (Jacoby, 1997).

These ß-lactams with the oxyimino side chain were not hydrolysed by TEM-1 or TEM-2 or SHV-1 ß-lactamases. However, resistance to these extended-spectrum ß-lactam antibiotics can be attributed to ß-lactamases emerged quickly after emergence of mutants in mid 1980s. These mutant enzymes conferred variable levels of resistance to cefotaxime, ceftazidime, and other broad spectrum-cephalosporins, and to monobactam such as aztreonam, but had no detectable activity against cephamycins and imipenem (Labia *et al*., 1988). Because of their increased spectrum of activity, those enzymes were called extended-spectrum ß-lactamases (ESBLs) (Gniadkowski, 2001). They were first recognized in a single strain of *K. pneumoniae* isolated in Germany (Knothe *et al*., 1983).

Besides *E. coli* and *K. pneumoniae*, TEM-type ESBLs have been reported in *Enterobacter aerogenes*, *Morganella morganii*, *Proteus mirabilis*, and *Salmonella* spp. (Perilli *et al*., 2000). TEM-type ESBLs were also found in non-*Enterobacteriaceae* Gram-negative bacteria. The majority of SHV-type ESBLs are found in strains of *K. pneumoniae* (Mangeney *et al*., 2000). However, several authors reported that these enzymes have also been found in *E. coli*, *Citrobacter diversus*, and *P. aeruginosa* (Naas *et al*., 1999).

Relatively most strains expressing ESBLs are susceptible to cephamycins (cefoxitin and cefotetan). However, Martinez-Martinez *et al.* (1996) reported that ESBLs-producing strains can become resistant to cephamycins due to the loss of an outer membrane porin protein. Porin loss as a cause of antimicrobial resistance has been shown in both laboratory mutants and clinical isolates (Jacoby *et al*., 2004).

A web site on the Internet has been created by Jacoby and Bush to catalog and update the amino acid substitutions by which individual enzymes are defined. In last modification of this site in February 2008, more than 200 different ESBLs have been described until now (Jacoby and Bush website).

1-2-10- ESBL Detection Methods:

ESBLs can be detected in the laboratories by two main methods; clinical microbiology method and molecular method. The two methods are shortly described below:

1-2-10-1- Clinical Microbiology Techniques:

Methods for detecting ESBL-producing bacteria have been evolved for more than 15 years, beginning with the description of the disk approximation test by Jarlier *et al*. (1988), which is also known as "double disk synergy "test, using a clavulanic acid-containing disk (usually amoxicillin-clavulanate) placed in proximity to disks containing extended-spectrum cephalosporins and aztreonam to demonstrate that the test strain contains ESBL whose activity is inhibited by clavulanic acid. ESBL production is inferred by enhancement of the zone of inhibition between the clavulanate-containing disk and one or more of the antibiotic-containing disks (Moland and Thomson, 1994).

This test may lack sensitivity because of the inability of clavulanate to inhibit all ESBLs, and the loss of clavulanate disk potency during storage (Moland and Thomson, 1994). Moreover, it has shown that the disk approximation test failed to detect some ESBL-producing strains (Coudron *et al*., 1997).

It is most practicable to screen with ceftazidime since virtually all ESBLs show obvious resistance to this compound (Bonafede and Rice, 1997). Testing with ceftazidime with and without clavulanate, as an inhibitor, enabling the detection of ESBL-production by zone comparison.

- The Vitek ESBL test which uses an automated growth monitoring system (Sanders *et al*., 1996), and E-test which uses a plastic drug-impregnated strips (Cormican *et al*., 1996), are two examples of such tests. The E-test was reported to be more sensitive than the double disk test and similar to the Vitek test (Essack, 2000).

- It was also suggested that determination of minimum inhibitory concentrations (MICs) of extended-spectrum cephalosporins with and without clavulanate, could be used for detection of ESBLs in clinical *Klebsiella* isolates. In this test, MICs for cefotaxime, ceftazidime, ceftriaxone, and aztreonam with and without clavulanic acid are determined by a two-fold microdilution technique. The test is considered positive if the MICs of the ß-lactams reduced ≥ 8-folds by clavulanate (Bedenic *et al.*, 2001).

The Clinical and Laboratory Standards Institute (CLSI, 2007) recommended an initial screening test for ß-lactamase-producing isolates in broth medium containing 1 µg/ ml of one of five expanded-spectrum ß-lactam antibiotics. A positive result was recorded as suspicious for the presence of ESBL. This screen is followed by

phenotypic confirmation test that consists of determining MICs of either ceftazidime or cefotaxime with and without of clavulanic acid. A decrease in the MICs of ≥ 8 two-fold dilutions in the presence of clavulanate is regarded as indication of ESBL presence (CLSI, 2007).

However, detection of organisms producing ESBLs can be difficult because the presence of ESBLs in a bacterial cell does not always produce a resistance phenotype when one is using the traditional MIC and disk diffusion interpretive criteria published by the NCCLS (Coudron *et al*., 1997). However the tests mentioned above were described presumptively identity the presence of ESBL, without identifying ESBL in clinical isolates specifically, hence molecular methods are described as quite satisfactory.

1-2-10-2- Molecular Methods:

Molecular methods use the DNA probes that specific for TEM and SHV enzymes (Arlet and Philippon, 1991). The first molecular method for the identification of β-lactamase was the oligotyping method developed by Ouellette *et al.* (1988), which was used to discriminate between TEM-1 and TEM-2 enzymes.

A number of different tests have been proposed later for the detection and identification of ESBLs, but nucleotide sequencing technique, remain the standard for determination of the specific β-lactamase gene present in a bacterial strain (Bradford, 2001; CLSI, 2007).

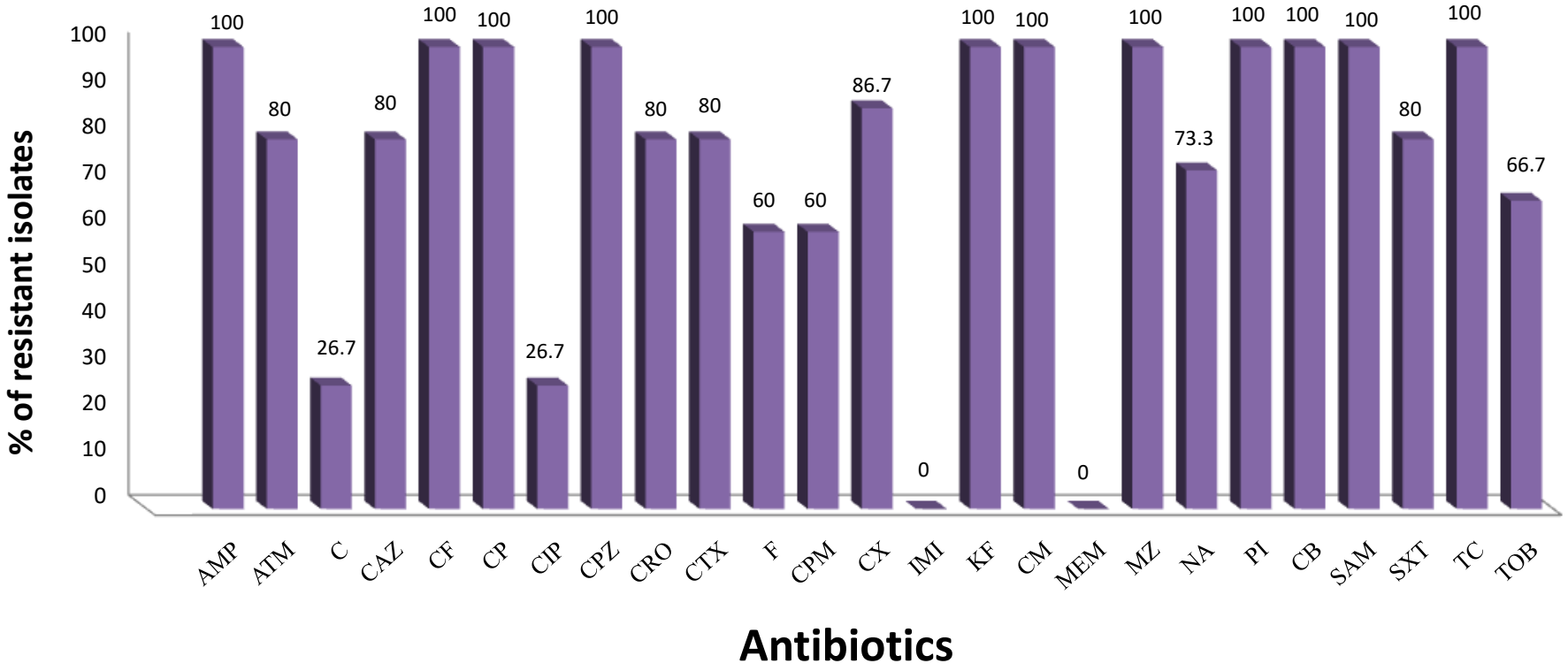

Figure (3-1): Antibiotic resistance pattern of β-lactamase-producing *Enterobacteriaceae* isolates

AMP: Ampicillin, **ATM:** Aztreonam, **C:** Chloramphenicol, **CAZ:** Ceftazidime, **CF:** Cefaclor, **CP:** Cefoperazone, **CIP:** Ciprofloxacin, **CPZ:** Cefprozil, **CRO:** Ceftriaxone, **CTX:** Cefotaxime, **F:** Nitrofurantoin, **CPM:** Cefepime, **CX:** Cefoxitine, **IMI:** Imipenem, **KF:** Cephalothin, **CM:** Cefamandole, **MEM:** Meropenem, **MZ:** Mezlocillin, **NA:** Nalidixic acid, **PI:** Pipracillin, **CB:** Carbencillin, **SAM:** Sulbactam + Ampicillin, **SXT:** Trimethoprim + Sulfamethaxazole, **TC:** Ticarcillin, **TOB:** Tobramycin.

2-1- Materials:

2-1-1- Instruments and Equipments:

The instruments and equipments used in the present study are listed in **Table (2-1)** below:

Table (2-1): Instruments and Equipments Used in the Study

Equipment	Company / Origin
Autoclave	Stermite, Japan.
Incubator	Memmert. Germany.
Distillator	GFL, Germany.
Sensitive Electronic Balance	A and D, Japan.
Hot air oven	Memmert.
Refrigerator	Concord, Italy.
Light Microscope	Olympus, Japan.
Millipore filter (0.22 µm)	Satorious membrane W., Germany.
pH meter	Weilheim, Germany.
Deep freezer	Ultima, Japan.
Shaker water bath	Memmert.
Micropipette	Oxford, USA.
Digital camera	Sony, Japan.
Spectrophotometer	Unico, Japan.
PCR system	Singapore.
Electrophoresis	Abnet, Taiwan.
UV- transilluminator	Taiwan.
Centrifuge	Memmert.
Bench centrifuge	Hettich, Germany.
Cold centrifuge	Hettich.

2-1-2- Chemical Materials:

Table (2-2): Chemical Materials Used in the Present Study

Chemical Materials	Company / Origin
KI	Fluka, Switzerland.
Iodine	BDH, England.
Agar-Agar	BBL, USA.
HCl	Sigma, USA.
Crystal violet	Sigma.
Tetramethyl p-phenyl diamine-dihydrochloride	Sigma.
Safranin	Sigma.
Sodium carbonate	BDH.
NaCl	BDH.
Glucose	BDH.
Sucrose	BDH.
Lactose	BDH.
D- Sorbitol	BDH.
D- Mannitol	BDH.
L- Arabinose	BDH.
NaOH	BDH.
Methyl red	BDH.
KOH	BDH.
α-naphthol	BDH.
Soluble starch	BDH.
EDTA	BDH.
KH_2PO_4	BDH.
Phenol red	Fluka, Switzerland.
Pepton	Michigan.
H_2SO_4	Fluka.
Ethanol	Fluka.
H_2O_2	Fluka.
Urea	Fluka.
$Na_2HPO_4.H_2O$	Fluka.

Table (2-2) (continued).

Chemical Materials	Company / Origin
Na_2HPO_4	Fluka.
$BaCl_2$. $2H_2O$	Fluka.
Agarose	BDH.
Chloroform	BDH.
Barium chloride ($BaCl_2$)	Fluka.
Bromophenol blue	Difco, USA.
Tris-(hydroxy methyl) methylamine (Tris-OH)	BDH.
Ethidium bromide	Sigma.
Isopropyl alcohol	Mast Diagnostic, USA.
Sodium dodecyl sulfate (SDS)	AppliChem, Germany.
Kovac's reagent	Himedia.

