Dissemination of β-lactamases in Iraqi hospitals: Molecular study of AmpC and carbapenemase-producing bacteria

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Dedication

To….

All martyrs and Iraqi peoples who died or vanished during violence waves that devastated Iraq in the last few years

We dedicate this work

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List of abbreviations

Abbreviation	Key
AmpC	Molecular class C β-lactamases
β	Beta
bla gene	β-lactamase gene
CDC	The Centers for Disease Control and Prevention
CIAT	Ceftazidime-imipenem antagonism test
CTX	β-lactamase active on cefotaxime
CTX-M	β-lactamase active on cefotaxime, first isolated at Munich
DHA	β-lactamase discovered at Dhahran hospital in Saudi Arabia
DDS	Double disc synergy
EDTA	Ethylene diamine tetra acetic acid
ESBLs	Extended-spectrum β-lactamases
ICUs	Intensive care units
IMP	β-lactamase active on imipenem
KPC-	*Klebsiella pneumoniae*-carbapenemase
MBLs	Metallo-β-lactamases
MHT	Modified hodge test
MICs	Minimum inhibitory concentrations
MR-VP broth	Methyl-red Voges-proskauer
MTD	Modified three dimensional test
MDR	Multidrug resistant
NCCLS	National Committee for Clinical Laboratory Standards
OXA	β-lactamase active on oxacillin
PABLs	Plasmid-mediated AmpC β-lactamases
PBPs	Pinicillin binding proteins
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulfate

PER	Pseudomonas extended resistant and also the initials of its discoverers: Patric, Esthel, and Roger
SHV	β-lactamase (Sulfhydryl reagent variable)
TBE	Tris-borate-EDTA buffer
TE buffer	Tris EDTA buffer
TEM	β-lactamase named after the patient (Temoneira) providing the first sample
Tris-OH	Tris-(hydroxymethyl) methylamine
UTIs	Urinary tract infections
VEB	Vietnam extended-spectrum β-lactamase
VIM	Verona integron-encoded metallo-β-lactamases

Chapter One

Introduction:

Resistance to β-lactam antibiotics has increased significantly in the last two decades and has been documented in both community and hospital settings (Mathor *et al*., 2005). Unfortunately, the indiscriminate use of β-lactams has exerted a tremendous evolutionary pressure on the targeted bacteria, causing the development of sophisticated molecular mechanisms to escape their deadly action (Tenover, 2006). Their most effective drug resistance mechanism is given by the expression of so-called β-lactamases (Babic *et al*., 2006). The production of β-lactamases is the predominant cause of resistance to β-lactam antibiotics in Gram-negative bacteria. These enzymes cleave the amide bond in the β-lactam ring, rendering β-lactam antibiotics harmless to bacteria (Bonnet, 2004), this resistance is due to chromosomal-and plasmid-mediated β-lactamases in Gram-negative bacilli which has become one of the major problems in human medicine (Bradford, 2001). β-lactamases have been isolated from a variety of *Enterobacteriaceae* (Schwaber *et al*., 2004).

The persistent exposure of bacterial strains to a multitude of β-lactams has led to overproduction and mutation of β-lactamases. These β-lactamases are capable of hydrolyzing penicillins, broad-spectrum cephalosporins, monobactams, cephamycins, carbapenems, in addition to other classes of antimicrobial agents. Thus, these are new β-lactamases and are called as extended-spectrum-β-lactamases (ESBLs), AmpC β-lactamases and carbapenemases (Jacoby and Munos-Price, 2005). β-lactamases-producing bacilli from the family *Enterobacteriaceae* are increasingly identified as pathogens, having become endemic in many healthcare settings and recently reported in hospital and community-acquired infections as well (Rodriguez-Bano *et al*., 2006).

During the past decades, extended-spectrum cephalosporins, including oxymino- β-lactam antibiotics, have been used worldwide, and antibiotic-resistant strains that produce β-lactamases have emerged among the *Enterobacteriaceae*, predominantly in *E. coli* and *K. pneumoniae* (Bush, 2001). *E. coli* and *K. pneumoniae* are major nosocomial pathogens causing intra-abdominal infection, urinary tract infection, and primary bacteremia (Eisenstein and Zaleznik, 2000). Resistance to the extended-spectrum cephalosporins can occur in *E. coli* and *Klebsiella* spp. via the production of β-lactamases that are capable of hydrolyzing the oxyimino- cephalosporins and monobactams. So these organisms become uniformly resistant to oxymino-β-lactam antibiotics (Ali Shah *et al*., 2004).

In Iraq, little attention has been paid to the genetic factors controlling β-lactamases producing isolates. However, in Hilla/ Babylon province, very little information are available regarding molecular studies of ESBLs or occurrence of β-lactamases (AmpC and carbapenemases)-producing *E. coli* and *Klebsiell*a species recovered from hospital environments. Therefore, there is an increase demand to investigate the role of these isolates in hospital infections, hence the aim of this study is to identify the prevalence of β-lactamases in *E. coli* and *Klebsiella* spp. isolates recovered from Merjan Teaching Hospital, as a representative of Iraqi hospitals. The present work was carried out to achieve the following objectives:

1-Studying the occurrence of multidrug resistance and antibiotic susceptibility patterns of β-lactamases-producing *E. coli* and *Klebsiella* spp. isolates. 2- Detection of genetic factors controlling β-lactamases production and attempting to detect the CTX-M and OXA β-lactamases in the isolates by typing $bl_{aCTX\text{-}M}$ and bl_{aOXA} genes.

Chapter Two

Literatures review:

2-1 Nosocomial infections:

Nosocomial infections, also called hospital-acquired infections are defined as infections acquired during hospital care which is not present or incubating at admission. Infections occurring more than 48 hours after admission are usually considered nosocomial (WHO, 2002).

The most important Gram-negative bacilli in hospital infections are *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Acinetobacter* spp. *and Serratia* spp. (Bicmen *et al*., 2004).

Such infections may occur as an outbreak (epidemic) or may become established as a regular occurrence (endemic) (Paterson and Bonomo, 2005). Endemic infections are most common, epidemic infections occur during outbreaks, an outbreak is defined as an unusual or unexpected increase of cases of a known nosocomial infection or the emergence of cases of a new infection (WHO, 2002). Outbreaks may be insidious and may be protracted causes of substantial morbidity and mortality. They occur in all health care settings and with all classes of infectious agents, especially antibiotic-resistant bacteria (Weinstein, 2001). At least 5-10 percent of infections occur in clusters, or outbreaks, that can be detected from careful review of surveillance information (Gaynes *et al*., 2001).

Antibiotic resistant bacteria have spread widely and have become a major cause of nosocomial infections associated with high mortality rates (Kim *et al.*, 2002). Large nosocomial outbreaks caused by β-lactamases producing Gram-negative bacilli have been reported, with *E. coli* and *K. pneumoniae* being the most frequently involved pathogens (Hernandez *et al*., 2005).

Four types of infection account for more than 80% of all nosocomial infections: urinary tract infection (usually catheter-associated) accounting

for about 35% of nosocomial infections but carry the lowest mortality, surgical-site infections are second in frequency (about 20%), bloodstream infection (usually associated with the use of an intravascular device), and pneumonia (usually ventilator-associated) are less common (about 15% each) but are associated with much higher mortality (Weinstein, 2001).

Reported risks for nosocomial infection, many of which are linked, include an increased length of stay in the hospital particularly in intensive care unit, severity of illness, prior administration of an oxyimino-β-lactam antibiotics, prior administration of any antibiotic (Ali shah *et al*., 2004). Some important factors related to acquisition and infection with β-lactamases producing organisms are seriously ill patients with prolonged hospital stays, in whom invasive medical devices are present (Paterson and Bonomo, 2005), several outbreaks have been reported in hospitals where patients transfer is high which enhances the dissemination of such organisms (Paterson, 2005).

Although β-lactamases producing organisms can be introduced into intensive care units, but infections can occur in almost any area of the hospital (Livermore, 2003), as well as in long-term care facilities and nursing homes (Kassis- Chikhani *et al.*, 2004).

The history of infectious disease can be divided into 3 eras: the pre-antibiotic era, the antibiotic era, and the era of emerging infectious diseases (Peterson, 2005). Several studies have found a relationship between concentrated use of third-generation cephalosporins which is the most prominent risk factor for emergence of β-lactamases-producing pathogens and acquisition of a β-lactamases-producing strain (Canton *et al*, 2008). Selective antibiotic pressure, particularly that caused by the intensive use of extended-spectrum cephalosporins and cross-transmission, has been associated with the emergence and dissemination

of β-lactamases producing members of the family *Enterobacteriaceae* (Graffunder *et al*., 2005).

2-2 β-lactam antibiotics:

β-lactams are a large group of antibiotics Table (2-1), all containing the β-lactam ring. There are four major groups, penicillins, cephalosporins, carbapenems and monobactems, which differ from one another in the nature of the additional ring attached to the β-lactam ring. In penicillins there is a five-membered thiazolidine ring, in cephalosporins a six-membered cephem ring, a double ring in carbapenems whereas in monobactams only the β-lactam ring is present. The various types of β-lactams within each group differ in the side chains attached to the core rings (Samaha-Kfoury and Araj, 2003).

Monobactams are active against Gram-negative rods but not against Gram-positive bacteria or anaerobes. The first such drug to become available was azetreonam (Rupp and Fey, 2003). Imipenem the first drug of the carbapenems, has good activity against many Gram-negative rods, Gram-positive organism and anaerobes. It is very stable in the presence of bacterial β-lactamase (Mandell and Petri, 1996). Clavulanic acid, a naturally occurring β-lactam, was the first inhibitor, which is produced by *Streptomyces clavuligerus* in 1977 (Reading and Cole, 1977). Subsequently, a few more inhibitors sulbactam, a penicillanic acid sulphone and tazobactam, etc., were found (Aswapokee Neu, 1978). Amoxicillin-clavulanate is a β-lactam- β-lactamase inhibitor combination that has antimicrobial activity against Gram-positive, Gram-negative, and anaerobic organisms (Appelbaum *et al.*, 1986).

The β-lactamases confer significant antibiotic resistance to their bacterial hosts by hydrolysis of the amide bond of the four-membered β -lactam ring **(**Gupta, 2007). Over the last decades many new β-lactams have been developed that were specifically designed to be resistant to hydrolytic

actions of β-lactamase (Ahmad *et al*., 1999). Development of the "third generation" cephalosporins in the early 1980s was based heavily on the ability of these agents to escape hydrolysis by all the common β-lactamases in both Gram-positive and Gram-negative bacteria (Sykes and Bush, 1983).

2-3 Mechanism of β-lactams action:

β-lactam antibiotics, including penicillins, cephalosporins, monobactams, and carbapenems target transpeptidase enzymes that synthesize the bacterial cell wall and act cytostatically on bacteria by inactivating peptidoglycan transpeptidases irreversibly. The desirable attributes of this class of antibiotic arise from the facts that these enzymes are localized to the outer leaflet of the bacterial cytoplasmic membrane (i.e. are relatively accessible) and that they are specific to bacteria (with no functional or structural counterpart in the human host) (Walther-Rasmussen and Hoiby, 2006**)**.

The transpeptidases catalyze the cross-linking of the peptidoglycan polymers in the bacterial cell wall (Lee *et al*., 2001). In the presence of the antibiotic, the transpeptidases form a lethal covalent penicilloyl-enzyme complex that serves to block the normal transpeptidation reaction and inhibition of the polymerization process. This results in weakly cross-linked peptidoglycan, which makes the growing bacteria highly susceptible to cell lysis and death (Wilke *et al*., 2005). The transpeptidases are members of the family of penicillin binding proteins (PBPs) from which β-lactamases are likely to have evolved (Massova *et al*., 1998).

2-4 Bacterial resistance to β-lactam antibiotics:

Resistance to β-lactam antibiotics in bacteria could be due to four mechanisms:

2-4-1 Antibiotic hydrolysis by β-lactamases:

Many antibiotics have hydrolytically susceptible chemical bonds (e.g. esters and amides). Several enzymes are known to destroy antibiotic activity by targeting and cleaving these bonds. These enzymes can often be excreted by the bacteria, inactivating antibiotics before they reach their target within the bacteria. The classical hydrolytic amidases are the β-lactamases that cleave the β-lactam ring of the penicillin and cephalosporin antibiotics. Many Gram-negative and Gram-positive bacteria produce such enzymes, and more than 470 different β-lactamases have been identified (Wright, 2005). They are most commonly detected in *E. coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*, but have also been found in other Enterobacteriaceae (Ali Shah *et al*., 2004).

2-4-2 Decreased affinity of the target PBPs:

The peptidoglycan component of the bacterial cell wall provides an excellent selective target for the antibiotics. The presence of mutations in the penicillin-binding domain of penicillin-binding proteins (PBPs) results in decreased affinity to β-lactam antibiotics (Dowson *et al*., 1994). This mechanism of resistance has arisen in a number of clinically important Gram-negative and Gram-positive bacteria (Dowson and Coffey, 2000). It is now a major cause of resistance in several pathogens including the problematic Gram-positive Staphylococcal and Streptococcal species (Wilke *et al*., 2005).

2-4-3 Outer membrane (OM) permeability changes:

Gram-negative bacteria possess an outer membrane consisting of an inner layer containing phospholipids and an outer layer containing the lipid A moiety of lipopolysaccharides (LPS). This composition of the outer membrane (OM) slows down drug penetration, and transport across the (OM) is achieved by porin proteins that form water-filled channels.

Antibiotics such as β-lactams and fluoroquinolones enter the Gram-negative outer membrane via porins (Nikaido, 2003).

2-4-4 Efflux pumps:

Both Gram-positive and Gram-negative bacteria can possess single-drug and/ or multiple drug efflux pumps (Langton *et al*., 2005). In some organisms (*P. aeruginosa* in particular), an active efflux system can reduce the intracellular accumulation of antibiotic and allow an enzyme with only limited hydrolytic capacity to inactivate the drug before it can reach its target. Although some are drug-specific, many efflux systems are multidrug transporters that are capable of expelling a wide spectrum of structurally unrelated drugs, thus contributing significantly to bacterial multidrug resistance (MDR) (Van Veen and Konings, 1997). Some bacteria produce an alternative metabolic pathway and become refractory to specific antibiotics by bypassing the inactivation of a given enzyme this mechanism of resistance is called: target bypass (Tenover, 2006).

These mechanisms depend on the nature of the antibiotic, its target site, bacterial species and whether it is mediated by a resistance plasmid or by a chromosomal mutation (Dzidic *et al*., 2008). Resistance to third-generation cephalosporins in *E. coli* and K. *pneumoniae* is often attributed solely to β-lactamases production. However, other factors must also be considered, the combined contributions of porin mutations, quantity of enzyme, activity and number of β-lactamases per strain (Bush, 2001).

2-5 Occurrence and spread of β-lactamases:

The first β-lactamase originating from enterobacteria was discovered and purified in 1964 from the *E. coli* strain isolated from the haemoculture of a man suffering from septicaemia. It was marked as TEM-1 β-lactamase, and in the following years TEM-1 as well as TEM-2 β-lactamases were

discovered in almost all *E. coli* strains resistant to penicillins and cephalosporins of the first generation. Soon SHV-1 β-lactamases were discovered in strains of *Klebsiella* species resistant to β-lactams. After discovering OXA-lactamases it was clear that Gram-negative bacteria possess several different β-lactamase production mechanisms (Livermore and Paterson, 2006).

By 1975, all three types of β-lactamases were discovered in almost all strains of enterobacteria resistant to penicillines and cephalosporins, and reports on the discovery of these strains have been submitted from all continents. For this reason the cephalosporins with an extended spectrum of action have been produced, the so-called oxyiminocephalosporins (cefotaxime, ceftazidime, ceftriaxone) which were resistant to TEM-1, SHV-1 and OXA β-lactamases (Thomson *et al*., 2001; Walsh, 2003). These antibiotics were used in treating patients suffering from intrahospital septicemia caused by enterobacteria resistant to older generations of penicillin and cephalosporins. However, as soon as 1983, the first strains of *E. coli* and *Klebsiella* spp. resistant to this new group of antibiotics were discovered. During investigations on this form of resistance, completely unknown enzymes belonging to the β-lactamase group were isolated, entitled ESBL, i.e. extended spectrum β-lactamase (due to the ability of dissolving cephalosporins and penicillins with extended spectrum of action). The production of ESBL is coded by mutated genes responsible for the production of TEM-1, TEM-2, SHV-1 and OXA β-lactamases (Livermore and Paterson, 2006).

Within the last 5 years, however, new variants of ESBL have been discovered, which do not originate from TEM, SHV and OXA lactamases, and are marked as CTX-M, PER, VEB and other β-lactamases. The genes responsible for the production of ESBL are most frequently located on plasmids by which they are easily transferred by

mechanisms of conjugation and transduction to other enterobacteria in nature. Bacterial strains which possess genes to produce ESBL most often are multiresistant and also carry genes responsible for the resistance to most other antibiotics, including aminoglycosides, sulfamethoxazole-trimethoprim and fluoroquinolones. Therefore, practically the biggest contemporary clinical problems are infections of humans and animals caused by ESBL-producing strains of *E. coli, Klebsiella, Enterobacter, Proteus, Serratia, Citrobacter, Salmonella* and *Shigella* species (Baraniak *et al*., 2002).

Enzymes from the ESBL group do not work on 7-α-methoxy cephalosporines, the so-called cephamycines (cefoxitin and cefotetan), as well as carbanepems which remain the only medicine of choice to treat infections caused by ESBL-producing strains, since ESBL strains resistant to imipenem, meropenem and ertapenem have not yet been discovered (Thomson *et al*., 2001).

The strains which produce ESBL show varying sensitivities to β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam). Enzymes belonging to the ESBL group do not work on cefepime, which belongs to 4th generation cephalosporins. Strains of *Enterobacter, Citrobacter, Morganella* and *Serratia* species have been discovered which are resistant also to oxyimino-cephalosporins, but this is an inducible resistance and relates to the development of chromosomal mutations responsible for the production of AmpC β-lactamases which do not belong to the ESBL (Weinbren and Borthwick, 2005).

The chromosomal carbapenemases may have evolved initially as a mechanism for bacteria to protect themselves from external threats to their cell wall (Bush, 1997), but in addition these β-lactamases may also play a role in the regulation of cell wall synthesis (Normark, 1995).

During the past decade, a number of acquired metallo-β-lactamases (MBLs) have been identified in Gram-negative pathogens and were categorized into 2 major types: IMP and VIM (Bush, 2002). IMP-1 was the first MBL identified in *P. aeruginosa* (Senda *et al*., 1996). The MBLs are considered to be potentially particularly dangerous because they are active toward the carbapenems, which are often the "last-resort" drugs for multidrug-resistant pathogens (Hall *et al*., 2003).

2-6 Mechanism of action of β-lactamases:

β-lactamases have been designated by the Nomenclature Committee of the International Union of Biochemistry as ‘‘enzymes hydrolyzing amines, amides and other CON bonds . . . separated on the basis of the substrate: . . . cyclic amides’’ (Payne *et al*., 1990). These enzymes are the major cause of bacterial resistance to β-lactam antibiotics. Once expressed, in Gram-negative bacteria these enzymes are secreted into the periplasmic space, where they attack the antibiotic before it can reach its receptor site, bound to the cytoplasmic membrane, or excreted (in Gram-positive bacteria) (Mark *et al*., 2005).

β-lactamases enzymes are able to hydrolyze the β-lactam ring using two types of nucleophile agents (Fisher *et al*., 2005), these are either a serine residue (as in class A, C and D β-lactamases) or a Zn(II)-bound water/hydroxide group (class B or metallo-β-lactamases, MBL’s). In serine β-Lactamases the enzyme first associates noncovalently with the antibiotic to yield the noncovalent Michaelis complex. The β-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 (Hata *et al*., 2006).

Metallo-β-lactamases, a less commonly encountered group of β-lactamases in which a divalent transition metal ion, most often zinc, linked to a histidine or cysteine residue react with the carbonyl group of the amide bond of most penicillins, cephalosporins, and carbapenems, but not monobactams (Walsh *et al*., 2005; Bebrone, 2007).

2-7 Classification of β-lactamases:

Because the number of TEM- and SHV-derived β-lactamases continues to increase, β-lactamases can be classified based on two major approaches. One is based on the biochemical and functional characteristics of the enzymes (Bush-Jacoby-Medeiros classification) and the second is based on the molecular structure of the enzyme (Ambler classification).

Functional classification of the β-lactamases is based on spectrum of antimicrobial substrate profile, enzyme inhibition profile, enzyme net charge, hydrolysis rate and other parameters. Bush *et al.* (1995) presented this classification based on 4 major groups (1-4) and subgroups (a-f). According to this classification, most ESBLs belong to group 2 be, which is β-lactamases inhibited by clavulanic acid, which can hydrolyze penicillins, narrow and extended spectrum cephalosporins and monobactams.

In molecular classification β-lactamases can be classified into four different molecular groups, A, B, C and D, according to amino acid sequence identities (Ambler, 1980; Huovinen *et al*., 1998). Class A, C (AmpC) and D β-lactamases use a catalytically active serine residue for inactivation of the β-lactam drug (Lamotte-Brasseur *et al*., 1994). The enzymes assigned to the molecular class B are metallo-enzymes requiring zinc for their catalytic activity, and they operate through a completely different mechanism (Majiduddin *et al*., 2002).

2-8 Genetic factors controlling production of β- lactamases:

2-8-1 Chromosomal β-lactamases:

Many *Enterobacteriaceae* have chromosomal-mediated β-lactamases. The most important are the AmpC β-lactamases which belong to class C β-lactamases. These AmpC β-lactamases occur in *P. aeruginosa*, most enterobacteria except *Salmonella* spp. and some *Klebsiella* spp. *E. coli* produces small amounts of AmpC β-lactamases regardless of whether β-lactam antibiotics are present. This small amount of enzyme is insufficient to inhibit the action of the β-lactam. Hyperproduction of AmpC β-lactamase in *E. coli* can arise as a result of two mutational events which occur rarely. AmpC β-lactamases are clinically important when hyperproduced because they may confer resistance to a wide variety of β-lactams such as cefoxitin, aztreonam, third generation cephalosporins, and β-lactam/clavulanic acid combinations (Shahid *et al*., 2004). Other species including *P. aeruginosa* and *Enterobacter* spp. exhibit inducible expression of AmpC β-lactamases and show much greater potential for resistance. In recent years evidence of plasmid-mediated AmpC β-lactamases has emerged. Such plasmids have "escaped" from the chromosomes of *Enterobacteriaceae* and have reached *K. pneumoniae* (Livermore and Brown, 2004).

Subclass B2 MBL consists entirely of chromosomally located genes of Gram-negative bacteria, while subclass B1 includes two chromosomally located genes of Gram-positive organisms (*Bacillus cereus* and *Bacillus anthracis*), suggesting the likelihood of horizontal transfer into those Gram-positive chromosomes. The subclass B3 MBL genes are all in Gram-negative organisms, and most are located within chromosomes (Hall *et al*., 2003).

Another type of chromosomal-mediated β-lactamase is K1. Production of K1 can arise in *Klebsiella oxytoca* via mutation. This enzyme is a class A

type. Unlike the AmpC enzyme, hyperproduction of K1 β-lactamases characteristically confers resistance to aztreonam, cefpodoxime and moderately resistance to ceftriaxone but is inactive against ceftazidime (Paterson and Bonomo, 2005).

2-8-2 Plasmid-mediated β-lactamases:

The first plasmid-mediated β-lactamase in Gram-negative bacilli, TEM-1, was described in the early 1960s and was originally found in a single strain of *E. coli* isolated from a patient in Greece (Datta and Kontomichalou, 1965). While the first plasmid-mediated β-lactamase capable of hydrolyzing extended-spectrum cephalosporins, SHV-2, was reported in 1983 (Knothe *et al.*, 1983). Today, TEM-1 is the most common β-lactamase in Gram-negative bacilli found in many different species of members of the family *Enterobacteriaceae*, *P. aeruginosa*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae* (Bush, 2001; Bradford, 2001).

Other plasmid-mediated β-lactamases found in Gram-negative bacilli include the OXA-type β-lactamases, which are mainly found in *P. aeruginosa* but in recent years their incidence in *Enterobacteriaceae* has increased. The most common OXA-type β-lactamase, OXA-1 has been found in 1 to 10% of *E. coli* (Paterson and Bonomo, 2005).

Plasmids are responsible for the spread of most of the new β-lactamases, but the genes encoding these enzymes may also be located on the bacterial chromosome. Currently, the most common new β-lactamases are ESBLs in the TEM, SHV, and CTX-M families are found most often in *Klebsiella* spp. and *E. coli*, although they have been detected in many other Gram-negative pathogens (Jacoby and Munoz-Price, 2005). The CTX-M family is associated with plasmid acquisition of β-lactamase genes that facilitate the spread of CTX-M genes between host bacteria (Bonnet, 2004; Canton and Coque 2006).