2-1-3- Culture Media:

Table (2-3): Ready-Made Culture Media Used in the Study

Culture Media	Company / Origin
Urea agar base	Biolife, Italy.
MR-VP broth	Biolife.
MacConkey agar	Himedia, India.
Nutrient agar	Himedia.
Nutrient broth	Himedia.
Brain-heart infusion broth	Himedia.
Mannitol salt agar	Mast diagnostic, U.K.
Mueller Hinton agar	Oxoid, U.K.
Simmons citrate agar	Oxoid.
S-S agar	Oxoid.
Kligler iron agar	Oxoid.
Eosin methylene blue (EMB)	Michigan, USA.

2-1-4- Antibiotics:

2-1-4-1- Antibiotic Powder:

Table (2-4): Antibiotic Powders Used in the Present Study

Antibiotic	Company / Origin
Penicillin G	Sarbach, France.
Ampicillin	SDI, Iraq.
Amoxicillin	Ajanta, India.
Amoxi-clav	Elsaad, Syria.
Cefotaxime	Eczacibasi, Turkey.
Ceftazidime	Julphar, UAE.
Imipenem	Switzerland.

2-1-4-2- Antibiotic Disks:

Table (2-5): Antibiotic Disks Used in the Present Study

Antibiotic Disks	Content	Assembly	Origin
Ampicillin	10 µg	AMP	Bioanalyse, Turkey
Amoxicillic-clavulanate	20 / 10 µg	AMC	
Piperacillin	100 µg	PI	
Ticarcillin	75 µg	TC	
Carbencillin	100 µg	CB	
Ampicillin-sulbactam	10 / 10 µg	SAM	
Mezlocillin	75 µg	MZ	
Aztreonam	10 µg	ATM	
Imipenem	10 µg	IMI	
Meropenem	10 µg	MEM	
Cephalothin	30 µg	KF	
Cefaclor	30 µg	CF	
Cefamandole	30 µg	CM	

Table (2-5) (continued).

Antibiotic Disks	Content	Assembly	Origin
Ceftriaxone	30 µg	CRO	Bioanalyse, Turkey
Cefprozil	10 µg	CPZ	
Cefoperazone	75 µg	CP	
Cefotaxime	30 µg	CTX	
Ceftazidime	30 µg	CAZ	
Cefoxitine	30 µg	CX	
Cefepime	30 µg	CPM	
Trimethprim-sulfamethaxazole	1.25 / 23.76 µg	SXT	
Ciprofloxacin	5 µg	CIP	
Chloramphenicol	30 µg	C	
Nitrofurantin	300 µg	F	
Tobramycin	10 µg	TOB	
Nalidixic acid	30 µg	NA	

2-1-5- Primers Used in PCR (Alpha DNA, Montreal):

Primer	Sequence
TEM (F)	5'-ATGAGTATTCAACATTTCCG-3'
TEM (R)	5'-CCAATGCTTAATCAGTGAGC-3'
SHV (F)	5'-CGCCGGTTATTCTTATTTGTCGC-3'
SHV (R)	5'-TCTTTCCGATGCCGCCGCCAGTA-3'

2-1-6- Master Mix Used in PCR (Promega, USA):

Go Tag DNA polymerase is supplied in 2x Green Tag reaction buffer pH 8.5, 400 µm dATP, 400 µm dGTP, 400 µm dCTP, 400 µm dTTP, and 3mM $MgCl_2$.

2-2- Methods:

2-2-1- Preparation of Culture Media:

2-2-1-1- Ready-Made Culture Media:

Culture media listed in **Table (2-3)** were prepared according to the manufacture instructions, autoclaved at 121 °C for 15-20 min, and used in appropriate tests.

2-2-1-2- Semi-Synthetic Culture Media:

The following culture media were prepared according to Collee *et al*. (1996), Finegold and Baron (1998) and MacFaddin (2000):

● Blood Agar Medium:

It was prepared by adding 5 % human blood to previously sterilized blood agar base after cooling the medium to 45 °C and then poured into sterile petri dishes.

● Urea Agar Medium:

It was prepared by adding 20 % of sterile urea solution (sterilized by filtration) to previously sterilized urea agar base. The contents were dispensed into sterile test tubes and prepared in test tubes as slants.

● Motility Medium:

It was prepared by dissolving 0.5 gm of agar-agar in 100 ml of brain-heart infusion broth, then the contents was dispensed into test tube and autoclaved.

● **Carbohydrate Fermentation Medium:** This medium was prepared as follow:

Medium Base:

It was prepared by dissolving 10 gm of Bacto-pepton, 1 gm of bacto-beef extract, 5 gm of sodium chloride, and 0.018 gm of phenol red in 1000 ml of D.W. After pH was adjusted to 7.4, contents were distributed into test tubes and a Durham tube was inserted in an inverted position to the bottom of the tube. The tubes were then autoclaved.

Sugar Solutions:

1 % of each of the following sugars was used: glucose, lactose, sucrose, L- arabinose, D- mannitol, and D- sorbitol. All sugars solutions were sterilized using chloroform vapor. 0.1 ml of each sterile sugar solutions was added to each tube containing medium base as mentioned above.

2-2-2- Preparation of Buffers and Solutions:

The following solutions and buffers were used in the present study. Those which require sterilization were autoclaved at 121 °C for 15- 20 min at 15 psi. Filtration using 0.22 µm Millipore filters was used for heat-sensitive solutions such as antibiotics and urea solutions. The pH of the solutions was adjusted using 0.1 N HCl.

2-2-2-1- Normal Saline Solution:

This solution was prepared by dissolving 0.85 gm of NaCl in 90 ml

Distilled water (D.W) and further completed to 100 ml with D.W.

2-2-2-2- McFarland Tube Standard (0.5):

A barium sulfate turbidity standard solution equivalent to a 0.5 McFarland standard was prepared as described by NCCLS (2003a), as follows:

- A 0.5-ml aliquot of 0.048 M $BaCl_2$ (1.175 % w/v $BaCl_2$. $2H_2O$) was added to 99.5 ml of 0.18 M H_2SO_4 (1 % v/v) with constant stirring to maintain a suspension.
- Correct density of the turbidity standard was verified by using reading the absorbance at 625 nm. The absorbance should be 0.08 to 0.1 for the McFarland standard.
- Barium sulfate suspension was distributed in 4 ml aliquots into screw cap tubes, which were tightly sealed and stored in the dark at room temperature.
- Barium sulfate turbidity standard was vigorously agitated on a mechanical vortex mixer before each use and inspected for uniformly turbid appearance.
- Barium sulfate standard should be replaced or their densities verified monthly.

2-2-2-3 Phosphate Buffer Solution (WHO, 1978):

This buffer consists of two solutions:

Solution (A): 0.907 gm of KH_2PO_4 was dissolved in 90 ml of D.W. then, completed to 100 ml with D.W.

Solution (B): 0.946 gm of Na_2PO_4 and 1.19 gm of Na_2PO_4. $2H_2O$, were dissolved in 90 ml of D.W, the volume was completed to 100 ml. Then, 87.6 ml of solution (A) was added to 12.3 ml of solution (B) and the pH was adjusted to 6.0. The buffer was used for detection of β-lactamase production.

2-2-2-4- β-Lactam Antibiotic Solutions:

β-lactam solutions were prepared as stock solutions with concentration of 10 mg/ml by dissolving 1gm of the antibiotic in a small volume of sterile phosphate buffer solution (pH 6.0), and further diluted with sterile D.W to volume of 100 ml, then stored at 4 °C. The following β-lactam solutions were prepared in appropriate concentrations: ampicillin, amoxicillin, ceftazidime, cefotaxime, and imipenem.

All β-lactams were solubilized and diluted as described above, except for ceftazidime which was solubilized in sodium carbonate and further diluted with sterile D.W. Anhydrous sodium carbonate was used at a weight of exactly 10 % of the ceftazidime to be used as recommended by CLSI (2007).

2-2-2-5- β-lactamase Detection Solutions (WHO, 1978):

- **Penicillin G Solution:** This solution was prepared by dissolving 0.5695 gm of penicillin G in phosphate buffer solution (**2-2-4-3**), and the resulting solution was stored at - 20 after sterilization by filtration and dispensed in small vials.

● **Iodine Solution:** 2.03 gm of iodine and 5.32 gm of KI were dissolved in 90 ml of D.W, and the volume was completed with D.W. to 100 ml then, stored in a dark bottle at 4 °C.

● **Starch Solution:** One gram of soluble starch was dissolved in 100 ml of D.W and boiled in a water bath for 10 min. The solution was stored in dark bottle at 4 °C.

2-2-2-6- Solutions Used in DNA Extraction:

The following solutions were prepared as described by Pospiech and Neumann (1995) with some modification according to the experimental conditions of this study:

2-2-2-6-1- Tris EDTA Buffer (TE buffer):

This buffer was prepared by dissolving 0.05 M of Tis-OH and 0.001 M of EDTA in 800 ml D.W, the pH was adjusted to 8.0 and completed to one litter by D.W., then autoclaved at 121 ºC for 15 minutes, and stored at 4 ºC until use.

2-2-2-6-2- SDS Solution (25 %):

SDS (25 mg) was dissolved in 100 ml of D.W, then sterilized in autoclave, and stored at 4 ºC.

2-2-2-6-3- NaCl Solution (5 M):

NaCl (14.625 gm) was dissolved in 50 ml D.W, sterilized in autoclave, and stored at 4 ºC.

2-2-2-6-4- Phenol: Chloroform: Isoamyl Alcohol (25:24:1):

The solvent was composed of 25 ml phenol, 24 ml chloroform, and 1 ml isoamyl alcohol.

2-2-2-7- Solutions Used in Gel Electrophoresis:

Solutions were prepared as described by Sambrook and Rusell (2001).

2-2-2-7-1- Tris-Borate-EDTA Buffer (TBE):

Tris-OH	0.08 M
Boric acid	0.08 M
EDTA	0.02 M

The pH was adjusted to 8.0, autoclaved, and stored at 4 ºC.

2-2-2-7-2- Ethidium Bromide Solution:

Stock solution (5 mg / ml) was prepared by dissolving 0.05 gm of ethidium bromide in 10 ml of D.W and stored in a dark reagent bottle.

2-2-2-7-3- DNA Loading Buffer:

Bromophenol blue	25 mg
Sucrose	4 mg
D.W	10 ml
Xylene xyanol	25 mg

2-2-3- Preparation of Reagents:

The following reagents were prepared as described in MacFaddin (2000):

2-2-3-1- Oxidase Reagent:

It was prepared freshly in a dark bottle by dissolving 0.1 gm of Tetramethyl-p- phenylene-diamine dihydrochloride in 10 ml D.W.

2-2-3-2- Catalase Reagent:

Hydrogen peroxide (3 %) was prepared from the stock solution in a dark bottle and used for detection of ability of isolates to produce catalase enzyme.

2-2-3-3- Methyl Red Reagent:

It was prepared by dissolving 0.1 gm of methyl red in 300 ml of 95 % ethyl alcohol, and then completed to 500 ml with D.W.

2-2-3-4- Voges-Proskauer Reagent:

This reagent consisted of two solutions:

Solution A: α-Naphthol: is prepared by dissolving 5 gm of α-naphthol in 100 ml of absolute alcohol.

Solution B: 40 % KOH: is prepared by dissolving 40 gm of KOH in 100 ml D.W.

2-2-4- Biochemical Tests:

2-2-4-1- Catalase Production Test:

Using sterile needle, 24 hours-old bacterial colony was placed on a clean glass slide and a drop of 3 % H_2O_2 solution was added to it.

Release of oxygen bubbles indicates a positive result (MacFaddin, 2000).

2-2-4-2- Oxidase Production Test:

A strip of filter paper (Whatman No. 1) was soaked with a little freshly made 1 % solution of tetramethyl p-phenylene-diamine dihydrochloride, and then the colony to be tested was picked up with a sterile wooden stick and smeared over the filter paper. A positive result was indicated by an intense deep-purple color which appeared within 5-10 seconds (MacFaddin, 2000).