In the late 1980s, inducible chromosomal AmpC genes were detected on plasmids (most without induction capabilities) and were transferred to organisms, which typically do not express chromosomal β-lactamases such as *Salmonella* spp. and some *Klebsiella* spp. (Hanson, 2003; Livermore and Brown, 2004).

Many genes may be found in integrons. Sometimes, β-lactamases markers are part of the integron that has gene cassettes encoding resistance genes for other classes of antibiotics as well (Poirel *et al*., 2000). Integrons are involved in the acquisition of AmpC type β-lactamases by plasmids (Philippon *et al*., 2002).

2-9 Types of β-lactamases:

2-9-1Extended spectrum β-lactamases (ESBLs):

Extended spectrum β-lactamases are known as extended-spectrum because they are able to hydrolyze a broader spectrum of β-lactam antibiotics than the simple parent β-lactamases from which they are derived. They are acquired plasmid-mediated β-lactamases. They have the ability to inactivate β-lactam antibiotics containing an oxyimino-group such as oxyimino-cephalosporins (e.g., ceftazidime, ceftriaxone, cefotaxime) as well as oxyimino-monobactam (aztreonam). They are not active against cephamycins and carbapenems. Generally, they are inhibited by β-lactamase-inhibitors such as clavulanate (Paterson and Bonomo, 2005). ESBLs are plasmid-mediated bacterial enzymes derived from older, narrow spectrum β-lactamases (e.g. TEM-1, TEM-2, SHV-1), which have an extended substrate profile that permits hydrolysis of all cephalosporins, pencillins, and aztreonam. These enzymes are most commonly produced by *Klebsiella* spp. and *E. coli* but may also occur in other Gram-negative bacteria (Paterson, 2006).

Extended spectrum cephalosporins (third generation cephalosporins such as ceftriaxone and cefotaxime) gained widespread clinical use in the early 1980s and were developed because of the increasing prevalence of ampicillin-hydrolyzing β-lactamases (TEM-1, TEM-2 and SHV-1) in *Enterobacteriaceae*, non-glucose fermenting Gram-negative bacilli, and some respiratory pathogens such as *Haemophilus influenzae* and *Moraxella catarrhalis* (Rupp and Fey, 2003). They were first recognized in a single strain of *K. pneumoniae* isolated in Germany (Knothe *et al*., 1983). ESBL are created by substituting one or more amino acids in the TEM, SHV and OXA lactamases molecules. So far over 200 new ESBL group enzymes have been discovered, which are responsible for the resistance to all cephalosporins including 3rd generation cephalosporins, as well as to all penicillins and aztreonam (Gupta, 2007).

The most important are the spectrum-extending mutations which result in the expansion of the active site that allows the increased activity against extended spectrum cephalosporins and may result in the increased susceptibility to β-lactamase inhibitors (Bush, 2001).

Each TEM-derived ESBL has a slightly different substrate profile in which one ESBL may hydrolyze a specific extended-spectrum cephalosporin more efficiently than another ESBL (Rupp and Fey, 2003). As with TEM, SHV-type ESBLs have one or more amino acid substitutions around the active site. More than 88 varieties of SHV are currently recognized on the basis of unique combinations of amino acid replacements (Jcoby, 2006).

2-9-2 AmpC β-lactamases:

AmpC β-lactamases are active-site serine enzymes that are primarily cephalosporinases, they confer resistance to cephalosporins in the oxyimino group, 7-α-methoxy cephalosporins and are not affected by available β- lactamase inhibitors (Thomson, 2001). Organisms expressing

these enzymes are not resistant to third-generation cephalosporins unless the AmpC β-lactamase is expressed at high levels (Navarro, 2006).

AmpC β-lactamases are of two types, plasmid-mediated and chromosomal or inducible. Chromosomal AmpC enzymes are seen in organisms such as *Citrobacter freundii*, *Enterobacter cloacae*, *Morganella morganii*, *Hafnia alvei* and *Serratia marcescens* and are poorly induced (if at all) by the third- or fourth-generation cephalosporins (Philippon *et al.*, 2002). In many species, inducible AmpC β-lactamases are normally produced at very low levels but are typically induced to several hundred fold higher by the presence of β-lactams (e.g. cefoxitin, cefotaxime, etc.) and certain β-lactam inhibitors (e.g. clavulanic acid) (Arora and Bal, 2005).

The AmpC enzyme in *E. coli* is poorly expressed and the AmpC gene is missing from the chromosome of *Klebsiella* and *Salmonella* species, but plasmid-mediated AmpC enzymes can give these organisms the same resistance profile as a β-lactam-resistant *Enterobacter* isolates (Jacoby and Munos- Price, 2005). More than 40 different AmpC β-lactamases have been found to be mediated by plasmids (Philippon *et al*., 2002).

2-9-3 Carbapenemases:

β-lactamase enzymes can be broadly divided into those with a serine residue at the active site and metallo-enzymes utilizing zinc as a co-factor (Garau *et al*., 2004). Ambler originally recognized these two groups in 1980 when he classified the serine β-lactamases as Class A and the metallo-β-lactamases as Class B (Ambler, 1980). Carbapenems-hydrolyzing β-lactamases can be metallo β-lactamases, extended-spectrum oxacillinases, or Ambler class A enzymes (Poirel *et al*., 2004; Queenan and Bush 2007). They belong to two major molecular families, distinguished by the hydrolytic mechanism at the active site, serine and metallo-Carbapenemases (Frere, 2005).

2-9-3-1 Serine carbapenemases:

In the mid- to late 1980s, another set of carbapenem-hydrolyzing enzymes emerged among the *Enterobacteriaceae* (Medeiros and Hare 1986), but EDTA did not inhibit their activity (Rasmussen *et al*., 1996). These enzymes utilized serine at their active sites and were inactivated by the β-lactamase inhibitors clavulanic acid and tazobactam include molecular class A and molecular class D serine carbapenemases (Queenan and Bush 2007).

2-9-3-1-1 Molecular class A serine carbapenemases:

Plasmid-mediated *Klebsiella pneumoniae* carbapenemase (KPC) family have the ability to hydrolyze a broad variety of β-lactams, including carbapenems, cephalosporins, penicillins, and aztreonam, and all are inhibited by clavulanate and tazobactam (Queenan and Bush, 2007).

2-9-3-1-2 Molecular class D serine carbapenemases:

The first OXA β-lactamase with carbapenemase activity was described by Paton *et al*. in 1993 (Paton *et al*., 1993). OXA (for "oxacillin-hydrolyzing") β-lactamases were functionally described as penicillinases capable of hydrolyzing oxacillin and cloxacillin (Bush, 1988). They are in general poorly inhibited by clavulanic acid and EDTA and known to have a large amount of variability in amino acid sequences (Bush *et al*., 1995). Currently there have been 102 unique OXA sequences identified (http://www.lahey.org /Studies), of which 9 are ESBLs and at least 37 are considered to be carbapenemases (Walther-Rasmussen and Hoiby 2006).

2-9-3-2 Metallo-carbapenemases:

Metallo-β-lactamases (MBL) belong to class B of the Ambler molecular classification (Bush, 1998). This class of β-lactamases is characterized by the ability to hydrolyze carbapenems and by its resistance to β-lactamase inhibitors (clavulanic acid and tazobactam) but susceptibility to inhibition

by metal ion chelators. In addition to the carbapenems, most of these enzymes hydrolyze cephalosporins and penicillins but lack the ability to hydrolyze aztreonam. The mechanism of hydrolysis is dependent on interaction of the β-lactams with zinc ions resulting in the distinctive trait of their inhibition by EDTA, a chelator of Zn (II) and other divalent cations (Walsh, 2005).

These enzymes contain at least one zinc atom at the active site that serves to facilitate hydrolysis of a bicyclic β-lactam ring, and are divided into three subclasses (B1, B2, and B3) based on a combination of structural features, zinc affinities for the two binding sites, and hydrolysis characteristics, subclasses B1 and B3 bind two zinc atoms for optimal hydrolysis, while enzymes in subclass B2 are inhibited when a second zinc is bound. Subclass B2 also differs in hydrolysis spectrum, it preferentially hydrolyzes carbapenems, in contrast to the broad hydrolysis spectrum observed for B1 and B3 enzymes (Frere, 2005).

2-9-4 Inhibitor resistant β-lactamases:

Another group of β-lactamases, called the inhibitor resistant β-lactamases, have been isolated with increasing frequency (Bradford, 2001). The inhibitor resistant β-lactamases are mostly TEM-derived, are resistant to the inhibition of clavulanic acid and sulbactam, but not tazobactam (Chaibi *et al*., 1999).

Chapter three

3-1 Materials and Methods

3-1-1 Equipments:

Type of equipment	Manufacture (Origin)
Autoclave	Arnold and Sons (USA)
Centrifuge	Memmert (Germany)
Bench centrifuge	Hettich (Germany)
Cold centrifuge	Hettich
Distillator	GFL (Germany)
Digital camera	Sony (Japan)
Electric oven	Memmert
Electrophoresis	Taiwan
Incubator	Memmert
Light microscope	Olympus (Japan)
Micropipette	Oxford (USA)
Millipore filter (0.22µm)	Difco (USA)
PCR system	Singapore
pH-meter	LKB (Sweden)
Standard loop 0.01 ml	Hemedia (India)
Sensitive balance	Memmert
Refrigerator	Ishtar (Iraq)
Ultra high speed centrifuge	Hettich
UV-transilluminator	Taiwan
Vortex	Memmert
Compound light microscope	Olympus (Japan)
Water bath	Memmert

3-1-2 Biological and Chemical Materials:

Material	Manufacturer (Origin)
Agarose	BDH (England)
Acetic acid	Difco (USA)
Beef extract	Difco
Bromophenol blue	Difco
Barium chloride ($BaCl_2$)	Fluka (Switzerland)

Cloroform	BDH
Disodium hydrogen phosphate (Na_2HPO_4)	BDH
EDTA	AppliChem (Germany)
Ethidium bromide	Sigma (USA)
Ethanol (96%)	BDH
Glucose ($C_6H_{12}O_6$)	Difco
Glycerol ($C_3H_8O_3$)	Fluka
Hydrochloric acid (HCl)	BDH
Isopropyl alcohol	Mast Diagnostic (USA)
Iodine	Mast Diagnostic
Kovac's reagent	BDH
Maltose	Difco
Methyl red	BDH
D-mannose ($C_6H_{12}O_6$)	Difco
α-naphthol ($C_{10}H_8O$)	BDH
Peptone	Difco
Phenol red	BDH
Potassium hydroxide (KOH)	BDH
Potassium diydrogen phosphate (KH_2PO_4)	BDH
Potassium iodide (KI)	Mast Diagnostic
Sucrose ($C_{12}H_{22}O_{11}$)	Difco
Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	BDH
Sodium chloride (NaCl)	BDH
Sodium hydroxide (NaOH)	BDH
Sodium carbonate (Na_2HCO_3)	BDH
Starch	Difco
Sodium dodecyl sulfate (SDS)	AppliChem
Tris-(hydroxymethyl) methylamine $NH_2.(CH_2OH)_3$ (Tis-OH)	BDH
Urea solution	Mast Diagnostic
Yeast extract	Difco
D-xylose	Difco
Xylene xyanol	Difco

3-1-3 Culture Media:

Medium	Manufacturer (Origin)
Brain heart infusion broth	Hemedia (India)
Blood agar	Hemedia
Eosin methylen blue agar	Hemedia
MacConkey agar	Hemedia
Muller-Hinton agar	Hemedia
MR-VP broth	Hemedia
Nutrient broth	Hemedia
Nutrient agar	Hemedia
Peptone water	Hemedia
Simmons citrate agar	Hemedia
Tryptic soy broth	Alpha (USA)
Triple sugar iron agar	Hemedia
Urea agar base	Hemedia

3-1-4 Antibiotics:

3-1-4-1 Antibiotic powders:

Antibiotic	Manufacturer (Origin)
Penicillin G	Jabir Bin Hayan (Iran)
Ampicillin	MAH Import Export GmbH (Germany)
Amoxicillin	Panpharma S.A. (France)
Co-amoxi-clav	Julphar (Ras AL-Khaimah)
Cefotaxime	Elsaad (Syria)
Ceftazidime	Julphar (Ras AL-Khaimah)
Ceftriaxone	XIER Pharmaceutical Co. (Germany)

3-1-4-2: Antibiotic Disks (Bioanalyse, Turkey).

Antibiotic disks				
Class	**Subclass**	**Agent used**	**Abbreviation**	**Content**
Penicillins	carboxypenicillin	Carbenicillin	PY	100 µg
	ureidopenicillin	Piperacillin	PRL	100 µg
β-Lactams /β-lactamase inhibitor combinations		Amoxi-clav	AMC	30 µg
Cephems	cephamycin	Cefoxitin	FOX	30 µg
	cephalosporin III	Ceftazidime	CAZ	30 µg
		Cefotaxime	CTX	30 µg
		Ceftriaxone	CRO	30 µg
	cephalosporin IV	Cefepime	FEP	30 µg
Penems	carbapenem	Imipenem	IMP	10 U
Monobactams		Aztreonam	ATM	30 µg
Aminoglycosides		Amikacin	AK	30 µg
		Gentamicin	CN	10 µg
		Tobramycin	TOB	10 µg
Quinolones	Quinolones	Nalidixic acid	NA	30 µg
	Fluoroquinolones	Ciprofloxacin	CIP	5 µg

3-1-5: Standard bacterial strain.

Standard Strain	Laboratory identifier	Key characteristics	Source
Escherichia coli	ATCC 25922	Susceptible to ampicillin, cephalosporins and gentamicin	Dept. of Microbiology, College of Medicine, Isfahan University for Clinical Sciences, Iran

3-2 Methods:

3-2-1 Collection of samples:

This study was conducted at Merjan Teaching Hospital, Babylon province, Iraq from April to July, 2009. A total of 210 hospital environment samples (floor, walls, doors, stairs, benches, beds, tables, waste containers, and medical equipment) were taken from intensive care unit, clinics, emergency department, wards, laboratories, laundry units, pharmacy, and kitchen. All samples were collected by sterile cotton swabs damped with normal saline.

Each sample was streaked on MacConkey agar and incubated at 37˚C for 24 hr, culture results were interpreted as being lactose fermenting and non-fermenting bacteria. Lactose-fermenters isolates were subcultured, incubated for additional overnights, then the colonies identified using classical morphological and biochemical tests.

3-2-2 Preparation of buffers and solutions:

Buffers and solutions which require sterilization were autoclaved at 121˚C for 15-20 minutes. Heat sensitive solutions like antibiotics and urea were sterilized by filtration using Millipore filters (0.22 µm). The pH of the solution was adjusted using 1M NaOH and 1M HCL. In the present study, the following buffers and solutions were used:

3-2-2-1 MacFarland turbidity standard (0.5):

MacFarland turbidity standard was prepared as follows: 0.5 ml of 0.048

M barium chloride was added to 99.5 ml of 0.36 N H_2SO_4. 5 ml of it was aliquoted into screw-capped tubes of the same size which were used in the procedure. The standard was stored in dark, at room temperature and was vortexed prior to use (NCCLS, 2003).

3-2-2-2 Saline solution:

Sodium chloride (0.85 gm) was dissolved in 90 ml D.W. and further completed to 100 ml with D.W (Collee *et al*., 1996).

3-2-2-3 Phosphate buffer solution (PBS):

This buffer consisted of two solutions and prepared as follows:

Solution A: 3.12 gm of $NaH_2PO_4.2H_2O$ was dissolved in 90 ml of D.W. and then completed to 100 ml with D.W.

Solution B: 2.839 gm of Na_2HPO_4 was dissolved in 90 ml of D.W. and the volume was completed to 100 ml. Then 87.7 ml of solution A was added to 12.3 ml of solution B, and the pH was adjusted to 6. The buffer was used for the detection of β-lactamase production (Collee *et al.*, 1996).

3-2-2-4 β-lactam antibiotic solutions:

The solutions were prepared as stock solutions with concentration of 10 mg/ml by dissolving 1gm of the antibiotic in a small volume of sterile PBS (pH 6) for ampicillin, amoxicillin and amoxi-clav, or by dissolving in sterile D.W. for cefotaxime and ceftriaxone, while ceftazidime was solubilized in Na_2HCO_3. Each of these solutions was further diluted with sterile D.W. to volume 100 ml, then stored at 4˚C (CLSI, 2007).

3-2-2-5 Solutions used for β-lactamase detection:

These solutions were prepared according to Collee *et al.* (1996) as follows:

Pencillin G solution: this was prepared by dissolving 0.569 gm of penicillin G in PBS (3-2-1-3**)**. The solution was sterilized, dispensed in small vials, and stored at -20˚C.

Starch solution: this solution was prepared by dissolving 1gm of soluble starch in 100 ml of D.W. and boiled in water bath for 10 minutes, and stored in a dark bottle at 4˚C.

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

3-2-2-6 DNA extraction solutions:

These solutions were prepared as described by Pospiech and Neumann (1995) with some modifications:

3-2-2-6-1 Tris EDTA buffer (TE buffer):

This buffer was prepared by dissolving 0.05 M Tris-OH and 0.001 M EDTA in 800 ml D.W., the pH was adjusted to 8 and completed to one litter by D.W. then sterilized by autoclaving and stored at 4˚C until used.

3-2-2-6-2 SDS solution (25%):

Sodium dodecyl sulfate (25 mg) was dissolved in 100 ml of D.W., then sterilized in autoclave at 121˚C for 15 minutes and stored at 4˚C.

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

Sodium chloride (14.625 gm) was dissolved in 50 ml D.W., autoclaved and stored at 4˚C.

3-2-2-6-4 Phenol: Chloroform: Isoamyl alcohol (25:24:1):

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

3-2-2-7 Solutions used in gel electrophoresis:

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

3-2-2-7-1 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, autoclaved, and stored at 4˚C.

3-2-2-7-2 Ethidium bromide solution:

Stock solution was prepared by dissolving 0.05 gm of ethidium bromide in 10 ml of D.W. and stored in dark reagent bottle.

3-2-2-7-3 DNA loading buffer:

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

3-2-3 Preparation of reagents:

The following reagents were prepared as described by MacFaddin (2000).

3-2-3-1 Methyl red reagent:

This reagent was prepared by dissolving 0.1 gm f methyl red in 300 ml of 96% ethanol and then completed to 500 ml with D.W. This reagent was used as indicator in methyl red test.

3-2-3-2 Voges-Proskauer reagents:

The reagents were prepared as follows:

Reagent A: 5% α-naphthol in 96% ethanol.

Reagent B: 40% KOH in D.W.

These reagents were used as indicators in Voges-Proskauer test.

3-2-4 Preparation of culture and diagnostic media:

3-2-4-1 Ready-prepared media:

Media used in this study listed in (3-1-3) were prepared in accordance with the manufacturer's instructions fixed on their containers. All the media were sterilized by autoclaving at 121˚C for 15 minutes. After sterilization, blood agar base was supplemented with 7% human blood, and urea agar base was supplemented with 20% sterile urea solution.

3-2-4-2 Laboratory prepared culture media:

3-2-4-2-1 Carbohydrates fermentation medium:

This medium was prepared according to MacFaddin (2000) as follows:

a- Basal medium:

Beef extract	1gm
Pepton	10 gm
NaCl	5 gm
Phenol red	0.018 gm
D.W.	1000 ml

The pH was adjusted to 7.4, then distributed in to test tubes and a durham tube was located at the bottom of each tube. The tubes were then autoclaved at 121˚C for 15 minutes, and cooled to 56 ˚C in the water bath.

b- Carbohydrate sources:

The following carbohydrate sources were used: sucrose, glucose, D-xylose, maltose, D-mannose, glycerol, and starch.

c- Carbohydrate solutions were sterilized by Millipore filters (0.22µm), they were separately added to the basal medium to give a final concentration of 1% of each source. The carbohydrate fermentation medium was used to test the ability of bacterial isolates to ferment a specific carbohydrate incorporated in a basal medium.

3-2-4-2-2 Motility medium:

Agar-agar (0.5 gm) was dissolved in to 100 ml of brain heart infusion broth, the contents were dispensed in to test tubes and autoclaved at 121˚C for 15 minutes (MacFaddin, 2000).

3-2-4-2-3 Maintenance medium:

This medium consisted of nutrient broth as a basal medium, supplemented with 15% glycerol. After autoclaving at 121˚C for 15 minutes, and cooling to 56˚C in water bath, 5 ml amounts were distributed in sterile tubes, and then kept at 4 ˚C until used. This medium was used to preserve the bacterial isolates at deep freeze for long term storage (Collee *et al*., 1996).

3-2-5 EDTA disks preparation:

Disks of EDTA were prepared by dissolving 190 mg of EDTA (EDTA powder must completely dissolved) in 1 ml of D.W., and adjusting it to pH 8, the mixture sterilized by autoclaving. EDTA solution was added to a 6-mm Whatmann filter No.1 disks (n=100) and allowed to dry. Each disk contain approximately 1,900 µg of EDTA and used to detection of metallo-β-lactamases producing isolates (Lee *et al*., 2003).

3-2-6 Biochemical tests:

3-2-6-1 Indole production test:

Peptone water was inoculated with a young agar culture and incubated at 37 ˚C for 24-48 hours. Few drops of Kovacs reagent were added to each tube. Formation of pink ring indicates a positive test (MacFaddin, 2000).

3-2-6-2 Methyl Red test:

Methyl Red-Voges Proskauer broth was inoculated with a young agar culture and incubated at 37˚C for 24 hr, five drops of methyl red solution was added to each tube, the result was read immediately. Changing the color to red is indicating a positive test (MacFaddin, 2000).

3-2-6-3 Voges-Proskauer test:

Methyl Red-Voges-Proskauer broth was inoculated with a young agar culture and incubated at 37˚C for 48 hr. One ml of 40% KOH solution and 3 ml of 5% solution of α-naphthol were added to each tube. A positive reaction was indicated by the development of a pink color in 20 minutes (MacFaddin, 2000).

3-2-6-4 Simmons citrate test:

Simmons citrate slant was inoculated with a young bacterial culture and incubated at 37˚C for 48-72 hr Changing the color from green to blue is indicating a positive test (MacFaddin, 2000).

3-2-6-5 Kliglers iron agar test:

With straight inoculation needle an inoculum was stabbed into the butt of the tube and streaked over the surface of the slant. Slants were incubated at 37˚C for 24 hr. Results were unformatted according to MacFaddin (2000) as follows:

Slant/Butt	Color
Alkaline/Acid	Red/Yellow
Acid/ Acid	Yellow/ Yellow
Alkaline/Alkaline	Red/Red
H_2S production	Black precipitate

3-2-6-6 Urease test:

The surface of urea agar slant was streaked with a young bacterial culture and incubated at 37˚C. Result was read after 6 hr, 24 hr, and every day for 6 days. Changing the color of medium to purple-pink indicate a positive result (McFaddin, 2000).

3-2-6-7 Carbohydrate fermentation test:

Carbohydrate fermentation broth tubes were inoculated with a young agar culture and incubated at 37˚C for 1-5 days. Changing the color of the indicator to yellow with or without gas production indicate a positive test (MacFaddin, 2000).

3-2-6-8 Motility test:

Motility medium was used for detection of bacterial motility. Tubes containing motility medium (3-2-3-2-2) were inoculated with a young bacterial culture by stabbing the center of the medium and incubated at 37˚C for 24-48 hr. Cloudy growth formation out of the line of stab indicates a positive result (MacFaddin, 2000).

3-2-6-9 Growth at 10˚C:

Bacterial culture was streaked on nutrient agar plate and incubated at 10˚C for 24 hr. Appearance of bacterial colonies indicates the ability of isolate to grow in this low temperature (MacFaddin, 2000).

3-2-7 Preservation and maintenance of bacterial isolates:

The bacterial isolates were preserved on nutrient agar slant at 4˚C. The isolates were maintained monthly by culturing on new culture media. Nutrient broth supplemented with 15% glycerol was used for long preservation and the isolates were maintained frozen for several months (long term maintenance) (Collee *et al*., 1996).

3-2-8 Identification of bacteria:

E. coli and *Klebsiella* spp. isolates were identified using the traditional morphological and biochemical tests (listed in 3-2-5) according to MacFaddin (2000). *Klebsiella* isolates were classified to the level of species using Baily and Scott's diagnostic microbiology book (Forbes *et al*., 2007).

3-2-9 β-lactam resistance testing:

Ampicillin and amoxicillin were added separately, from stock solution, to the cooled Muller-Hinton agar at final concentration of 100 and 50µg/ml, respectively. The medium poured into sterilized petridishes, then stored at 4˚C. Preliminary screening of *E. coli* and *Klebsiella* spp. isolates resistance to both antibiotics was carried out using pick and patch method on above agar plates (CLSI, 2007).

3-2-10 Production of β-lactamase:

All bacterial isolates that were resistant to β-lactam antibiotics were tested for β-lactamase production by rapid iodometric method as follows:

Several colonies of a young bacterial culture on MacConkey agar, were transferred to Eppendorf tube containing 100 µl of penicillin G solution, and the tubes were incubated at 37˚C for 30 minutes. Then, 50 µl of starch solution was added and mixed well with the content of the tube, 20 µl of iodine solution was added to the tube which cause the appearance of dark blue color, rapid change of this color to white (within few second to 2 minutes) indicated a positive result (Collee, 1996).