2-2-4-3- Sugar Fermentation Test:

Tubes of sugar fermentation medium were inoculated and incubated at 37 °C for 1-5 days. The color of the indicator is changing to yellow with or without gas production indicates a positive result with this test (MacFaddin, 2000).

2-2-4-4- Indole Production Test:

Peptone water was inoculated with a young culture and incubated at 37 °C for 24-48 hrs. Few drops of Kovac's reagent were added to each tube. Formation of pink ring is indicating a positive result (MacFaddin, 2000).

2-2-4-5- Methyl Red Test:

Methyl red - Voges proskauer broth was inoculated with a young agar culture and incubated at 37 °C for 24 hrs. Five drops of methyl red

solution were added, mixed, and the result was read immediately. Changing the color to red is indicating a positive result (complete hydrolysis of sugar and production of acid) (MacFaddin, 2000).

2-2-4-6- Voges-Proskauer Test:

Methyl red - Voges Proskauer broth was inoculated with a young agar culture and incubated at 37 °C for 48 hrs. 0.2 ml of 40 % KOH solution and 0.6 ml of 5 % solution of α-naphthol were added to each tube. A positive result was indicated by the development of a pink color in 20 minutes (MacFaddin, 2000).

2-2-4-7- Simmons Citrate Test:

Simmons citrate slant was inoculated with a young bacterial culture and incubated at 37 °C for 48-72 hrs. Changing the color of the medium from green to blue indicates a positive result (MacFaddin, 2000).

2-2-4-8- Kligler Iron Agar Test:

A heavy inoculum was streaked over the surface of the slope and stabbed into the butt; incubated at 37 °C for 24 hrs. Results were unformatted according to MacFaddin (2000) as follows:

Slant / Butt	Color
Alkaline / Acid	Red / yellow
Alkaline / Alkaline	Red / Red
Acid / Acid	Yellow / Yellow

H_2S production	Black precipitate
Gas production	Breaking or hole in the medium

2-2-4-9- Urease (Christensen's) Production Test:

Urea agar slant was streaked with bacterial culture and incubated at 37 °C. Result was read after 6 hrs, 24 hrs, and every day for 6 days. Changing the color of medium to purple-pink indicates a positive result (MacFaddin, 2000).

2-2-4-10- Motility Test:

Hanging-drop method was used for observation of bacterial motility. Motility medium was used for detection of bacterial motility. Tubes were inoculated with bacterial culture by stabbing and incubated at 37 °C for 24-48 hrs. Formation of cloudy growth out of the line of the stab indicates a positive result.

2-2-5-Preservation and Maintenance of Bacterial Isolates:

The bacterial isolates were preserved on nutrient agar slants at 4°C. The isolates were maintained monthly during the study by subculturing on new culture media. For long preservation, Nutrient broth supplemented with 15 % glycerol was used and the isolates were maintained, frozen for 6-8 months (-20 °C) (Collee *et al*., 1996).

2-2-6- Patients:

This study includes a total of 313 patients (children) with ages (1 day to 14 years) who admitted to Pediatric and Maternity Hospital in

Hilla city during the period from October 2008 to February 2009. Information regarding the patients includes the patient's name, age, and sex was recorded. The febrile children in the present study were all with history of previous use of antibiotics (defined as one applied at least 24 hours and up to 30 days prior to the installation of bacteremia).

2-2-7- Sample Collection:

A total of 313 blood samples were collected from children (2-5 ml of blood from each child), and withdrawn by disposable syringe under aseptic technique. The blood samples were immediately inoculated into sterilized blood culture bottle containing brian heart infusion broth (BHI broth) (each 1 ml of blood was added to 9 ml of BHI broth). After that, the blood culture bottle was incubated at 37 ºC for 24-72 hours. If any sign of bacterial growth is indicated **(1-2-2),** the bacteria is further cultured on MacConkey and blood agar, Gram-stain procedure. Other morphological and biochemical tests should be done to confirm the diagnosis of bacterial isolates.

2-2-8- Identification of Bacterial Isolates:

Bacterial isolates belonging to *Enterobacteriaceae* family were identified to the level of species or subspecies using the traditional morphological and biochemical diagnostic tests (listed in **2-2-4**), according to the methods of MacFaddin (2000) and Forbes *et al.* (2007).

2-2-9- Screening Test for β-Lactam Resistance (CLSI, 2007):

- Muller- Hinton agar was prepared, autoclaved, and then cooled to 45 °C.

- Ampicillin and amoxicillin were separately added, from stock solution, to the cooled Mueller- Hinton agar at final concentrations of 100 and 50 µg/ml, respectively. The media poured into sterilized petridishes, then stored at 4 °C.

- Preliminary screening of isolates belonging to *Enterobacteriaceae* being resistance to β-lactam antibiotics was carried out using pick and patch method on the above plates.

2-2-10- Detection of β-Lactamase Production (Rapid Iodometric Method):

This test was performed for all bacterial isolates that were resistant to β-lactam antibiotics as follows:

Several colonies of 18 hours-old bacterial cultures on MacConkey agar were transferred to Eppendrof tubes containing 100 µg of penicillin G solution, and the tubes were incubated at 37 °C for 30 minutes. Then, 50 µg of starch solution was added and mixed well with the content of the tube. A portion of 20 µg of iodine solution was added to each tube which causes the appearance of dark blue color. Rapid

change of this color to white (within few seconds to 5 minutes) indicates a positive result (Collee *et al*., 1996).

2-2-11- Antibiotic Disk Susceptibility Test:

Resistance of the bacterial isolates to antibiotics was determined by using disk diffusion method, and all isolates that were positive to β-lactamase production, were tested for their antibiotic susceptibility.

The antibiotics used in this experiment were listed in **Table (2-5)**; tests were performed on plates of Muller Hinton agar as follows:

● A portion of 0.1 ml of bacterial suspension equal to a 0.5 McFarland suspension was spreaded on plates, then dried in an incubator at 35 °C for 15 minutes.

● Antibiotic disks were placed on the agar with a sterile forceps (6 disks for each plate). The agar plates were incubated at 35 °C for 16-20 hours.

● Results were recorded by measuring the inhibition zone (in millimeters) and interpreted according to Clinical and Laboratory Standards Institute documents (CLSI, 2007).

2-2-12- Detection of ESBL Production:

Three methods were performed for detection of ESBLs in isolates that cause bacteremia. All bacterial isolates that were positive to β-lactamase production were tested for their ability to produce ESBL enzymes. These tests included:

2-2-12-1- Disk Approximation Method:

This method was carried out as modified by Coudron *et al*. (1997) as follows:

Muller-Hinton agar plate was inoculated with an overnight blood agar culture of the test bacterial isolate as recommended for standard disk diffusion susceptibilities test. Disks containing 30 µg cefotaxime, ceftazidime, ceftriaxone, and aztreonam were placed 15 mm (edge to edge) from a disk of augmentin (20 µg amoxicillin plus 10 µg clavulanate) and then incubated for 16-20 hrs at 35 °C. Any enhancement of the zone of inhibition between a β-lactam disks and augmentin disk gave an indication that the test isolate contains ESBL whose activity is inhibited by clavulanic acid (Rolinson, 1994).

2-2-12-2- Inhibitor-Potentiated Disk-Diffusion Test (IPDDT):

This test is based on a modification of the standard disk- diffusion method as described by the Clinical and Laboratory Standards Institute documents (CLSI, 2007), as follows:

Muller- Hinton agar supplemented with 4 mg/L of clavulanate was prepared the day before testing, with the addition of clavulanate after cooling to 50 °C in water bath. Antibiotic disks containing cefotaxime (30 µg), ceftazidime (30 µg), and ceftriaxone (30 µg), were placed on clavulanate-containing and clavulanate-free Muller- Hinton agar plates.

After 16-20 hrs of incubation at 37 °C, the diameters of the inhibition zones around the antibiotics were measured. Augmentation zone widths were obtained by subtracting inhibition zone diameters produced by the β-lactam disks on clavulanate-containing medium. An augmentation zone width of ≥ 10 mm was considered positive for ESBL production.

2-2-12-3- Detection of ESBLs with Clavulanate:

This method was recommended by CLSI (2007) as follows:

- Minimum inhibitory concentrations of cefotaxime, and ceftazidime with and without clavulanic acid (4 µg / ml) were determined by using agar dilution method.

- The test was considered positive (ESBL-producing isolate), if the MIC against the tested β-lactam antibiotic was reduced ≥ 8-fold by clavulanate.

2-2-13- Determination of MICs of ESBL-Producing Isolates (NCCLS, 2003b):

- The two- fold agar dilution susceptibility method was used for determination of MICs of a number of β-lactam antibiotics.
- The ranges of appropriate dilutions of antibiotic for MIC determination (µg/ml) were used as described by Miles and Amyes (1996), as follows:

Suggested Ranges for MIC

B-lactam Antibiotic	**Determination (µg / ml)**
Ampicillin	0.25 - 128
Ceftazidime	0.004 - 128
Cefotaxime	0.004 - 128
Imipenem	0.06 - 4

- Appropriate dilutions of β-lactam antibiotic solutions were prepared according to the report of the international collaborative study by Ericsson and Sherris (1971), in which one part of the antimicrobial solution was added to nine parts of liquid Muller-Hinton agar.
- The prepared dilutions of the β-lactam solutions were added to the molten Muller-Hinton agar media that have been allowed to equilibrate in a water bath to 45-50 ºC.
- The agar and antimicrobial solutions were mixed thoroughly and the mixture was poured into a sterile petri dish. The agar was allowed to solidify at room temperature.
- A standardized inoculum for agar dilution method was prepared by growing bacteria to the turbidity of 0.5 McFarland standard (**2-2-4-2**). The 0.5 McFarland suspension was diluted 1:10 in sterile normal saline.
- The agar plates were marked for orientation of the inoculum spots. 1-µL aliquot of each inoculum was applied to the agar surface with standardized loop.
- Antibiotic free media were used as negative controls and inoculated;

the inoculated plates were allowed to stand at room temperature (for no more than 30 min) until the moisture in the inoculum spots is absorbed by the agar. The plates were inverted and incubated at 35 °C for 16 to 20 hours.

- To determine agar dilution break points, the plates were placed on a dark surface, and "the MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibits growth "(disregarding a single colony or a faint haze caused by the inoculums) or that concentration (in µg/ml) at which no more than two colonies were detected. The MIC values were compared with the break points recommended by CLSI (2007).

2-2-14- Extraction of DNA:

The method that used for extraction of DNA was:

2-2-14-1- Salting Out Method:

This method was prepared according to Pospiech and Neumann (1995) with some modification according to experimental conditions of this study, as follows:

Bacterial cells of 50 ml culture were precipitated by centrifugation (10000 rpm for 10 minutes). Rewashed (2-3 times) in TE buffer. Then, the pellet was resuspended in 5 ml TE buffer. A volume of 600 µL of freshly made 25 % SDS was added, mixed by inversion to the cell suspension, and incubated for 5 minutes at 55 °C. Then 2 ml of 5 M

NaCl solution was added to the lysate, mixed thoroughly by inversion, and let to be cooled to 37 °C.

5 ml of phenol: chloroform: isoamylalcohol (25: 24: 1 v/v) was added to the lysate and mixed by inversion for 30 minutes at 25 °C. It was spun by centrifuge 4500 rpm at -20 °C for 10 minutes. Then the aqueous phase was transferred to a fresh tube, which contains nucleic acid. Isopropanol (o.6 volume) was added to the extract and mixed by inversion, after 3 minutes, DNA spooled on to a sealed pasture pipette. DNA rinsed in 5 ml of 70 % ethanol, air dried, and dissolved in 1-2 ml TE buffer at 55 °C, then DNA extract was kept in -20 °C until use.

2-2-15- Detection of *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ Genes by PCR:

DNA (extracted from bacterial cells) (**2-2-14-1)** was used as a template in specific PCR for detection of *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ genes. DNA was purified from bacterial cells using the salting out reported by Pospiech and Neumann (1995). A pair of TEM primers was used for the amplification of a fragment that covers the entire *bla*$_{\text{TEM}}$ genes and a pair of SHV primers was also used for amplification of a fragment that covers the entire *bla*$_{\text{SHV}}$ genes (**2-1-5**).