3-2-11 Antibiotic susceptibility testing:

Antimicrobial susceptibility testing of β-lactam resistant *E. coli* an *Klebsiella* spp. isolates was carried out against the antibiotics shown in Table (3-1-4-2) using the disk diffusion method on Muller-Hinton agar medium. The cultures were incubated at 37C for 18hr under aerobic conditions, and bacterial growth inhibition zones around the disks were measured and interpreted as recommended by the NCCLS guidelines. *E. coli* ATCC 25922 was used as the reference strain for antimicrobial susceptibility testing (CLSI, 2007).

3-2-12 Extended-spectrum β-lactamase production:

All bacterial isolates that were resistant to β-lactam antibiotics were tested for ESBL production by two methods, these tests included:

3-2-12-1 Disk approximation test:

All β-lactam resistance isolates were detected using the double disk approximation test as modified by Coudron *et al*. (1997). 30 µg antibiotic disks cefotaxime, ceftazidime, ceftriaxione, and azetreonam were placed 15 mm (edge to edge) around a central disk of amoxi-clav (20 µg amoxicillin plus 10 µg clavulanate) on Muller-Hinton agar plates seeded with organism being tested for ESBL production. Any augmentation (increase in diameter of inhibition zone) between the central amoxi-clav disk and any of the β-lactam antibiotic disks showing resistance or intermediate susceptibility was recorded, and the organism was thus considered as an ESBL producer.

3-2-12-2 Detection of bla_{CTX-M} and bla_{OXA} genes:

3-2-12-2-1 DNA preparation:

DNA preparation from bacterial cells was performed by salting out method (Pospiech and Neumann, 1995) with some modification as follows: bacterial cells of 50 ml culture were precipitated by centrifugation (1000 rpm for 10 minutes). Rewashed three 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: isoamylacohol) (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 for 10 minutes. Then the aqueous phase was transferred to a fresh tube, which contains nucleic acid. Isopropanol (0.6 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 300µl TE buffer, and then DNA extract was kept in deep freeze until use.

3-2-12-2-2 Master Mix Used in PCR:

Master mix 2x	Source
Go Tag DNA polymerase is supplied in 2x Green Taq Reaction buffer pH 8.5, 400µm dATP, 400µm dGTP, 400µm dCTP, 400µm dTTP, and 3mM $MgCl_2$	Promega (USA)

3-2-12-2-3 PCR amplification:

Polymerase chain reaction was used to amplify the entire sequence of bla_{CTX-M} and bla_{OXA} genes in plasmid preparations. The primers (Alpha DNA, Montreal) used for the amplification of these genes were: CTX-M/F (5′-GCTTTATGCGCAGACGAGTG-3′) and CTX-M/R (5′-TCATTGGTGGTG-3′); OXA/F (5′-ATATCTCTACTGTTGCATCTCC-3′) and OXA/R (5′- AAACCCTTCAAACCATCC-3′). Amplification reaction mixture was carried out in a 50µl volume using 25 µl Master mix (reaction buffer, 1 µl; nucleotide mixture dNTP, 10 mM; and Taq DNA polymerase, 0.5µl), 5 µl DNA extract, 2µl of 10 pmol/µl of upstream primers specific and, 2µl of 10 pmol/µl of downstream primers specific in the reaction buffer recommended by the enzyme manufacturer. Cycling parameters of bla_{CTX-M} were as follows: an initial denaturation at 94˚C for 5 minutes followed by 30 cycles of denaturation at 94˚C for 30 sec., annealing at 60˚C for 30 sec., elongation at 72˚C for 60 sec., and final elongation step at 72˚C for 7 minutes. For bla_{oxa} amplification, the reactions were accurse under the following conditions: an initial denaturation at 94˚C for 3 minutes, followed by 32 cycles of denaturation at 94˚C for 30 sec., annealing at 67˚C for 30 sec., elongation at 72˚C for

1 minutes, and finally, 10 minutes at 72˚C. The resulting PCR products were run in 1.5% agarose gels.

3-2-12-2-4 Agarose gel electrophoresis:

Agarose gel was prepared by dissolving 1.5 gm of agarose powder in 100 ml of TBE buffer (pH 8) in boiling water bath, allowed to cool to 50˚C, then ethidium bromide (at concentration of 0.5 mg/ml) was added. A tape was placed across the end of the gel tray, the comb was fixed at one end of the tray for making wells used for loading DNA samples. The agarose was poured gently in to the tray, and allowed to solidify at room temperature for 30 minutes. Then the comb was removed gently from the tray and the tap was also removed from the ends of the tray. The latter was fixed in electrophoresis chamber which was filled with TBE buffer has covered the surface of the gel. 5µl of each DNA sample was transferred to Eppendorf tube. 5µl of loading buffer was added to the tube and the mixture was loaded into the wells in agarose gel.

The electric current was allowed at 70 volt for 2 hr. UV transilluminater was used at 320-336 nm for the observation of DNA bands, and the gel was photographed using digital camera.

3-2-13 Determination of MICs of ESBL-producing isolates:

The two-fold agar dilution susceptibility method was used for determination of MICs of β-lactam antibiotics (3-1-4-1). Appropriate dilutions of β-lactam antibiotic solutions were prepared according to the report of 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 (Table 3-1). The prepared dilutions of 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°C. The agar and antimicrobial solution were mixed thoroughly and the mixture was

poured into petridishes, 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. 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 minutes) until the moisture in the inoculum spots was absorbed by the agar. The plates were inverted and incubated at 35 °C for 16 to 20 hr.

To determine agar dilution break points, the plates were placed on a dark surface, and the MIC was recorded as the lowest concentration of the antimicrobial agent that completely inhibits growth (disregarding a single colony or a faint haze caused by the inoculum) 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) documents.

Table (3-1): Scheme for preparing dilutions of antimicrobial agents to be used in agar dilution susceptibility test (Ericsson and Sherris, 1971)

Antimicrobial solution						
Step	**Concent.**	**Source**	**Volume +**	**D.W =**	**Intermediate Concentration =**	**Final Concent. at 1:10 dilution in agar**
1	5120 µg/ml	Stock	-	-	5120 µg/ml	512 µg/ml
2	5120	Step 1	1 ml	1 ml	2560	256
3	5120	Step 1	1	3	1280	128
4	1280	Step 3	1	1	640	64
5	1280	Step 3	1	3	320	32
6	1280	Step 3	1	7	160	16
7	160	Step 6	1	1	80	8
8	160	Step 6	1	3	40	4
9	160	Step 6	1	7	20	2
10	20	Step 9	1	1	10	1
11	20	Step 9	1	3	5	0.5
12	20	Step 9	1	7	2.5	0.25
13	2.5	Step12	1	1	1.25	0.125

3-2-14 Detection of AmpC β-lactamases:

3-2-14-1 Modified three dimensional test (MTDT):

This test was carried out according to Manchanda and Singh (2003) as follows: fresh overnight growth from Muller-Hinton agar plate was transferred to a pre-weighed sterile Eppendorf tube. The tube was weighed again to ascertain the weight of the bacterial mass. The technique was standardized so as to obtain 15 mg of bacterial wet weight

for each sample. The growth was suspended in peptone water and pelleted by centrifugation at 3000 rpm for 15 minutes. Bacterial growth washed with normal saline 2 to 3 times, crude enzyme extract was prepared by repeated freeze-thawing (approximately 10 cycles). Lawn cultures of *E. coli* ATCC 25922 were prepared on Muller-Hinton agar plates and cefoxitin (30µg) disks were placed on the plate. Linear slits (3 cm) were cut using a sterile surgical blade 3 mm away from the cefoxitin disk. Small circular wells were made on the slits at 5 mm distance, inside the outer edge of the slit by stabbing with a sterile Pasteur pipette on the agar surface. Approximately 30µl of extract was loaded in the wells, the plates were kept upright for (5-10) minutes until the solution dried, and were then incubated at 37ºC overnight. The isolates showing clear distortion of the zone of inhibition of cefoxitin were taken as AmpC producers. The isolates with no distortion were taken as AmpC non producers and isolates showing minimal distortion were taken as indeterminate strains.

3-2-14-2 AmpC disk test:

All isolates subjected to MTDT were also simultaneously checked by AmpC disk test. A lawn culture of *E. coli* ATCC 25922 was prepared on Muller-Hinton agar plate. Sterile disks (6 mm) were moistened with sterile saline (20µl) and inoculated with several colonies of test organism. The inoculated disk was then placed beside a cefoxitin disk (almost touching) on the inoculated plate. The plates were incubated overnight at 37ºC. A positive test appeared as flattening or indentation of the cefoxitin inhibition zone in the vicinity of the test disk. A negative test had an undistorted zone (Singhal *et al.*, 2005).

3-2-14-3 Cetazidime-imipenem antagonism test (CIAT):

This test was carried out for detection of inducible AmpC β-actamases according to Cantarelli *et al*. (2007) as follows:

Screening for the inducible AmpC β-lactamase was done by cetazidime-imipenem antagonism test (CIAT), which consisted of an imipenem disk (10U) placed 20 mm apart (edge-to-edge) from a ceftazidime disk (30µg) on a Muller-Hinton agar plate previously inoculated with a 0.5 McFarland bacterial suspension, and incubate for 24 hr at 35ºC. For comparison, a cefoxitin disk was also placed 20 mm a part from the ceftazidime disk. Antagonism indicated by a visible reduction in the inhibition zone around the ceftazidime disk adjacent to the imipenem or cefoxitin disks, was regarded as positive for the inducible AmpC β-lactamase production.

3-2-15 Detection of carbapenemases:

3-2-15-1 Modified hodge test (MHT) for serine carbapenemase detection in Enterobacteriaceae:

The test was done as described by Lee *et al.* (2001). A 0.5 McFarland dilution (3-2-1-1) of *E. coli* ATCC 25922 was prepared in 5 ml of tryptic soy broth, a lawn was streaked to a Muller-Hinton agar plate and allow to dry (3-5) minutes, imipenem susceptibility disk (10U) was placed in the center of the test area. In a straight line the test organism streaked from the edge of the disk to the edge of the plate, the plates were incubated overnight at 37ºC. Up to four organisms can be tested on the same plate with one drug. MHT positive test has a clover leaf-like indentation of *E. coli* ATCC 25922 growing along the test organism growth streak within the disk diffusion zone. MHT negative test has no growth of *E. coli* ATCC 25922 along the test organism.

3-2-15-2 Imipenem-EDTA double disk synergy test:

Metallo-β-lactamase detection was performed by double disk synergy method according to Lee *et al*. (2003). A (10U) imipenem disk was placed in the center of a Muller-Hinton agar plate inoculated with a 0.5 McFarland dilution of the test isolate. An EDTA disk (1,900 µg) was placed at a distance of 15 mm center to center from the imipenem disk. The plate was incubated at 37ºC overnight. The zone around the imipenem disk would be extended on the side nearest the EDTA disk for a metallo-β-lactamase producer.

Chapter Four

Results and Discussion

4-1 Bacterial isolates:

The goal of the present study was to evaluate the incidence of *E. coli* and *Klebsiella* spp. and to ascertain the detection rate of these microorganisms from the other Gram-negative enteric pathogens presented in Merjan Teaching Hospital. Since the present study focused on detection of *E. coli* and *Klebsiella* spp. rather than other microorganisms, *E. coli* and *Klebsiella* were detected in 89 (42.4%) of the hospital environment samples, there were 47(22.4%) *Klebsiella* spp. and 42(20%) *E. coli* . However, the frequency of the bacterial isolates, and their site of isolation are listed in Table (4-1). A large number of bacteria are potential pathogens in hospitalized patients. *E. coli*, *Klebsiella*, *Proteus*, *Morganella*, *Enterobacter*, *Citrobacter*, *Serratia*, *Acinetobacter* and *Pseudomonas* spp. are commonly associated with the hospital environment and may also be isolated from patients with underlying disease (Kuçukates and Kocazeybek, 2002). A report by Jabeen *et al.* (2003), noted that out of 471 *Enterobacteriaceae* isolates collected from tertiary care hospital in Karachi, Pakistan, the most isolates were *E. coli* (74%) followed by *K. pneumoniae* (13%). In a study conducted in India, Datta *et al*. (2004), found that of the 300 clinical isolates collected from patients admitted to different hospital wards, *K. pneumoniae* (35.7%) was the most common isolates followed by *E. coli* (29%).