A single reaction mixture contained 2-5 µL DNA extract, 25 µL Master Mix (reaction buffer, 1 µL; nucleotide mixture dNTP, 10 mM; and Tag DNA polymerase, 0.5 µL), 2 µL of 10 pmol/ µL of up stream primers specific, and 2 µL of 10 pmol/ µL of down stream primers specific in a total volume of 50 µL. The amplification of *bla*$_{\text{TEM}}$ was

run under the following conditions: 5 minutes at 94 °C followed by 30 cycles of 1 minute at 94 °C, 1 minute at 55 °C, and 1 minute at 72 °C, and finally, 10 minutes at 72 °C. For *bla*$_{SHV}$ amplification, the reactions were occurs under the following conditions: 5 minutes at 94 °C, followed by 30 cycles of 30 sec. at 94 °C, 30 sec. at 68 °C, 50 sec. at 72 °C, and finally, 10 minutes at 72 °C. The resulting PCR products were run in 1.5 % agarose gels.

2-2-16- Agarose Gel Electrophoresis:

Agarose gel was prepared by dissolving 1.5 gm of agarose powder in 100 ml of TBE buffer (pH 8.0) in boiling water bath, allowed to cool to 50 °C, and ethidium bromide at concentration of 0.5 gm/ml was added. Then a tape was placed across the end of the gel tray. The comb was fixed at one end of the tray for making wells used for loading DNA samples. The agarose gel was poured gently in to the tray, and allowed to solidify at room temperature for 30 minutes.

Then the comb was removed gently from the tray and the tape was also removed from the ends of the tray. The latter was fixed in electrophoresis chamber which was filled with TBE buffer has covered the surface of the gel. 25 µL of each DNA sample was transferred to Eppendrof tube. 5 µL of loading buffer was added to the tube and the mixture was loaded into the wells in agarose gel. The electric current was used at 70 volt for 2 hrs. UV transilluminator was used at 320 or

336 nm for the observation of DNA bands, and the gel was photographed using digital camera.

3-1- Isolation and Identification of Bacterial Isolates:

The present study included the collection of 313 blood samples, collected from febrile children. The febrile children in the present study were all with history of previous use of antibiotics.

Morphological and biochemical characterization indicated that only 43 samples (13.7 %), out of 313 blood samples, gave positive growth for bacteria; each sample of these 43 samples gave single bacterial isolate. Types and percentage of bacterial isolates recovered from blood samples are shown in **Table (3-1).**

Table (3-1): Types of Bacterial Isolates Recovered from Blood Samples Collected from Febrile Children

Bacterial Isolates	No. of Isolates	Percentage (%)
Enterobacteriaceae	**34**	**10.9**
Staphylococcus aureus	**6**	**1.9**
Pseudomonas aeruginosa	**3**	**0.9**
No growth (negative)	**270**	**86.3**
Total	**313**	**100**

The results obtained in this study revealed that Gram-negative bacilli were the most dominant in pediatric bloodstream infections, responsible for approximately 86% of the all positive cases (out of 43 bacterial isolates obtained, 37 isolates diagnosed as Gram-negative bacilli). This finding was similar to that reported by Chang *et al.*

(2003). Of these 313, 34 isolates (10.9 %) were identified as members of *Enterobacteriaceae* as shown in **Table (3-2).** Various members of *Enterobacteriaceae* have been reported to be the major pathogens in neonates, infants, and children (Réjiba and Kechrid. 2007).

The results showed that *Klebsiella* spp. (50%) and *E. coli* (32.4%) were the most common isolated organisms among *Enterobacteriaceae*. This may be attributed to the presence of many mechanisms of antibiotics resistance by *E. coli* and *Klebsiella* spp., which enable them to reach blood stream. This finding was in accordance with the results reported by Al Zamil, (2008).

Table (3-2): The Frequency and the Percentage of the Members of Bacterial Isolates Belong to the Family *Enterobacteriaceae*

Bacterial Isolates	No. of Isolates	Percentage (%)
***Klebsiella* spp.**	**17**	
K. peumoniae* subsp, *pneumonia	**(16)**	**(47.1)**
K. oxytoca	**(1)**	**(2.9)**
Escherichia coli	**11**	**(32.4)**
***Enterobacter* spp.**	**4**	
E. aerogenes	**(3)**	**(8.8)**
E. cloacae	**(1)**	**(2.9)**
P. mirabilis	**2**	**(5.9)**
Total	**34**	**100**

A study of bacteremia in febrile children by Lai *et al.* (2003) in Taiwan performed on febrile children admitted to a medical center, who showed that among 190 episodes of febrile children, 17 (8.9%) bacterial isolates (isolated from blood) belonging to family *Enterobacteriaceae* were detected. Of these, *Klebsiella peumoniae* and *K. oxytoca* (35.3%), *E. cloacae* (29.4%), *E. coli* (23.5%), and *Salmonella* spp. (11.8%).

In India, a study was performed by Jain *et al*. (2003) that involved 728 blood samples of neonates, who found that of these 728, 221 (30.4 %) bacterial isolates can be identified as *Enterobacteriaceae*. Of these, *Klebsiella* spp. (39.3%), *Enterobacter* spp. (36%), *E. coli* (22%), and *Citrobacter* spp. (2.7%).

3-2-The Distribution of Patients with Bacteremia According to Sex and Age:

In the present study, the prevalence of bcteremia by bacterial isolates belonging to family *Enterobacteriaceae* among female patients was 18/34 (52.9%), while among the male patients was 16/34 (47.1%). The results indicated that the susceptibility of both males and females to blood stream infection was nearly similar, since there was no significant difference (P value > 0.05). This result may be in accordance with that reported by Omoregie *et al*. (2007) who states that the prevalence of bacteremia in female (46.5%) was not significantly different from that in male (53.5%). Moreover, the

present study was agreed with another study in Nigeria (Ukaga *et al.*, 2006).

This finding was disagreed with that of Mugalo *et al.*, (2006), they reported significantly a higher prevalence of bacteremia in the females than males. The reason for this difference is unclear, but may be due to geographical locations.

It was found that, neonates (12 isolates) and infants (17 isolates) were at increased risk for blood stream infection by members of family *Enterobacteriaceae* in comparison to other pediatric age groups **Table (3-3)**.

Table (3-3): The Distribution of Pediatrics According to their Age Groups and the Distribution of *Enterobacteriaceae* Isolates among these Pediatrics

Age Groups	Age Ranges	No. of Pediatrics	No. of Isolates
Neonate	**1 day - 28 days**	**12**	**12**
Infant	**1 month - 1 year**	**17**	**17**
Toddler	**1 year - 4 years**	**5**	**5**
Preschool	**4 years – 6 years**	**0**	**0**
School	**6 years - 12 years**	**0**	**0**
Adolescent	**12 years – 14 years**	**0**	**0**
Total		**34**	**34**

This may be attributed to the immaturity of their immune system. Furthermore, children in this age may be febrile for 1 of 4 major reasons: fever of unknown reason, occult bacteremia, serious bacterial infections, and sepsis. Serious bacterial infection includes bacteremia, sepsis, infections of the soft tissues or joints, meningitis, bacterial enteritis, bacterial pneumonia, and urinary tract infections (Al Zamil, 2008). In a similar study, it was found that children below one year were more susceptible for bacteremia than other pediatric age groups (Kellogg *et al*., 2000).

3-3- Primary Screening Test for β-Lactam Resistant-Isolates:

In this study, an attempt was performed to evaluate the frequency of β-lactam resistance in all bacterial isolates, belonging to the family of *Enterobacteriaceae*, obtained from blood of febrile children. All the bacterial isolates (34) were grown on Muller- Hinton agar supplemented with ampicillin and amoxicillin antibiotics (each antibiotic was added separately).

The results obtained from this study revealed that all bacterial isolates (34) of the family *Enterobacteriaceae* were resistant to both ampicillin and amoxicillin. All these isolates were able to grow normally in the final concentrations of 50 µg/ml for amoxicillin and 100 µg/ml for ampicillin.

These two β-lactam antibiotics were selected because they are the most widely used antibiotics in the treatment of different bacterial

infections in comparison to other β-lactam antibiotics. Apart from their therapeutic usage, these antibiotics can provide a comprehensive primary screening for β-lactam resistant isolates because the isolate that is resistant to carbenicillin and cephalosporin is already resistant to ampicillin and amoxicillin (Bush *et al.*, 1995).

The development of antibiotic resistance is often associated with the overuse, and misuse of the antibiotics prescribed. Iraq is one of the developing countries, in which all types of antibiotics are sold over the counter, an attitude that encourage self-medication. Ampicillin, amoxicillin and other penicillin derivatives frequently prescribed for pediatric patients usually predispose them to colonization with *K. pneumoniae*, which is naturally resistant to these antibiotics (Stock and Wiedemann, 2001).

The resultant high percentage of resistance (100 %) to these two antibiotics was not only attributed to the production of β-lactamase enzyme, but it could be also due to other mechanisms of resistance such as decreased the affinity of the target (PBPs) or decreased the permeability of the drug into the bacterial cell (Jacoby and Munoz-Price**,** 2005). Similarly, in a local study in Hilla, Al-Charakh (2005) was found that 73.8% of *Klebsiella* isolates that obtained from clinical samples were resistant to both antibiotics. In another local study in Najaf, Hadi (2008), her results showed that 84.5% of both *E. coli* and *Klebsiella* that recovered from urine samples were resistant to these

two antibiotics. Moreover, Aiyegoro *et al.* (2007) reported that 66.7% of *K. pneumoniae* isolates were resistant to ampicillin and amoxicillin.

It was found that some bacteria have two types of mechanisms of resistance, enzymatic and that related to permeability, which together provide a strong defence mechanism for these bacteria, making their therapy and eradication were difficult to achieve (Sanders and Sanders, 1992; Piddock *et al.*, 1997).

3-4- The Production of ß-Lactamase:

Rapid iodometric method was used for the detection of ß-lactamase production in the bacterial isolates (of the family *Enterobacteriaceae*) that resist ß-lactam antibiotics as mentioned in the screening test. The principle of this method depends on the detection of penicilloic or cephalospoic acid that resulted from breakdown of amide bond in the β-lactam ring of each of penicillins or cephalosporins (Sykes and Matthew, 1976; Livermore, 1995).

The ß-lactamase-producing bacterial isolates of the family *Enterobacteriaceae* that showed resistance to ß-lactam antibiotics (as mentioned previously) will be illustrated in **Table (3-4).** It was found that, out of the 34 bacterial isolates that showed resistance to ß-lactam antibiotics, only 15 isolates (44%) gave positive result for ß-lactamase production by rapid iodometric method.

Table (3-4) revealed that *Klebsialla* species constitutes the largest percentage of ß-lactamase production (26.4%) as compared with *E.*

coli which constitutes the second percentage (14.7%), then followed by (2.9%) for *Enterobacter cloacae*, finally *Proteus mirabilis* which was the lowest percentage obtained among others (0%).

Table (3-4) ß-Lactamase-Producing and Non-Producing Bacterial Isolates Among Members of *Enterobacteriaceae*.

Types of Bacterial Isolates	No. of ß-Lactamase-Producers (%)	No. of ß-Lactamase-Non Producers (%)
K. peumoniae subsp. Peumoniae	**8 (23.5)**	**8 (23.5)**
K. oxytoca	**1 (2.9)**	**0 (0)**
E. coli	**5 (14.7)**	**6 (17.6)**
***Enterobacter* spp.**	**1 (2.9)**	**3 (8.8)**
Proteus mirabilis	**0 (0)**	**2 (5.9)**
Total	**15 (44)**	**19 (56)**

The prevalence of ß-lactamase enzymes, primarily among *Klebsiella* spp. and *E. coli* in the present study, may be attributed to the fact that these two bacteria were naturally resistant to ampicillin and amoxicillin by the production of plasmid-mediated and chromosomally-encoded ß-lactamase enzymes (Sanders and Sanders, 1992).

Additionally, it was also found that among the 15 isolates obtained as ß-lactamase-producer, 11 isolates (73.3%) gave rapid

positive results in rapid iodometric method within seconds (not more than one minute), while the remaining isolates gave positive results within minutes (from 1-5 minutes). The interpretation of these results may be attributed to the concentration of the ß-lactamase enzyme in the periplasmic space (Livermore, 1995). Furthermore, other factors such as temperature and pH may also play an important role in the enhancement or inhibition of the enzymetic activity (Empel *et al*., 2008)

In a related study, Al-Charrakh (2005) reported that out of 65 ß-lactam-resistant *Klebsiella* spp., 38 (58.5%) isolates were able to produce ß-lactamase enzymes, and he also tested these 38 ß-lactamase-producing *Klebsiella* isolates, and was found that 26 isolates (68%) gave rapid positive reaction within few seconds, whereas 9 isolates gave positive reaction after 1 minute and finally, 3 isolates were detected as ß-lactamase-producer after 2-5 minutes. Rapid positive results obtained (within few seconds to few minutes) by this test indicate that ß-lactamase enzymes may be of constitutive type.