It has been noticed that the *Klebsiella* spp. is the most common Gram-negative bacteria isolated from Merjan hospital. However, 8(40%) are obtained from kitchen, 18(36%) from waste containers, 12(30%) from bathrooms, 2(16.7%) from floor, 3(15%) from benches and tables, and 2(13.3%) from each of medical equipment and beds (Table 4-1).

Table (4-1): *E. coli* and *Klebsiella* spp. isolates recovered from Merjan Teaching Hospital

Source of sample	No. of samples	No. (%) of isolates			
		Klebsiella spp.		*E. coli*	Total
		K. pneumoniae	*K. oxytoca*		
Benches and tables	20	1(5%)	2(10%)	5(25%)	8(40%)
Medical equipment	15	2(13.3%)	0	1 (6.7%)	3(20%)
Doors	14	0	0	0	0
Beds	15	2(13.3%)	0	2(13.3%)	4(26.7%)
Floor	12	1(8.3%)	1(8.3%)	1(8.3%)	3(25%)
Walls	12	0	0	3(25%)	3(25%)
Stairs	12	0	0	0	0
Waste containers	50	10(20%)	8(16%)	13(26%)	31(62%)
Bathrooms	40	6(15%)	6(15%)	13(32.5%)	25(62.5%)
Kitchen	20	6(30%)	2(10%)	4 (20%)	12(60%)
Total	210	28(13.3%)	19(9%)	42 (20%)	89(42.4%)

Klebsiella spp. are regarded simply as transient members of the flora. Their carrier rates change drastically in the hospital environment, where colonization rates increase in direct proportion to the length of stay. Even hospital personnel have elevated rates of *Klebsiella* carriage (Podschun *et al.*, 2001). According to the statistics of the Centers for Disease Control and Prevention, *Klebsiella* spp. account for 8% of endemic hospital infections and 3% of epidemic outbreaks (Stamm *et al.*, 1981). Other studies found that *K. pneumoniae* is the most

frequently occurring species in hospital-acquired infections, accounting for 75 to 86 % of *Klebsiella* spp. reported (Hansen *et al.*, 1998).

In this study, all *E. coli* and *Klebsiella* isolates were identified using the traditional biochemical tests (Table 4-2). It was found that 28(59.6%) isolates were belonged to *K. pneumoniae* and 19(40.4%) were belonged to *K. oxytoca*.

In the present study, out of the 210 hospital samples, 42(20%) *E. coli* isolates were obtained, 13(32%) were isolated from bathrooms, 13(26%) from waste containers, 3(25%) from walls, benches and tables each alone, 4(20%) from kitchen, 1(8.3%) from floor, and 1(6.7%) from medical equipment (Table 4-1). In previous study, Ali Shah *et al.* (2003) reported that of the 200 nosocomial *Enterobacteriaceae* isolates collected from different hospital wards, *E. coli* was the most frequent (35%) followed by *K. pneumoniae* (25%). In another study, out of 387 *Enterobacteriaceae* isolates collected from six Singapore hospitals, 189(48.9%) were *E. coli*, 194(50.1%) were *K. pneumoniae* and 4(0.01%) were *K. oxytoca* isolates (Tan *et al*., 2008). In the United Arab Emirates, of the 130 *Enterobacteriaceae* isolated from patients in six general hospitals, *E. coli* were predominant 83(63.8%) (Al-Zarouni *et al*., 2008).

In this study, the low detection rates in stairs, walls and floor may be due to the frequent use of disinfectants in cleaning. Although most nosocomial infections result from a patients endogenous flora or person to person transmission, contaminated surfaces, medical equipments (contamination due to faulty hygienic procedures) and blood products play a very important role in the development of hospital acquired infection such as bloodstream and urinary

Table (4-2): Morphological and biochemical tests of *E. coli* and *Klebsiella* spp. isolates

Test	Type of isolate		
	***E. coli* (n=42)**	***K. pneumoniae* (n= 28)**	***K.* oxytoca (n= 19)**
Gram stain	-	-	-
Catalase	+	+	+
Oxidase	-	-	-
H_2S	-	-	-
Indole	+	-	+
Methyl red	+	-	-
Vogas-Proskaures	-	+	+
Citrate utilization	-	+	+
Urease	-	+	+
Motility	+	-	-
Growth at 10˚C	-	-	+
Acid from Glucose	+	+	+
D-mannose	+	+	+
Sucrose	V	+	+
D-xylose	+	+	+
Lactose	+	+	+
Glycerol	V	+	+

V, Variable reaction

tract infections (Wang *et al.*, 2005). It was found that the hands of the nurses frequently colonized by *E. coli*, *Klebsiella* and other coliform organisms and suggested as transmitters of the epidemic strains in hospitals (Goetz *et al.*, 1995).

4-2 Primary screening of β-lactam resistant isolates:

In this investigation, a total number of 89 consecutive environmental isolates of *E. coli* (n=42) and *Klebsiella* spp. (n=47) were screened for β-lactam resistance on Muller-Hinton agar supplemented with ampicillin and amoxicillin (each alone) at final concentrations of 100 and 50 µg/ml, respectively. Compared to other β-lactam antibiotics, ampicillin and amoxicillin are the most

commonly used in the therapy of bacterial infections and can provide a comprehensive primary screening of β-lactam resistant isolates, because the isolate that is resistant to carbenicillin and cephalosporins, is already resistant to ampicillin and amoxicillin (Bush *et al*., 1995).

During the study period, a total of 82(92%) *E. coli* and *Klebseilla* spp. were resistant to ampicillin and amoxicillin (Table 4-3). The results suggest that the development of antibiotics resistance in these isolates may be related to the overuse and misuse of the prescribed antibiotics, the study also suggests that the frequency of β-lactam resistant isolates was higher than has been suspected. However, the rate of β-lactam resistant *E. coli* and *Klebsiella* isolates was higher than previously reported by Hadi (2008), who found that out of 71 *E. coli* and *Klebsiella* isolates, 60(84.5%) were resistant to β-lactam antibiotics in Najaf, on the other hand, Akram *et al*. (2007) reported that all *E. coli* and *Klebsiella* isolates were resistant to ampicillin in Turkey. Antibiotic resistance arises quickly and spreads rapidly, especially when resistance genes are horizontally transferred via plasmids and integrons among individuals, among species, and even among bacterial kingdom **(**Hall and Barlow, 2004).

However, the present study indicated that 95.2% of *E. coli* isolates are resistant to both ampicillin and amoxicillin. This rate is higher than that obtained by Ahmed *et al*. (2000) who found that 75% of *E. coli* isolates collected from urine samples in the Sudan were resistant to ampicillin and 72% to amoxicillin. While this rate is close to that reported in Korea by Kang *et al*. (2005) who found that 91% of *E. coli* isolates were resistant to ampicillin. However, it is lower than that reported in Tehran, Iran by Hosseini-Mazinani *et al*. (2007) who reported that all the clinical isolates of *E. coli* were resistant to ampicillin and amoxicillin. Resistance to β-lactam antimicrobial agents in *E. coli* is primarily mediated by β-lactamases, which hydrolyze the β-lactam ring

and thus inactivate the antibiotic. The TEM-1 is the most commonly encountered β-lactamase in Gram-negative bacteria; up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Bradford, 2001).

(Table 4-3): Ampicillin and amoxicillin resistant *E. coli* and *Klebsiella* spp. isolates collected from Merjan hospital

Susceptibility to ampicillin and amoxicillin	**No. (%) of isolates**			**Total No. (%)**
	K. pneumoniae	***K. oxytoca***	***E. coli***	
Resistant	25 (89.3%)	17 (89.5%)	40 (95.2%)	82 (92%)
Sensitive	3 (10.7%)	2 (10.5%)	2(4.8%)	7 (7.9%)
Total	28 (59.6%)	19(40.4%)	42 (47%)	89 (100%)

Results of this study also revealed that 42(89.4%) *Klebsiella* isolates are resistant to both ampicillin and amoxicillin. The SHV-1 β-lactamase is most commonly found in *Klebsiella* and is responsible for up to 20% of the plasmid mediated ampicillin resistance (Babin and Livermore, 2000). In previous study, Al-Charrakh (2005) found that 73.8% of *Klebsiella* isolates obtained from environmental and clinical samples in Hilla were resistant to both antibiotics. In India, ampicillin resistance was detected in (94.1%) of *Klebsiella* isolates (Datta *et al*., 2004). Aiyegoro *et al*. (2007) reported that (66.7%) of *K. pneumoniae* isolates were resistant to ampicillin and amoxicillin.

In this study, the high ratio of resistance for these two drugs could be attributed not only to the sensitivity of these antibiotics to β-lactamases, but also may be to other resistance mechanisms such as the decreased affinity of the target PBPs or decreased permeability of the drug into the cell.

4-3 Survey of β-lactam resistant isolates to antibiotics:

Antibiotic resistance is a major clinical problem in treating infections caused by bacteria. In this study, all the 82 β-lactam resistant *E. coli* (n=40) and *Klebsiella* spp. (n=42) isolates were screened for their antibiotic resistance against selected antimicrobial agents of different classes (Table 4-4) (Appendix 1). A strain is considered a multidrug resistant (MDR) if an isolate is resistant to representatives of three or more classes of antibiotics (penicillins, cephalosporins, aminoglycosides, monobactams, fluoroquinolones, sulfon-amides, tetracyclins, and carbapenems) (Falagas and Karageorgopoulos, 2008). In this study, all the tested isolates are resistant to a minimum of 3 classes of antibiotics to which they are tested. Hence the isolates are considered to be multidrug resistant. Similar results with MDR isolates have been reported with other authors in Iraq, Al-Mohana (2004) found that 56.8% of clinical *E. coli* isolates in Najaf were resistant to more than five antimicrobial agents. Hadi (2008) reported that all 60 (100%) of the β-lactam resistant *E. coli* and *K. pneumoniae* isolates obtained from patients with significant bacteriurea were resistant to at least five antimicrobial agents in Najaf, Iraq too.

However, MDR rates vary from country to another. Over all Gram-negative isolates from Latin American countries revealed the lowest susceptibility rates to all antibiotics followed by Asian-Pacific isolates and European strains. Strains from Canada exhibit the best global susceptibility testing results (Gales *et al*., 2001). Widespread use of antimicrobial therapy has often been held responsible for co-resistance to four or more unrelated families of antibiotics and the occurrence of multiply resistant *E. coli* and *Klebsiella* strains in hospitals (Maynard *et al*., 2003). Transference of resistance determinants by mobile genetic elements including plasmids, transposons, and

gene cassettes in integrons between and across different bacterial species with relative ease are important factors that can contribute to the increase in multiresistant bacteria (Livermore, 2007).

Table (4-4): Susceptibility profiles of β-lactam resistant *E. coli* and *Klebsiella* spp. isolates

Antibiotic	No. (%) of resistant isolates		Total (%)
	E. coli **(n=40)**	***Klebsiella*** **spp. (n=42)**	
Carbenicillin	38 (95 %)	42 (100 %)	80(97.6%)
Piperacillin	34 (85%)	34(81%)	68(82.9%)
Cefoxitin	22 (55%)	11(26.2%)	33(40.2%)
Ceftazidime	28 (70%)	27(64.3%)	55(67.1%)
Cefotaxime	31 (77.5%)	31(73.8%)	62(75.6%)
Ceftriaxone	30 (75%)	31(73.8%)	61(74.4%)
Cefepime	30 (75%)	27(64.3%)	57(69.5%)
Imipenem	0(0%)	1(2.4%)	1(1.2%)
Aztreonam	30 (75%)	27(64.3%)	57(69.5%)
Amikacin	2 (5%)	5(11.9%)	7(8.5%)
Gentamicin	19 (47.5%)	22(52.4%)	41(50%)
Tobramycin	22 (55%)	21(50%)	43(52.4%)
Nalidixic acid	23 (57.5%)	23(54.8%)	46(56.1%)
Ciprofloxacin	20 (50%)	12(28.6%)	32(39%)

In the present study, carbenicillin was found to be the most resistant antibiotic in *E. coli* with resistance of 95%. The next most resistant antibiotic was piperacillin (85%), followed by cefotaxime (77.5%); ceftriaxone, cefepime, and aztreonam (75% each alone); ceftazidime (70%); nalidixic acid (57.5%); cefoxitin and tobramycin (55% each); ciprofloxacin(50%); gentamicin (47.5%); and amikacin (5%). The present results revealed high rate of resistance in *E.*

coli isolates to carbenicillin, this rate is in agreement with those reported by Kang *et al*. (2005) who found that 91% of *E. coli* isolates obtained from hospitalized patients in Korea to be resistant to carbenicillin, while they are much higher than those given in the Sudan (Ahmed *et al*., 2000). In Iran, 76.3% of urinary isolates of *E. coli* have been reported as carbenicillin resistant (Hosseini-Mazinani *et al*., 2007). This high rate of resistant is probably due to the extensive use of carbenicillin in Hilla hospitals. The present study indicated that among antibiotics tested, *E. coli* revealed high resistance against third generation cephalosporins, cefotaxime (77.5%), ceftriaxone (75%) and ceftazidime (70%), these rates of resistance are markers for the production of β-lactamases.