Other studies have reported that most *E. coli* strains posses a chromosomally-encoded class C ß-lactamase enzyme which is often called AmpC type (philippon *et al*., 1994), and most *Klebsiella peumoniae* strains have class A chromosomally-encoded SHV-1 ß-lactamase enzymes. All these enzymes are constitutive and usually produced at low to moderate levels which in turn are sufficient to

protect against ampicillin, carbencillin and ticarcillin (Heritage *et al*., 1999).

In this investigation, 34 isolates those were resistant to ß-lactam antibiotics in primary screening test, 19 isolates (56%) were failed to produce ß-lactamase enzymes by the rapid iodometric method. This may be attributed either to the production of relatively low quantities of these enzymes, making their detection more difficult to achieve (Livermore, 1995), or these tested isolates may lack the ability to produce ß-lactamase enzymes.

Iodine reacts with starch to form dark blue complex, which stays without change in the absence of β-lactamase enzyme. In case of the presence of β-lactamase-producing bacteria, the resulting penicilloic or cephalospoic acid (resulting from breakdown of amide bond as mentioned above) will reduce iodine into iodide; consequently, decolorization of starch-iodine complex will occurs (changing the color directly to white) if an isolate is a β-lactamase producer (Sykes and Matthew**,** 1979).

The rapid iodometric assay was characterized by availability of materials that was required for achieving the test, it's simplicity to perform it, and short period for yielding the results. This assay relies on the fact that the β-lactamase under test is a penicillinase and achieved by measuring the production of penicilloic acid, which is produced when β-lactamases hydrolyze benzylpenicillin. This is likely to be true when estimating β-lactamase production in *Haemophilus*

influenza, *Moraxella catarrhalis*, and *Neisseria gonorrhoeae*, it may not be the case with other bacterial species (Eliasson *et al.*, 1992; Miles and Amyes, 1996).

The more sensitive technique was done by measuring β-lactamase activity with a chromogenic cephalosporin, usually nitrocephin (O'Callaghan *et al*., 1972) which changes from yellow to pink/red on hydrolysis. In addition, for many β-lactamases, nitrocephin is the substrate that is most readily hydrolyzed by the enzyme. This property makes it often the most sensitive detection system and it provides a very rapid and continent method for detection of β-lactamases. Nitrocephin, can be used as a solution or as disks upon which test cultures are smeared (Livermore, 1995; Livermore and Brown, 2001).

The rapid iodometric method is cheaper than other methods such as chromogenic cephalosporin method (that used nitrocephin) but it might give false-positive results, which probably related to the non-specific reaction of iodine with the bacterial proteins after one hour of the reaction. However, this disadvantage has been overcome and the test has been improved in the present study by reducing the time of the reaction to 5 minutes, thus only rapid β-lactamase-producing bacterial isolates can be detected.

Other disadvantage of the iodometric method is that, low levels of inducible chromosomal β-lactamases, are often inadequate to give a color reaction. In many species of the family *Enterobacteriacea*e,

including *Enterobacter* spp., *Citrobacter freundii*, *Providencia* spp., *Morganella morganii*, *Hafnia alvei*, *Serratia marcescens*, the expression of chromosomal *ampC* genes is low and inducible in response to β-lactam addition, which is related to a *LysR* regulator located upstream and divergently from the cephalosporinase gene.

E. coli behaves differently since its chromosomal AmpC is normally undetectable due to a weak promoter as well as a transcriptional attenuator. (Sanders and Sanders, 1992; Mammeri and Nordmann 2007). Only trace amounts of β-lactamase of these bacteria are made in the absence of antibiotics that produce delayed or negative reaction if they tested by the rapid iodometric assay (Livermore, 1995).

3-5- The Antibiotic Susceptibility Patterns:

As mentioned previously in **Table (3-4)**, 15 bacterial isolates were found to be ß-lactamase-producer. All these 15 isolates were tested for their susceptibility for a number of antibiotics (listed in **Table 2-5**) by using disk diffusion method (**Figure 3-1**).

It was found that, all these isolates were resistant to at least 11 antibiotics from a total of 25 antibiotics used in the present study. As a result of these findings, all these isolates were considered to be a multi-drug resistant isolates. Bacterial resistance to antibiotics is widespread nowadays and constitutes serious clinical threats (Mathur *et al*., 2002).

The bacterial resistance to antibiotics can be either hereditary in the microorganism (natural), such as the outer membrane in Gram-negative bacteria which acts as a permeability barrier against the antibiotics, or acquired resistance which can be achieved by two genetic processes in bacteria: mutation (vertical evolution) and exchange of genes between strains and species (horizontal evolution) (Ibezim, 2005).

Results from **Figure (3-1)** showed that all isolates 100% were found to be resistant to ampicillin, piperacillin, ticarcillin, carbencillin, ampicillin + sulbactam, mezlocillin, cephalothin, cefaclor, cefamandole, cefprozil and cefoperazone. The relatively high percentages of antibiotic resistance among these ß-lactamase-producing isolates indicated that these isolates possess an enzymetic mechanism of resistance categorized by the production of ß-lactamase enzymes.

These findings were disagreed with that obtained by the study of Al-Charrakh (2005), which stated that among *Klebsiella* spps., low level of resistance to piperacillin, cephalothin and cefaclor were obtained, this may be attributed to the fact that the samples used in this study were blood samples. In addition to that, all patients were on antibiotic treatment and this gave an indication that these isolates were sharing mechanisms of antibiotic resistance that enable them from reaching blood stream. For these reasons, the bacterial isolates

recovered in the present study were more resistant than these obtained from Al-Charrakh (2005).

It was found that the major mechanism of antibiotics resistance in Gram- negative bacteria that causing clinically significant infection was the expression of β-lactamase enzymes, of which there are several classes including plasmid-encoded and chromosomally encoded enzymes (Sanders and Sanders, 1992; Frère, 1995; Livermore, 1998).

The parental ß-lactamase enzymes (TEM-1, TEM-2, and SHV-1) hydrolyze penicillins and early cephalosporins, such as cephalothin, cefaclor, and cefazolin, but do not hydrolyze the later, more stable cephalosporins, like cefuroxime, cefixime, cefotaxime, ceftriaxone, ceftazidime, cefepime, and the monobactam, aztreonam. The mutations extend the substrate profiles, permitting hydrolysis of at least some of the more stable drugs listed above (Bush *et al.*, 1995).

Results from **Figure (3-1)** revealed that these isolates were 86.7% resistant to cefoxitin (belonging to cephamycins group). In spite of the structural stability of cefoxitin to ESBL-mediated hydrolysis as compared with other cephalosporins, resistance has been emerged to this antibiotic recently. The resistance to cefoxitin may be as a result of the development of porin-deficient mutants (Martinez-Martinez *et al.* 1996). In addition, increasing numbers of ESBL-producing strains express multiple β-lactamases including Amp C type of enzyme may also increase the chance for resistance to cephamycins (cefoxitin) (Bradford *et al.*, 1997).

AmpC class β-lactamases are cephalosporinases that are resistant to inhibition by clavulanate or other β-lactamase inhibitors in vitro. They can be differentiated from other ESBLs by their ability to hydrolyze cephamycins as well as other extended-spectrum cephalosporins (Livermore and Woodford, 2006). Furthermore, AmpC- β-lactamases demonstrated to be chromosomally or plasmid mediated, have been described in pathogens e.g., *K. pneumoniae, E. coli, Salmonella* spp., *Proteus mirabilis, Citrobacter freundii, Acinetobacter, Enterobacter* spp. and *Pseudomonas aeruginosa*. (Manchanda and Singh, 2003)

As shown in **Figure (3-1),** 80% of bacterial isolates were found to be resistant to ceftriaxone, cefotaxime, ceftazidime (third generation, extended-spectrum cephalosporins), and aztreonam (monobactam). Cefotaxime and ceftriaxone resistance, mainly, are an important indicator for the presence of extended-spectrum β-lactamases (ESBLs).

The relatively high rates of antimicrobial resistance may also attributed to additional factors such as poorly directed therapy, drug overuse and over-the-counter sales. Other workers, Mathur *et al*. (2002) also reported high rates of antimicrobial resistance among ESBL-producer. It is important to demonstrate that all bacterial isolates that had been resistant to ceftriaxone, cefotaxime and ceftazidime (extended-spectrum cephalosporins) were found later to be ESBL-producers (as it will be discussed later). On other hand, the

bacterial isolates that were sensitive to these antibiotics were detected, at later time, as non-ESBL-producers.

At the same time, it was also demonstrated that, the overall bacterial isolates were 60% resistant to cefepime (fourth generation cephalosporins) as illustrated in **Figure (3-1)**. This resistance perhaps due to the fact that the expression of extended-spectrum AmpC ß-lactamases (ESACs) might confer reduced susceptibility to all cephalosporins including not only ceftazidime, but also cefepime (Livermore and Woodford, 2006).

Regading susceptibility to imipenem and meropenem antibiotics (carbapenems) **(Figure 3-1)**; it was found that all isolates 100% were sensitive to these antibiotics. These findings were in accordance with that results being reported by Herna´ndez *et al.* (2005) and Hadi (2008). These results may occur as a consequence of the inability of these isolates to produce carbapenemase enzyme (an enzyme that has the ability to inactivate the carbapenem antibiotics).

The relatively high sensitivity to carbapenems may also reflect good permeability of these compounds across the cell wall of Gram-negative bacteria (Labombardi, 2007). In addition to that, the newer introduction of Carbapenem antibiotics in Iraq may also responsible for this elevated sensitivity to carbapenem antibiotics; consequently the resistant has not developed yet.

The bacterial resistance to other non ß-lactam antibiotics were also detected **(Figure 3-1)**. 80% of isolates showed resistance to co-

trimoxazole (trimethoprim + sulphamethoxazole), 73.3% of resistance was to nalidixic acid (quinolone), 66.7% of resistance was to tobramycin (aminoglycosides), and 60% of resistance was to nitrofurantoin. Finally, only 26.7% of resistance to both ciprofloxacin (fluoroquinolone) and chloramphenicol was detected.

In spite of low level of resistance to ciprofloxacin antibiotic (26.7%) (i.e. relatively higher levels of sensitivity, 73.3%), this antibiotic was not recommended for children below 18 years of age because of the possibility of the erosion to the growing cartilage (arthropathy) during the use of ciprofloxacin for children below 18 years (Harvey and Champe, 2006). Therefore, ciprofloxacin can be used to treat infections caused by these bacterial species but in patients older than 18 years old.

As a result of this study, it was found that, among the bacterial species tested for their antibiotics susceptibility, *K. peumoniae* subsp. *pneumoniae* and *E. coli* were found to be the most common species that showed higher levels of antibiotics resistance or what they are referred to as multi-drug resistant, especially to third generation cephalosporins (e.g. cefotaxime, ceftriaxone, and ceftazidime).

In spite of the resistance to many antibiotics displayed by other species (*K. oxytoca* and *Enterobacter cloacae*), these species showed relative sensitivity to many other drugs especially to third generation cephalosporins.

According to these results, the first two species (*K. peumoniae* subsp. *pneumoniae* and *E. coli*) were considered to be an ESBL-producer (as it will be discussed later). Furthermore, the spread of most broad-spectrum β-lactamases is facilitated by transferable and transconjugable plasmids, which often carry other resistance genes by means of their integron architecture (i.e. in addition to their resistance to ß-lactam antibiotics, there is a co-resistance to other antibiotics such as aminoglycosides and trimethoprim-sulfamethoxazole) (Katsanis *et al*., 1994; Jacoby and Munoz-Price, 2005).

3-6- The Production of Extended-Spectrum β-Lactamases:

Extended-spectrum ß-lactamases (ESBLs) are enzymes capable of hydrolyzing pencillins, broad spectrum cephalosporins and monobactams, and are generally derived from TEM and SHV-type enzymes. ESBLs are often encoded by plasmids that are transferable from strain to strain and among bacterial species (Thompson and Moland, 2001).