However, levels of resistance are (52.4%) and (50%) against tobramycin and gentamicin, respectively. The activity of amikacin against *E. coli* isolates remains high (95% sensitive), this better activity of amikacin may be due to its less vulnerability to bacterial enzymes than other aminoglycosides. In a study conducted in Turkey, Kucukates and Kocazeybek (2002) found that gentamicin, tobramycin and amikacin were relatively effective against *E. coli* isolates from various clinical samples, susceptibility rates were 35.9%, 32.1% and 39.6%, respectively. Arora and Devi (2007) found that the resistance to gentamicin and amikacin was detected in 28.89% and 20% of *E. coli* isolates, respectively, and maximum resistance was seen against piperacillin 73.33%.

Also there was a reduce activity of fluoroquinolones including ciprofloxacin in *E. coli* isolates (50% resistant). Fluoroquinolones- resistance is typically encoded chromosomally. This resistance against fluoroquinolones in this study may reflect antibiotic pressure in Merjan hospital rather than co-carriage of the resistance gene on the plasmids. Rates of *E. coli* resistance to nalidixic acid (57.5%) in this study suggesting the presence of quinolones-

resistant *E. coli* strains in the hospital. In some cases, the fluoroquinolones-resistant strains like those resistant to β-lactams, have emerged because of clonal spread (Ho *et al*., 1999) or increasing selection pressure for resistance. In a study from Spain, Pena *et al*. (1995) reported that the percentages of ciprofloxacin resistant episodes, among 855 cases of *E. coli* bacteremia, increased during the study period: 0% in 1988, 0% in 1989, 1.9% in 1990, 5.5% in 1991, and 7.5% in 1992, they also found that there was an increase in nosocomially acquired cases (2.4% in 1990, 8.6% in 1991, and 10% in 1992) and noticed significant associations were found between the isolation of ciprofloxacin resistant *E. coli* and both prior quinolone use and the presence of chronic underlying diseases.

Susceptibility to ciprofloxacin was detected in 47.2% of *E. coli* isolates collected from clinical specimens of hospitalized patients in Istanbul, Turkey (Kucukates and Kocazeybek 2002). Quinolone resistance is higher in developing countries because the use of less active quinolones, such as nalidixic acid, and / or the use of low dosages of more potent compounds such as ciprofloxacin resulting in selection of mutant isolates (Acar and Goldstein, 1997).

This study indicated that the resistance rates of the 42 *Klebsiella* spp. isolates were as follows: carbenicillin (100%) ; piperacillin, 81% ; cefotaxime and ceftriaxone, (73.8% each); ceftazidime, aztreonam and cefepime 64.3% each; nalidixic acid, 54.8%; gentamicin 52.4%; tobramycin, 50%; ciprofloxacin, 28.6%; cefoxitin 26.2% ; and amikacin 11.9% (Table 4-4). The emergence of chromosomally encoded resistance to ampicillin, amoxicillin, and carbenicillin has been reported by many studies, it was also found that the β-lactamase enzyme SHV-1 derivatives widely distributed among *Klebsiella*

species conferring resistance to these β-lactam antibiotics (Tzouvelekis and Bonom , 1999; Babin and Livermore, 2000).

In this investigation, there was relatively high resistance to third generation cephalosporins and aztreonam in *Klebsiella* isolates. It might be possible that this high level of resistance was most probably due to acquisition of β-lactamases by these isolates, possibly during therapy. The results of three surveillance studies from ICUs in Turkey published in different years revealed that the resistance rates of *Klebsiella* spp. isolates to cefotaxime and ceftazidime were 96.7% and 85.5% (Gunseren *et al*., 1999); 58.73% (Aksaray *et al*., 2000); and 57.65% (Leblebicioglu, *et al*., 2002). In Korea, Lee *et al*. (2006) reported that 34% of *K. pneumoniae* isolates collected from 44 KONSAR group hospitals were resistant to ceftazidime and 28% to cefotaxime. A report by Tan *et al*. (2008) documented that susceptibility to ceftriaxone and cefepime was equal, 30.8% in *Klebsiella* spp. isolates collected from Singapore hospitals.

In the present study, amikacin was effective against *Klebsiella* spp. isolates. In 2002, Kucukates and Kocazeybek found that amikacin was relatively effective against *Klebsiella* spp. isolates from various clinical samples, 40.6% of *Klebsiella* spp. were susceptible to amikacin. In a study from Singapore, noted that all *Klebsiella* isolates were susceptible to amikacin (Tan *et al*., 2008).

In this study, resistance rates to quinolones (nalidixic acid) in *Klebsiella* isolates may be due to the frequent use of this class of antibiotics for treating infections in Hilla hospitals. Results revealed that fluoroquinolones (ciprofloxacin) was more effective against *Klebsiella* isolates than quinolones (nalidixic acid). The Surveillance Network database (http://www. mrlworld.com) found resistance trends to ciprofloxacin in bloodstream isolates

from 250 United States hospitals as follows: *Klebsiella* spp., 7.1% in 1996 and 6.7% in 1999. However, resistance rate to nalidixic acid among *Klebsiella* isolates in the present study was much lower than that reported by Enabulele *et al*. (2006) who found that 84.9% of *Klebsiella pneumoniae* isolates in Nigeria was resistant to nalidixic acid. Although mutations are the most important mechanism in quinolone- resistant isolates, other mechanisms such as porin and non porin pathways (Cohen *et al*., 1988) and the active efflux mechanism of fluoroquinolones (Piddock and Wise, 1986) may contribute in the emergence of such isolates.

The results also revealed that imipenem has the widest coverage. All the *E. coli* and 98% of *Klebsiella* spp. isolates in the present study were found to be sensitive to imipenem which represents the most successful β-lactam antibiotic. Imipenem is highly stable to hydrolyses by TEM and SHV β-lactamases (Jacoby and Munoz-Price, 2005). This drug should be administrated as empirical therapy for Gram-negative infections that are not life threatening because their overuse can pose a significant problem.

However, other mechanisms of resistance such as plasmid-mediated AmpC β-lactamases and /or reduced outer membrane permeability could be involved in the resistance to β-lactams since approximately 55% of the *E. coli* and 26% *Klebsiella* spp. isolates were also resistant to cefoxitin. In one study, Lee *et al*. (2006) reported that resistance rates of *E. coli* and *Klebseilla* isolates to cefoxitin were 8% and 32%, respectively.

It was observed that the difference in the resistance rates for amikacin and other aminoglycosides between *E. coli* and *Klebsiella* isolates, this could be due to the extensive use of this class of antimicrobials in hospitals environment. Multiple antibiotic resistance to useful classes of the antibiotics, including β-lactams, aminoglycosides and fluoroquinolones has gradually increased among

a number of Gram-negative hospital pathogens, especially *Klebsiella* spp. and *E. coli* (Struelens, 1999). The driving force of antibiotic resistance is the widespread use of antimicrobial drugs. In Iraq , antibiotics are extensively used and this situation may lead to uncontrolled resistance emergence at the hospital level, therefore the therapeutic strategies to control infections due to *E. coli* and *Klebsiella* strains has to be carefully formulated.

4-4 Detection of β-lactamase producing isolates:

In this investigation, β-lactamase production in 82 β-lactam resistant *E. coli* and *Klebsiella* spp. isolates was detected by using rapid iodometric method. The results revealed that 54.9% of *E. coli* and *Klebsiella* spp. tested isolates were positive with the rapid iodometric method (Table 4-5), which could be attributed to the production of plasmid-mediated or chromosomally encoded β-lactamases. β-lactamase producing isolates in this study were different in the time required to give positive reaction. Out of 45 isolates, 31(68.9%) indicated rapid reaction within few seconds, while the remaining 14(31.1%) isolates were detected after two minutes. This result is in consistent with that previously reported by Hadi, (2008) who found that out of 60 *E. coli* and *K. pneumoniae* isolates collected from patients with significant bacteriurea in Najaf, 34 (56.7%) were positive with rapid iodometric method and all the isolates gave positive results within few seconds to two minutes.

Additionally it was found the majority (57.1%) of *Klebsiella* spp. isolates were positive with rapid iodometric method. In a related study, Al-Charrakh (2005) found that out of 65 β-lactamase resistant *Klebsiella* spp., 38 (58.5%) isolates were able to produce β-lactamase enzymes.

Table (4- 5): β-lactamases producing *E. coli* and *Klebsiella* spp. isolates using iodometric method

Type of isolate	No. of organism	No. (%) of β-lactamases producers
E. coli	40	21 (52.5 %)
K. pneumoniae	25	16(64%)
K . oxytoca	17	8(47.1%)
Total	82	45 (54.9%)

Out of 40 β-lactam resistant *E. coli* isolates, 21(52.5%) gave positive results. In a study conducted in Najaf, Hadi (2008) revealed that out of 38 *E. coli* clinical isolates, only 21(55.3) isolates were detected as β-lactamase producers. However, 37(45.1%) *E. coli* and *Klebsiella* spp. isolates were found to be negative and failed to produce β-lactamase, suggesting that these isolates either producing these enzymes with low quantities or they have no β-lactamases. Most *E. coli* strains produce class C plasmid-mediated β-lactamases (Jacoby, 2009), and most *K. pneumoniae* isolates produces chromosomally mediated SHV-1 β-lactamases, these enzymes are constitutive and are usually produced at low or moderate levels, but are sufficient to protect against ampicillin, amoxicillin, carbenicillin, and ticarcillin (Babin and Livermore, 2000).

4-5 Extended spectrum β-lactamases (ESBLs) production:

4-5-1 Third generation cephalosporins and aztreonam resistance:

Resistance mediated by ESBLs can be difficult to detect depending on the antimicrobial agents tested (Steward *et al.*, 2001). Currently the Clinical and Laboratory Standards Institute recommended that more than one of the

5 indicators (ceftazidime, cefotaxime, ceftriaxone, aztreonam, and cefpodoxime) should be used to screen expression of ESBL in *K. pneumoniae*, *K. oxytoca*, *E. coli* and *P. merabilis* (CLSI, 2007).

This study demonstrated the presence of ESBL-mediated resistance in 82 β-lactam resistant *E. coli* and *Klebsiella* isolates in Merjan hospital in Hilla. Despite the rise in the prevalence of ESBL in many countries, there are very few reports from Iraq (Al-Charrakh, 2005; Hadi, 2008). ESBL detection is not carried out in many microbiology units in developing countries, included Iraq, and this could be attributed to the lack of awareness and the lack of resources and facilities to conduct ESBL identification. However, an initial screening for reduced susceptibility to third generation cephalosporins and aztreonam was done by the standard Kirby-Bauer disk diffusion method (Bauer *et al*., 1966). The isolate was considered positive for screening test when the zone diameter of any of the indicators met the NCCLS criteria (NCCLS, 2003_a) and additional phenotypic tests are mandatory in order to ascertain the production of ESBL.

The results of this study revealed that resistance to screened agents is as follows: cefotaxime, 62(75.6%); ceftriaxone, 61(74.4%), aztreonam, 57 (69.5%); and ceftazidime, 55(67.1%) (Table 4-6). Possible ESBL production has been reported to occur in up to 9% of European *E. coli* isolates (Winokur *et al*., 1997). In India, Sridhar Rao *et al*. (2009) found that 98.5% of Gram-negative bacterial isolates obtained from culture of various clinical specimens were detected as screen positive isolates.

In this study, the resistance of *E. coli* isolates to third generation cephalosporins and aztreonam was high and ranged from 77.5% for cefotaxime, 75% for ceftriaxone and aztreonam each, and 70% for ceftazidime, (Table 4-4). These rates of resistance might be as markers for

the production of ESBLs by these isolates. The results of three surveillance studies from ICUs in Turkey published in different years revealed that the rates of resistance of *E. coli* to cefotaxime and ceftazidime were 30.4% and 28.6% (Gunseren *et al*., 1999); 20.3% and 26.1% (Aksaray *et al*., 2000); and 11.12% (Leblebicioglu, *et al*., 2002). In a study conducted in Korea, resistance rates has been reported as 14% and 10% for cefotaxime and ceftazidime in *E. coli* isolates (Lee *et al*., 2006). 0ut of 189 *E. coli* isolates collected from Singapore hospitals, resistance to extended spectrum cephalosporins was detected in 27% of *E. coli* strains (Tan *et al*., 2008).