The majority of the ESBLs are derived through single amino acid substitutions in three non-ESBL parental ß-lactamase enzymes, TEM-1, TEM-2 and SHV-1. Since TEM- and SHV-ESBLs have been uniformly susceptible to ß-lactamase inhibitors (e.g. clavulanic acid, sulbactam and tazobactam), the use of inhibitor /ß-lactam combination was considered to be an alternative (Jacoby, 1997).

3-6-1- Disk Approximation Method:

This method is also known as double disk synergy test; it was first described by Jarlier *et al.* (1988). In this test, as discussed previously (**2-2-12-1**), any enhancement of the zone of inhibition between a β-lactam disks and augmentin disk gave an indication that the test strain contains ESBL whose activity is inhibited by clavulanic acid (**Figure 3-2)**.

It was found that out of 15 ß-lactamase-producing isolates; only 7 isolates (46.6%) were detected as ESBL-producers by using disk approximation method as shown in **Table (3-5).** Only *K. pneumoniae* subsp. *Pneumoniae* and *E. coli* were the species that gave positive results by using this method.

These results showed that the frequency of ESBL-producing isolates was higher than that reported by other researchers. Al-Charrakh (2005) examined 38 ß-lactamase-producing *Klebsiella* spp. isolates obtained from different clinical samples, and found that only 4 *Klebsiella* isolates (10.5%) were identified as ESBL-producers by using disk approximation method.

In another local study by Hadi (2008), her results revealed that out of 60 ß-lactam resistant *E. coli* and *K. pneumoniae* subsp. *pneumoniae* isolates, only 11 isolates (18.3%) were detected as ESBL-producers by using disk approximation method.

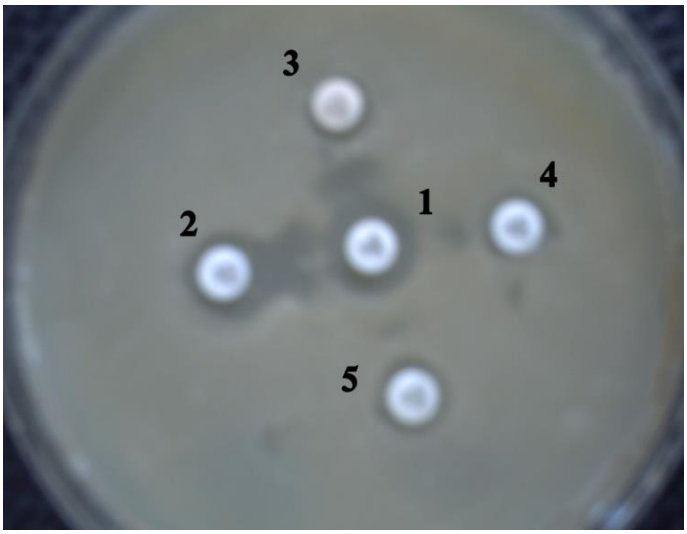

Figure (3-2): Disk Approximation Method for Detection of ESBL-Producing *Klebsiella pneumoniae* subsp. *pneumoniae* 10 Isolate.

1- Amoxicillin-clavulanate disk (20/10 µg).

2- Ceftazidime disk (30 µg).

3- Ceftriaxone disk (30 µg).

4-Cefotaxime disk (30 µg).

5- Aztreonam disk (30 µg).

Disk approximation method has served as the reference method for the detection of ESBL-producing strains for a number of years (Katsanis *et al*., 1994). The detection of ESBL-mediated resistance in Gram-negative bacilli is one of the major problems in the clinical microbiology laboratories (Arpin *et al.*, 2003). Furthermore, many studies showed that there is a clinical and financial benefit of rapid bacterial identification and early determination of antibacterial susceptibility (Livermore and Brown, 2001).

Table (3-5): The Frequency of ESBL-Producing and Non-Producing Isolates by Disk Approximation Method.

Bacterial Isolates	No. of Isolates	No. of ESBL-Producers (%)	No. of Non ESBL-Producers (%)
K. pneumoniae* subsp. *pneumoniae	**8**	**5 (33.3)**	**3 (20.0)**
K. oxytoca	**1**	**0 (0)**	**1 (6.7)**
E. coli	**5**	**2 (13.3)**	**3 (20.0)**
Enterobacter cloacae	**1**	**0 (0)**	**1 (6.7)**
Total	**15**	**7 (46.6)**	**8 (53.4)**

Kumar *et al.* (2006) reported that from a total of 1699 *Enterobacteriaceae* isolates, only 336 isolates (19.8%) were identified as potential ESBL producers by the double disk synergy test. On the other hand, Chanawong *et al.* (2001) showed that 35% of examined *Enterobacteriaceae* in Thailand were resistant to third generation cephalosporins.

The β-lactamase inhibitor (clavulanic acid) was first detected in 1976 in strain of *Streptomyces clavuligerous* (Rolinson, 1980). Clavulanic acid has the ability to inhibit a wide range of β-lactamases, including the plasmid-mediated enzymes such as TEM and SHV which have a wide-spread among the *Enterobacteraceae*.

ESBLs are also highly susceptible to inhibition by clavulanic acid (Rolinson, 1994). As a result, several tests have been developed for detection of ESBLs based on the sensitivity of ESBLs to clavulanic acid. These tests have become an important issue in the clinical laboratories since they provide a rapid and an accurate detection of ESBL-producing isolates (Moland and Thomson, 1994; Bedenic *et al.*, 2001; Livermore and Brown, 2001; CLSI, 2007).

In spite of the fact that the disk approximation method is the most widely used test because it is easy to be used and it can be performed in every laboratory. However, this test may lack sensitivity for some reasons such as: the optimum disk placement is not standardized, the inability of clavulanate to inhibit all ESBLs, the loss of clavulanate disk potency during storage, and the inability of the test to detect ESBLs in strains that also able to produce chromosomal cephalosporinases (Bush *et al.*, 1991; Thomson and Sanders, 1992; Moland and Thomson, 1994).

Some other studies have also reported that the disk approximation test was found to be inefficient to detect some ESBL-producing strains (Thomson and Sanders, 1992; Coudron *et al*., 1997). Most of clinical laboratories have some difficulties in the detection of extended-spectrum beta-lactamases (ESBLs) and plasmid-mediated AmpC beta-lactamases. Failure to detect these enzymes may be attributed to their uncontrolled spread and sometimes to the therapeutic failures (Manchanda and Singh, 2003).

3-6-2- Inhibitor-Potentiated Disk Diffusion Test (IPDDT):

In this test, as discussed previously (**2-2-12-2**), a difference in the ß-lactam zone of inhibition width of ≥ 10 mm on the two media (with and without clavulanate) was considered positive result for ESBL production.

All 15 ß-lactamase producing isolates were also tested for their ESBL production capability by using IPDDT. However, it was found that among these 15 ß-lactamase producing isolates; 10 isolates (66.6%) gave positive result for ESBL production by using IPDDT as shown in **Table (3-6).** It was also found that 7 of these 10 isolates were detected as ESBL-producer with disk approximation method.

This finding corresponding to that reported by Ho *et al.* (1998), who illustrated that the inhibition zones diameters of antibiotics were increased in the presence of known ESBL-producing control strains; but, not with the ESBL-negative strains and they also found that the sensitivity of IPDDT reached to 100%. The results obtained from this study were higher than that recorded by Hadi (2008), who showed that out of 60 ß-lactam-resistant *Klebsiella* spp. and *E. coli* isolates, only 13 isolates (21.7%) were detected as ESBL-producers by this test.

Early detection of ESBLs is necessary in achieving excellent infection control. As a result, routine screening for ESBL-producing isolates is needed. Thus, IPDDT is more convenient than double disk synergy test and much less expensive than the E-test ESBL screen (Ho *et al.*, 1998).

Table (3-6): The Frequency of ESBL-Producing and Non-Producing Isolates by Using IPDDT.

Bacterial Isolates	No. of Isolates	No. of ESBL-Producers (%)	No. of Non ESBL-Producers (%)
K. pneumoniae subsp. pneumoniae	**8**	**7 (46.6)**	**1 (6.7)**
K. oxytoca	**1**	**0 (0)**	**1 (6.7)**
E. coli	**5**	**3 (20.0)**	**2 (13.3)**
Enterobacter cloacae	**1**	**0 (0)**	**1 (6.7)**
Total	**15**	**10 (66.6)**	**5 (33.4)**

The test was described as a highly sensitive and specific method for the detection of ESBL-producing strains (Ho *et al.*, 1998). The accurate detection and reporting of ESBL production in clinical isolates are crucial in human infection with ESBL-producing strains, and a slight increase in the minimum inhibitory concentration (MIC) of oxyimino cephalosporins has been reported to be sufficient to result in treatment failure (Rice *et al.*, 1991).

In this study, the inhibitor-potentiated disk diffusion test gave a higher percentage (66.6%) for detection of ESBL production in comparison with the results obtained by disk approximation method (46.6%). The explanation for this variation may reflect the fact that

the results interpretation of the disk approximation method is more difficult and requires experienced investigators to obtain reliable results, than that required for IPDDT. On the other hand, the procedure of IPDDT includes the spreading of clavulanic acid solution in the Mueller-Hinton agar plates, and this provide a greater chance for clavulanic acid to inhibit ESBL enzymes than that mediated by disk approximation method.

Bedenic *et al*. (2001) modified this test by supplementing Mueller-Hinton agar with 8 µg/ml clavulanate instead of 4 µg/ml that described by Ho *et al.* (1998), and they found that the IPDDT to be a very sensitive method but it lacks specificity.

False positive and false negative results (disadvantages) may occur with IPDDT because ESBL production may vary on repeated testing (Sanders *et al.,* 1996). IPDDT is based on a biochemical reaction between the ESBL enzyme and their inhibitor (clavulanic acid); thus, this test might fail in the event that involved the absence of gene expression, or gene expression has been occur but the resultant ESBL is found in undetectable quantities.

The expression of an AmpC ß-lactamase together with an ESBL may be associated with masking the production of ESBL enzyme. However, silent genes may be activated or minimally functional genes may be enhanced as a result of selective pressure of antibiotic therapy (Jain *et al*., 2003).

3-6-3- Clavulanate Method:

In this method, an 8-fold or larger reduction of the MIC for either cefotaxime or ceftazidime in the presence of clavulanate (4 µg/ml) was indicated as positive result for ESBL production by tested isolates (CLSI, 2007).

The results shown in **Table (3-7)** indicated that among the 15 ß-lactamase producing isolates, 12 isolates (80%) were found to be ESBL-producers by this method. Also it was found that, the numbers of folds decrease the MICs of these ß-lactams were ranged from 10-16 folds which indicated that these isolates were ESBL-producers. Moreover, all ESBL-producing isolates were belonging to *K. pneumoniae* subsp. *pneumoniae* (n=8) and *E. coli* (n=4). Many studies used this test for detection of different types of ESBL- producing isolates (Bedenic *et al*., 2001).

It was found that ceftazidime was a good substrate for production of most ESBLs and these criteria make ceftazidime an appropriate indicator to detect such enzymes (Bonafede and Rice, 1997), and this may reflect the fact that almost all ESBL-producers are resistant to ceftazidime.

Bedenic *et al*. (2001) compared five different methods for detection of different types of TEM and SHV-ESBLs. They found that the MIC determination of ß-lactam with and without clavulanate was the preferable method regarding sensitivity for detection of ESBL-producing strains, regardless of the type of ß-lactamase.

Table (3-7): The MICs of Cefotaxime and Ceftazidime with and without Clavulanic Acid for ESBL- Producing Isolates

Bacterial Isolates	MICs (µg/ml)					
	Cefotaxime (CTX)			Ceftazidime (CAZ)		
	Without	With	No. of Folds Decrease	Without	With	No. of Folds Decrease
***K. pneumoniae* subsp. *pneumoniae* 1**	**64**	**0.125**	**10**	**128**	**0.064**	**12**
***K. pneumoniae* subsp. *pneumoniae* 7**	**64**	**0.032**	**12**	**64**	**0.008**	**14**
***K. pneumoniae* subsp. *pneumoniae* 8**	**64**	**0.125**	**10**	**> 128**	**0.008**	**16**
***K. pneumoniae* subsp. *pneumoniae* 9**	**32**	**0.016**	**12**	**128**	**0.004**	**16**
***K. pneumoniae* subsp. *pneumoniae* 10**	**32**	**0.016**	**12**	**128**	**0.004**	**16**
***K. pneumoniae* subsp. *pneumoniae* 20**	**> 128**	**0.008**	**16**	**> 128**	**0.008**	**16**
***K. pneumoniae* subsp. *pneumoniae* 24**	**64**	**0125**	**10**	**> 128**	**0.064**	**12**
***K. pneumoniae* subsp. *pneumoniae* 32**	**> 128**	**0.032**	**14**	**128**	**0.016**	**14**
***E. coli* 16**	**64**	**0.032**	**12**	**32**	**0.004**	**14**
***E. coli* 19**	**32**	**0.064**	**10**	**64**	**0.002**	**16**
***E. coli* 23**	**> 128**	**0.008**	**16**	**> 128**	**0.032**	**14**
***E. coli* 25**	**64**	**0.125**	**10**	**32**	**0.064**	**10**

Although MIC determination of β-lactam antibiotic with and without clavulanate provides an accurate method for detection of ESBLs, it is time a consuming, and for this reason it is very rarely used in the clinical laboratories. It has been reported that detection of ESBL producers can be difficult because the presence of ESBLs in a bacterial cell does not always produces a resistance phenotype when one is using the traditional MIC and disk diffusion interpretive criteria published by the NCCLS (Thomson and Sanders, 1992; Katsanis *et al*., 1994; Coudron *et al*., 1997).

Other disadvantages include, increased the expression of non-ESBL SHV-1 β-lactamase may occasionally result in elevated MICs of some of the extended-spectrum cephalosporins and these MICs may be reduced by 8-fold or more in the presence of clavulanate. In addition to the apparent synergistic effect between these antibiotics and clavulanate, all these factors collectively may result in false positive results of ESBL production (Miro *et al*., 1998). In another study, Wu *et al*. (2001) reported from an outbreak of *K. pneumoniae*, that co-existence of TEM-1 and SHV-1 as well as the reduced expression of an outer membrane protein in the same strain may lead to false positive identification of ESBL enzymes.

The co-existence of ESBLs with plasmid-mediated AmpC ß-lactamases in strains of *K. pneumoniae* and *E. coli* has been reported in Taiwan (Wu *et al*., 2005). The AmpC enzymes, which are not inhibited by clavulanate, may potentially interfere with the inhibitory

effect of clavulanate on ESBLs and thus contribute to maintaining elevated MICs to all groups of cephalosporins, provided that the AmpC enzymes are produced in large amounts. This may lead to false-negative result for ESBL production (disadvantages). False-negative results are supposed to occur if the AmpC activity is larger than that of ESBL activity (Yan *et al*., 2002).

Among the three methods used for ESBL detection in the present study, it was found that the determination of MIC with and without clavulanate was the most accurate method in the detection of ESBL-producing isolates since in this method, 12 isolates were identified as an ESBL-producers in comparison with the results obtained by the other two methods (7 isolates by disk approximation method and 10 isolates by IPDDT) (**Table 3-7)**. Consequently, disk approximation method may be associated with the lowest recovery in the detection of ESBL enzymes.

It is important to note that the clinical microbiology laboratory is obviously the first line of defence in detection and control of ESBLs spread. Therefore, it is imperative that laboratory personnel are well skilled in the detection of these organisms since the failure in their detection may lead to treatment failures (Patterson *et al*., 2001). Unfortunately, many clinical laboratories may lack to understand regarding ESBLs and AmpC ß-lactamases and their detection. This has been documented in a study in USA, where it was found that 21% of laboratories failed to detect ESBL (Tenover *et al*., 1999).

Table (3-8): A Comparison Between the Results of Disk Approximation, IPDDT, and Clavulanate Method for Detection of ESBLs Production.

Isolates	Disk Approximation	IPDDT	Clavulanate Method
***K. pneumoniae* subsp. *pneumoniae* 1**	+	+	+
***K. pneumoniae* subsp. *pneumoniae* 7**	+	+	+
***K. pneumoniae* subsp. *pneumoniae* 8**	–	+	+
***K. pneumoniae* subsp. *pneumoniae* 9**	+	+	+
***K. pneumoniae* subsp. *pneumoniae* 10**	+	+	+
***K. pneumoniae* subsp. *pneumoniae* 20**	–	+	+
***K. pneumoniae* subsp. *pneumoniae* 24**	+	+	+
***K. pneumoniae* subsp. *pneumoniae* 32**	–	–	+
***K. oxytoca* 12**	–	–	–
***Enterobacter cloacae* 31**	–	–	–
***E. coli* 11**	–	–	–
***E. coli* 16**	+	+	+
***E. coli* 19**	–	+	+
***E. coli* 23**	+	+	+
***E. coli* 25**	–	–	+
Total 15 (100 %)	7 (46.6%)	10 (66.6%)	12 (80%)

3-7- MICs of ESBL-Producing Isolates:

MICs of four ß-lactam antibiotics (ampicillin, cefotaxime, ceftazidime and imipenem) were determined for ESBL-producing isolates by using two-fold agar dilution susceptibility method. The values of MICs were based on break point recommended by CLSI (2007) for estimation of the response. The break point represents the optimum concentration of the drug that can reach the serum and provide high level of therapy. The microorganism was considered sensitive if the estimated MICs were less than the break point while in the case of the MIC value was higher than the break point, the microorganism was considered to be resistant to the selected drug.

As illustrated in **Table (3-8),** all 12 ß-lactamase-producing isolates were highly resistant to ampicillin with concentrations reached beyond the break point values. It was found that the MICs values of ampicillin for all ESBL-producing isolates were >128 µg/ml.

Livermore (1995) showed that TEM or SHV enzymes of Gram-negative bacteria confers resistance to ampicillin, amoxicillin, ticarcillin, and carbenicillin, with MIC values exceeding 256 µg/ml in comparison to (1-4) µg/ml for *E. coli* isolates that lack the ability to produce these enzymes. Results from a local study reported that Gram-negative enteric rods were 100 % resistant to ampicillin and amoxicillin (Al-Jubouri, 1997).

Al-Charrakh (2005) found that the MICs values of ampicillin for 8 ESBL-producing *K. pneumoniae* subsp. *pneumoniae* isolates were ranged from 64 to > 128. The results obtained in the present study were similar to those reported by Hadi (2008) who showed that all 11 ESBL-producing *Klebsiella pneumoniae* and *E. coli* isolates were highly resistant to ampicillin with MICs >128 μg/ml. The higher ratio of ampicillin resistance with subsequently higher MICs values may be as a result of frequent and over use of this antibiotic.

The results shown in **Table (3-8)**, illustrated that the MICs values for cefotaxime were less than that of ampicillin. MICs values for 12 ESBL-producers ranged from 32 to >128 μg/ml. Furthermore, it was reported that 9 isolates (6 *Klebsiella pneumoniae* and 3 *E. coli*) were found to be able to grow in a concentration more than or equal to the break point (64 to >128 μg/ml), and 3 isolates (2 *Klebsiella pneumoniae* and 1 *E. coli*) were able to grow in a concentration below the break point value (32 μg/ml).

The results obtained by Casellas and Goldberg (1989) showed that 46 ESBL-producing *Klebsiella pneumoniae* and *E. coli* isolates, the MIC of cefotaxime for 50% of isolates tested was < 16 μg/ml. Al-Charrakh (2005) tested 8 ESBL-producing *Klebsiella pneumoniae* isolates and found that the MIC values of cefotaxime were less than those in penicillins and first generation cephalosporins and also reported that, the MIC values for 7 isolates ranged from 16 to 32 μg/ml which were less than the break point value (≥ 64 μg/ml). Only

one clinical isolate was able to grow in concentration equal to break point (64 μg/ml).

Table (3-9): The MICs of some β-Lactam Antibiotics for ESBL-Producing *Klebsiella pneumoniae* and *E. coli* Isolates.

Bacterial Isolates	MIC (μg/ml) of :			
	AMP (≥ 32)	CTX (≥ 64)	CAZ (≥ 32)	IMI (≥ 16)
***K. pneumoniae* subsp. *pneumoniae* 1**	>128	64	128	1
***K. pneumoniae* subsp. *pneumoniae* 7**	>128	64	64	0.5
***K. pneumoniae* subsp. *pneumoniae* 8**	>128	64	>128	0.5
***K. pneumoniae* subsp. *pneumoniae* 9**	>128	32	128	1
***K. pneumoniae* subsp. *pneumoniae* 10**	>128	32	128	0.5
***K. pneumoniae* subsp. *pneumoniae* 20**	>128	>128	>128	1
***K. pneumonia e* subsp. *pneumoniae* 24**	>128	64	>128	0.25
***K. pneumoniae* subsp. *pneumoniae* 32**	>128	>128	128	2
***E. coli* 16**	>128	64	32	0.25
***E. coli* 19**	>128	32	64	0.25
***E. coli* 23**	>128	>128	>128	0.25
***E. coli* 25**	>128	64	32	1

*Numbers between brackets refer to the break points recommended by CLSI (2007).
AMP: Ampicillin, CTX: Cefotaxime, CAZ: Ceftazidime, IMI: Imipenem

In a recent study, Hadi (2008) illustrated that the MIC values (for cefotaxime) of 11 *K. pneumoniae* and *E.coli* isolates were ranged from 16 to 64 μg/ml, 7 isolates were able to grow in a concentration equal to the break point (64 μg/ml) and 4 isolates were grown in concentration below the break point value (16-32 μg/ml).

Bedenic and Zagar (1998) revealed that the SHV-5 β-lactamase of *K. pneumoniae* isolates resulted in higher ceftazidime MICs (256 μg/ml) than cefotaxime (64 μg/ml) and ceftriaxone (32 μg/ml). They attributed these differences of varying affinities for some essential PBPs or to the different rates of diffusion across the outer membrane, or both, which minimize the hydrolytic effect of this enzyme on cefotaxime and ceftriaxone.

In the case of ceftazidime, it was found that all ESBL-producers included in this study were 100% resistant to ceftazidime (able to grow in concentrations equal or above the break point values, ≥ 32 μg/ml). 4 isolates (3 *K. pneumoniae* and 1 *E. coli*) were able to grow in a concentration of > 128 μg/ml, 4 isolates (all *K. pneumoniae*) were able to grow in a concentration of 128 μg/ml, 2 isolates (1 *K. pneumoniae* and 1 *E. coli*) were able to grow in a concentration of 64 μg/ml, while only 2 isolates of *E. coli* were able to grow in a concentration equal to the break point value, 32 μg/ml.

These results were in accordance with results being reported by Al-Charrakh (2005) who showed that the ESBL-producing *K. pneumoniae* isolates were 100% resistant to ceftazidime with MIC

values equal or exceed the break point values. In another local study, Hadi (2008) showed that among 11 ESBL-producing isolates (*K. pneumoniae* and *E. coli*), 8 isolates (72.7%) were resistant to ceftazidime with MIC values equal or exceed the break point values.

Relatively higher MIC values of ceftazidime compared to those of cefotaxime suggested the ceftazidimase activities of the ESBLs in these isolates. Yuan *et al*. (2000), found that the MICs of cefotaxime, ceftazidime, ceftriaxone, and aztreonam of *K. pneumoniae* isolates were ranged widely between 4- to 32-fold above that recommended for ESBL-negative *K. pneumoniae* but, all of these isolates were susceptible to imipenem.

In this study, MIC determination was done as well for imipenem antibiotic which was a very effective drug against many ESBL-producers. It was obvious from **Table (3-9)** that all ESBL-producers were fully sensitive (100%) to imipenem with MIC values, extremely below the break point value (≥ 16 μg/ml), ranging from 0.25 to 2 μg/ml. As a result, the MIC values for all tested ESBL-producing *K. pneumoniae* and *E. coli* were found to be ≤ 2 μg/ml. Therefore, this finding gave an indication that all these isolates were highly sensitive to imipenem antibiotic and accordingly, carbapenems should be regarded as the drugs of choice for serious infections with ESBL-producing organisms. The basis of this conclusion is just not the almost uniform susceptibility in vitro of these compounds but also increasingly extensive clinical experience (Endimiani *et al*., 2004).

Similarly, Herna´ndez *et al*. (2005) found that all ESBL-producing *E. coli* and *K. pneumoniae* isolates tested were 100% sensitive to imipenem with MIC values ranging from 0.25 to 0.5 µg/ml. The higher sensitivity to imipenem antibiotics may be attributed to the fact that this drug is recently introduced in Iraq, and the resistance to this antibiotic was not developed yet.

3-8- Extraction of DNA:

DNA was extracted by using salting out method described by Pospiech and Neumann (1995) (**2-2-14-1**). In the present study, the DNA extraction procedure was performed for 14 ß-lactamase producing isolates (**Table 3-4**) (both 12 ESBL- and 2 non ESBL-producing isolates were selected). Because of the unavailability of standard plasmid (or lambda phage) of known molecular weight (size markers), the molecular weight of plasmid DNA isolated was not determined in the present study.

3-9- Detection of *bla*$_{\mathbf{TEM}}$ and *bla*$_{\mathbf{SHV}}$ Genes by PCR:

The detection of ß-lactamase producing isolates and the subsequent detection of ESBL-producers were performed. However, such detection not provided any information about the type of these enzymes. An attempt was made to evaluate the frequency of TEM and SHV enzyme type among 14 ß-lactamase-producing isolates (9 *Klebsiella* spp. and 5 *E. coli*).

DNA extracted from bacterial cells (as mentioned above) was used as a template in specific PCR technique for detection of *bla*$_{TEM}$ and *bla*$_{SHV}$ genes. The frequency of TEM and SHV enzymes type among ß-lactamase producing isolates was summarized in **Table (3-10)**. Based on the types of ß-lactamase enzymes, all 14 ß-lactamase-producing isolates were divided into four categories (**Table 3-10**): the first category included 1 isolate (*K. pneumoniae* subsp. *pneumoniae* 32) produced amplification products with TEM-PCR specific primers (**Figure 3-3**). The second category included 4 isolates (1 *K. pneumoniae* subsp. *pneumoniae*, 2 *E. coli* and 1 *K. oxytoca*) gave PCR products with SHV-specific primers (**Figure 3-3**).

The third category represented by 7 isolates (6 *K. pneumoniae* subsp. *pneumoniae* and 1 *E. coli*), which produce both SHV and TEM enzymes (**Figure 3-3**). Finally, the fourth category represented by 2 isolates (both of them *E. coli*), which they do not produce neither TEM nor SHV enzymes.

According to the results obtained, the isolate in first category (1 isolate) and third category (7 isolates) were detected previously as ESBL-producers **Table (3-7)**, except one isolate (*E. coli* 11) which was detected as non ESBL-producer; but, ß-lactamase-producer and this isolate was showed relatively moderate level of antibiotic resistance as compared with the other 7 ESBL-isolates (**Table 3-10**).

The spread of TEM ß-lactamases was worldwide and was found in many *Enterobacteriaceae*. *Klebsiella* spp. displayed reduced

susceptibility to first and second generation cephalosporins by the production of plasmid-mediated, TEM ß-lactamases. Since 1980s, the emergence of resistance to third generation cephalosporins has been reported in strains of *K. pneumoniae* (Knothe *et al.*, 1983).

The native TEM-1 β-lactamase confers resistance to ampicillin, penicillin and first-generation cephalosporins such as cephalothin. This enzyme has been reported to be responsible for 90% of ampicillin-resistance in *E. coli* isolates. TEM-13 also has a similar hydrolytic profile to TEM-1 and TEM-2. Moreover, TEM-1, TEM-2, and TEM-13 are not ESBLs (Livermore, 1995).

Mutations within the $bla_{\text{TEM-1}}$ structural gene, presumably through antibacterial selection, have allowed the enzyme to expand the hydrolysis capabilities to particular extended-spectrum cephalosporins and aztreonam, while maintaining its original hydrolysis capabilities. Additionally, most of produced enzymes are mutant of TEM-1 and TEM-2 such as TEM-3, TEM-4, TEM-10, TEM-27, and TEM-92 (Bradford, 2001).

The first ESBL observed at the teaching hospitals of Clemont-Ferrand, France in July 1984, the cefotaximase TEM-3/ CTX-1 was detected in *K. pneumoniae* (Sirot *et al*., 1987). In the United States, the enzymes which occur commonly in outbreak caused by *K. pneumoniae* were TEM-10, TEM-12, and TEM-26 (Urban *et al*., 1994).

Table (3-10): The Frequency of TEM and SHV Enzymes Type According to PCR Technique with Antibiotics Resistance Pattern for ß-Lactamase Producing Isolates.

Bacterial Isolates	Type of ß-Lactamase Enzyme		No. of Antibiotics Resistant by each Isolates
	TEM type	SHV type	
***K. pneumoniae* subsp. *pneumoniae* 1** *	+	+	**21**
***K. pneumoniae* subsp. *pneumoniae* 7** *	+	+	**16**
***K. pneumoniae* subsp. *pneumoniae* 8** *	+	+	**19**
***K. pneumoniae* subsp. *pneumoniae* 9** *	+	+	**16**
***K. pneumoniae* subsp. *pneumoniae* 10** *	–	+	**20**
***K. pneumoniae* subsp. *pneumoniae* 20** *	+	+	**21**
***K. pneumoniae* subsp. *pneumoniae* 24** *	+	+	**21**
***K. pneumoniae* subsp. *pneumoniae* 32** *	+	–	**23**
***K. oxytoca* 12** **	–	+	**11**
***E. coli* 11** **	+	+	**14**
***E. coli* 16** *	–	–	**19**
***E. coli* 19** *	–	–	**23**
***E. coli* 23** *	–	+	**20**
***E. coli* 25** *	–	+	**19**

*: ESBL- producer. **: Non ESBL- producer.

Figure (3-3): Electrophoresis of PCR Test to Determine the Frequency of TEM and SHV Enzyme Types in Isolates of *Enterobacteriaceae*.

TEM: TEM enzyme, **SHV:** SHV enzyme.

A: *K. pneumoniae* subsp. *pneumoniae* 1

B: *K. pneumoniae* subsp. *pneumoniae* 7

C: *K. pneumoniae* subsp. *pneumoniae* 8

D: *K. pneumoniae* subsp. *pneumoniae* 9

E: *K. pneumoniae* subsp. *pneumoniae* 10

F: *E. coli* 11

G: *K. oxytoca* 12

H: *E. coli* 16

I: *E. coli* 19

J: *K. pneumoniae* subsp. *pneumoniae* 20

K: *E. coli* 23

L: *K. pneumoniae* subsp. *pneumoniae* 24

M: *E. coli* 25

N: *K. pneumoniae* subsp. *pneumoniae* 32

The results presented in **Table (3-10)** showed that, only 4 isolates were able to yield amplification products with SHV-PCR specific primers, 1 *K. pneumoniae* subsp. *pneumoniae* 10 isolates, 2 *E. coli* 23, 25 isolates and *K. oxytoca* 12 isolates. Furthermore, all these isolates were also detected as ESBL-producers (**Table 3-7**), with high level of antibiotic resistance **Table (3-10)**, except one isolate (*K. oxytoca* 12) which was detected as non ESBL-producer**.** Most of *K. pneumoniae* isolates have chromosomally or plasmid-mediated SHV-1 ß-lactamase, which has a narrow-spectrum ß-lactamase with activity against pencillins (Bush *et al*., 1995).

It has been reported that more than 50 variants of SHV which were important worldwide and currently recognized on the basis of unique combination of amino acid replacement (Jacoby and Munoz-Price, 2005). SHV-2 and SHV-5, plasmid-mediated ß-lactamase enzymes, have been recorded in at least five countries. Additionally, SHV-5 enzyme was widespread in Greece (Gianneli *et al*., 1994).

In another study, Tasli and Bahar (2005) found that of 91 strains of *K. pneumoniae* being isolated from clinical samples in Turkey, 42 isolates revealed SHV-PCR positive. The majority of SHV enzymes are found in strains of *K. pneumoniae*. Nevertheless, these enzymes have also been found in *E. coli* (Bradford *et al*., 1995). It was recently reported by Tasli and Bahar (2005) that 15% of *E. coli* isolates obtained from clinical samples in Turkey were able to produce SHV enzymes depending on PCR test.

Among the 14 isolates tested for their ß-lactamase enzyme type by PCR technique (**Table 3-10**), only 2 isolates (*E. coli* 16 and *E. coli* 19) were unable to yield amplification products with TEM and SHV-PCR specific primers. In spite of PCR results suggested that the TEM and SHV enzymes were not presenting in these 2 isolates, it is possible that these isolates contain other types of enzymes such as AmpC, CTX-M, OXA or others. In addition to that, this possibility came from the fact that these isolates were detected previously as ß-lactamase-producers (**Table 3-3**), and ESBL-producers (**Table 3-7**) with high level of antibiotic resistance, particularly toward ß-lactams.

Regarding the isolates (n=12) that exhibited positive results with TEM, SHV enzyme (or with both) and the isolates (2 isolates) that gave negative results for TEM and SHV enzyme by PCR test, also there is a possibility of presence of other types of ESBL enzyme because bacterial isolate can acquire more than one plasmid. Moreover, the bacterial isolates may contain large plasmid called (mega plasmid), which had the ability to carry different types of antibiotic resistant genes including genes that coded for one or may be more than one type of ESBL enzymes. Additionally, the spread of most broad-spectrum β-lactamases is facilitated by transferable and transconjugable plasmids, which often carry other resistance genes by means of their integron architecture (Jacoby and Munoz-Price, 2005).

Consequently, all above factors gave an explanation about the high level of resistance to many antibiotics shared by these isolates

and hence, named as “multi-drug resistant”. In a study in the United States, it was estimated that 3-4% of *K. pneumoniae* isolates recovered from clinical samples were able to carry plasmid-mediated AmpC enzymes (Black *et al*., 2003).

PCR test is very sensitive and accurate; but, it is relatively laborious and expensive. As a result, it is not recommended from practical view to use them in routine laboratories. However, it can serve as an important detector of TEM and SHV ß-lactamase enzymes in the epidemiological studies.

Regarding these results, many risk factors may be associated with the development of blood stream infection by multi-drug resistant Gram-negative bacteria in paediatrics, such as: the presence of underlying diseases, the duration of hospitalization prior to the occurrence of bloodstream infection, severity of the clinical conditions, and the presence of central venous catheter (Kang *et al*., 2005)

Chapter One

Introduction & Literature Review

Chapter Two

Materials & Methods

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List of Abbreviations

Abbreviation	Description
SHV	Sulfhydryl Variable
TEM	Temoneira
ESBL	Extended-Spectrum Beta-Lactamase
ESBL-P-PM	ESBL -Producing *Proteus.mirabilis*
PCR	Polymerase Chain Reaction
BSI	Blood Stream Infection
PCMB	P-Chloromercuribenzoate
S-S agar	Salmonella- Shigellas agar
MIC	Minimum Inhibitory Concentration
CLSI	Clinical and Laboratory Standard Institute
NCCLS	National Committee for Clinical Laboratory Standards
EDTA	Ethylene Di-amine Tetra acetic Acid
MR-VP broth	Methyl-Red Voges-Proskauer Broth
IPDDT	Inhibitor-Potentiated Disk Diffusion Test
AMP	Ampicillin
AMC	Augmentin

Abbreviation	Description
CB	Carbencillin
TC	Ticarcillin
PI	Pipracillin
SAM	Sulbactam +Ampicillin
ATM	Aztreonam
MZ	Mezlocillin
IMI	Imipenem
MEM	Meropenem
KF	Cephalothin
CM	Cefamandole
CPZ	Cefprozile
CF	Cefaclor
CP	Cefoperazone
CX	Cefoxitin
CRO	Ceftriaxone
CAZ	Ceftazidime
CTX	Cefotaxime
CPM	Cefepime
C	Chloramphenicol
CIP	Ciprofloxacin
F	Nitrofurantoin
SXT	Trimethoprim + Sulfamethoxazole
TOB	Tobramycin
NA	Nalidixic acid
TE buffer	Tris- EDTA buffer
SDS	Sodium Dodecyl Sulfate
TBE	Tris-Borate-EDTA Buffer
BHI broth	Brian Heart Infusion Broth
ICU	Intensive Care Units
ESACs	Extended-Spectrum AmpC ß-Lactamases

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Chapter Three

Results

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Discussion

Conclusions & Recommendations

References