In this investigation, there was also high resistance to third generation cephalosporins and aztreonam in *Klebsiella* isolates, the resistance rates ranged from 73.8% for ceftriaxone and cefotaxime each, and 64.3% for ceftazidime and aztreonam each (Table 4-4). These high levels of resistance most probably due to acquisition of ESBLs by these isolates, possibly during therapy. The results of three surveillance studies from ICUs in Turkey published in different years revealed that the resistance rates of *Klebsiella* spp. isolates to cefotaxime and ceftazidime were 96.7% and 85.5% (Gunseren *et al*., 1999); 58.73% (Aksaray *et al*., 2000); and 57.65% (Leblebicioglu, *et al*., 2002). Datta *et al*. (2004) found that the resistance to ceftriaxone, ceftazidime and cefotaxime were 83.1%, 81.5% and 76.4% in *Klebsiella* spp. isolates collected from patients in different hospital wards in India. A report by Tan *et al*. (2008) documented that susceptibility to ceftriaxone was 30.8% in *Klebsiella* spp. isolates collected from Singapore hospitals.

In this investigation, 58(70.7%) of the 82 β-lactam resistant isolates were detected as potential ESBL- producers (resistant to at least one of the screening agents) of which 31(77.5%) were *E. coli* and 27(64.3%) were

Klebsiella spp. [19/25(76%) *K. pneumoniae* and 8/17(47.1%) *K. oxytoca*] isolates (Table 4-6).

Table (4-6): Incidence of potential ESBL production for *E. coli* and *Klebsiella* spp. isolates by Kirby- Bauer disk diffusion method

Type of organism	No.	No.(%) of potential ESBL producer
E. coli	**40**	31(77.5%)
K. pneumoniae	**25**	19(76%)
K. oxytoca	**17**	8(47.1%)
Total	**82**	58(70.7%)

The resistance to the third generation oxyimino-cephalosporins is mediated by ESBLs which are derivatives of narrow spectrum TEM-1, TEM-2 and SHV-1 β-lactamases (Bradford, 2001). Presumably, the selective pressure imposed by the use and overuse of these antibiotics in the treatment of infections caused by *E. coli* and *Klebsiella* spp. isolates and access to antibiotics without a physician's prescription in many countries has resulted in the emergence of new variants of β-lactamases. The Centers for Disease Control and Prevention (CDC) reported that more than 70% of the bacteria that cause hospital acquired infections are resistant to at least one of the antibiotics commonly selected to treat them. The problem of dissemination of resistant clones and genes in hospital environments can also occur due to contact with healthcare workers or to patients transferring between hospital units (NIAID, 2008).

4-5-2 Disk approximation test:

The Jarlier disk approximation or double disk synergy (DDS) was the first detection test described in 1980's (Jarlier *et al.*, 1988). DDS is a disk diffusion test in which 30µg antibiotic disks of ceftazidime, ceftriaxone, cefotaxime and aztreonam are placed on the plate, 30 mm (center to center) from the amoxicillin/clavulanate (20µg/10µg) disk. A clear extension of the edge of the antibiotic's inhibition zone toward the disk containing clavulanate is interpreted as synergy, indicating the presence of an ESBL. The efficacy of β-lactam group of antibiotics was reduced due to the production of β -lactamases by the resistant bacterial strains. Therefore, search for their inhibitors was initiated to protect the antibiotic activity in vivo against β -lactam resistant pathogens. DDS test remains a reliable, convenient and inexpensive method of screening for ESBLs. However, the interpretation of the test is quite subjective. Sensitivity may be reduced when ESBL activity is very low leading to wide inhibition zones around the cephalosporin and aztreonam (Vercauteren *et al*., 1997).

The results of the present study indicate that ESBL production was confirmed in 36/82 (43.9%) of *E. coli* and *Klebsiella* spp. isolates, while the remaining 46/82 (56.1%) were no ESBL producers (Table 4-7) (Figure 4-1). The overall ESBL production rate for *Enterobacteriaceae* was 10.5%, the highest rates were encountered in Egypt (38.5%) and Greece (27.4%) and lowest in the Netherlands (2%) and Germany (2.6%) (Bouchillon *et al*., 2004). In Saudi Arabia, although the exact prevalence of ESBL is currently unknown, the PEARLS study (2001- 2002) reported that the overall ESBL production rate from *Enterobacteriaceae* was 18.6% (Kader *et al*., 2004). Other reports from Saudi Arabia suggest that ESBL producers are common and began to disseminate between hospitals (Bouchillon *et al*., 2004; El-

Khizzi and Bakheshwain, 2006). Other studies reported that a high endemic of ESBL- producing *Enterobacteriaceae* have been determined in Latin American countries especially in Brazil (Marry *et al*., 2006 and Martins *et al*., 2006). In recent study, 35.2% of *E. coli* and *K. pneumoniae* isolates obtained from nosocomial blood stream infections were ESBL producer (Superti *et al*. , 2009).

Table (4-7): Frequency of ESBL production in *E. coli* and *Klebsiella* spp. isolates by disk approximation test.

Type of isolate	No. of isolates	No.(%) of ESBL producer
E. coli	**40**	18 (45%)
K. pneumoniae	**25**	12 (48%)
K. oxytoca	**17**	6 (35.3%)
Total	**82**	36 (43.9%)

It was found that in many parts of the world almost 10- 40% of strains of *E. coli* and *K. pneumoniae* carry genes encoding ESBLs (Ali Shah *et al*, 2004). The factors that may affect the frequency of ESBLs include methods of detection and the proportion of nosocomial strains or epidemic clones in the collected bacterial samples (Paterson, 2004).

The prevalence of ESBLs among *Klebsiella* spp. isolates vary greatly worldwide and in geographical areas and are rapidly changing over the time (Livermore, 1995). However, the occurrence of ESBL producers in Merjan hospital isolates of *Klebsiella* in present study was found to be 18/42(43%) (Table 4-7). This result is higher than that reported in other studies. In

Croatia, more than 30% of *Klebsiella* strains isolated in Zagreb hospitals were ESBL producers (Tambic Andrasevic *et al*., 2002). In Spain, a shift in the proportion of ESBL-producing *Klebsiella* isolates recovered from outpatients (7% to 31%) and ICU patients (41% to 25%) was observed between the periods 1989 to 2000 and 2001 to 2004 (Valverde *et al*., 2008).

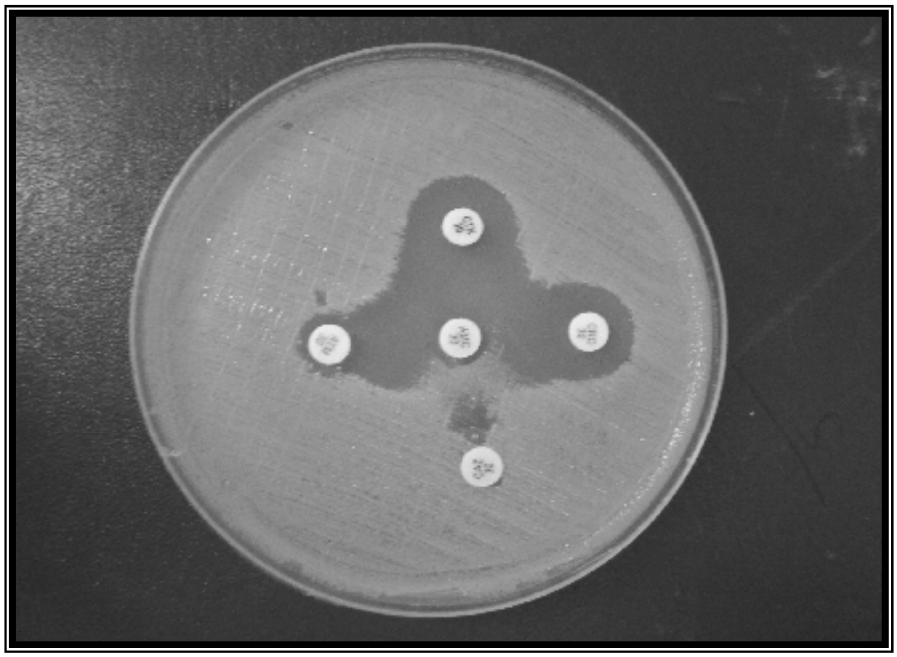

Figure (4-1): Disk Approximation Test for detection of ESBL in *K. pneumoniae* E15 isolate. AMC, Amoxi-clav; ATM, Aztreonam; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime.

However, the data generated in this study indicated that 12/25(48%) of *K. pneumoniae* and 6/17(35.3%) of *K. oxytoca* isolates were phenotypically confirmed to be ESBL producers. These rates are much higher than those reported by the European arm of SENTRY Antimicrobial Surveillance Program from 1997-1999, ESBL production was confirmed in: 10.5% of *K. oxytoca*, 5.8% of *K. pneumoniae* in Spain; 23.2% of *K. pneumoniae* were found to be ESBL producers, whereas no ESBLs were detected in *K.*

oxytoca isolates in Portugal; while no ESBLs were found in Albania (Nijssen *et al*., 2004). However, ESBL production in *K. pneumoniae* isolates in this study is much lower than that reported by Dropa *et al*. (2009) who found that ESBL production was higher (75.6%) in *K. pneumoniae* than in *K. oxytoca* (1.6%) clinical isolates in Brazil.

Among the 40 *E. coli* isolates analyzed, 18(45%) were detected as ESBLs producers. In the first study for clinical isolates of ESBL-producing bacteria carried out in Najaf, the prevalence of ESBL-producing *E. coli* isolates was 18.3% (Hadi, 2008). However, in a survey of nosocomial isolates of *Enterobacteriaceae* collected from different wards in Pakistan, 28.57% of *E. coli* isolates were ESBL producers (Ali Shah, 2003). Similarly, high rates of ESBL-producing *E. coli* isolates have been observed in some parts of the world such as Egypt, where several studies have pointed to a high prevalence of ESBLs in *E. coli* isolated from hospitalized patients varying from 38% to 66.6% (El-Kholy *et al*., 2003; Al-Agamy *et al*., 2006). While in the United States, ESBL production was detected in 57.69% *E. coli* isolates tested by the Clinical Microbiology Laboratory in Rochester (Robberts *et al*., 2009).

Table (4-7) revealed that ESBL production was higher among *K. pneumoniae* (48%) than *E. coli* (45%) and *K. oxytoca* (35%) isolates. In 2005, Tonkic *et al*. found that 36.8% of *K. pneumoniae* isolates were positive for ESBL, while 4.7% of *E. coli* were ESBL producers. El-Astal and Ramadan (2008) stated that the prevalence of ESBL-producing organisms was higher among *K. pneumoniae* than *E. coli* isolates, 35% and 9% respectively. The high prevalence of ESBL producing isolates in this study may probably due to the large amount of third generation cephalosporins consumption, which has been reported as a risk factor for

infection with ESBL-producing isolates bacteria (Rice *et al*., 1996; Saurina *et al*., 2000), suggests that ESBL producing isolates are already endemic in Merjan hospital.

In this investigation, specific samples have been analyzed. However, out of 36(43.9%) ESBL-producing isolates, 21(58.3%) were from waste containers, 8(22.2%) from bathrooms, 3(8.3%) from tables and patients beds each alone, and 1(2.8%) were from D.C. shock device (Table 4-8). However, ESBL-producing *E. coli* was found the most prevalent organism in tables and beds 3/18(16.7%) each and medical equipment 1/18(5.6%), whereas ESBL-producing *Klebsiella* spp. were most common in waste containers 12/18(66.7%), bath rooms 6/18(33.3%). ESBL-producing bacteria are usually found in those areas of hospitals where patients are critical and antibiotic use is frequent, particularly intensive care units, surgical wards, neonatology wards and oncologic wards (Ali Shah *et al.*, 2004). Detection of ESBL production is of importance in hospital isolates. Firstly, these strains are probably more prevalent than currently recognized. Secondly, ESBLs constitute a serious threat to currently available antibiotics. Thirdly, institutional outbreaks are increasing because of selective pressure due to heavy use of extended- spectrum cephalosporins and lapses in effective control measures.

However, in this study not all screen positive *E. coli* and *Klebsiella* spp. isolates were ESBL producers. Thus, there may be other mechanisms of resistance to third generation cephalosporins and aztreonam. In organisms that produce both ESBL and AmpC, clavulanate may induce hyperproduction of the AmpC β-lactamase leading to hydrolysis of the third generation cephalosporin thus masking any synergy arising from inhibition of the ESBL, producing false negative result in the ESBL detection test

(Thomson, 2001). There are a number of instances whereby the screening tests are positive but the confirmatory tests are negative or indeterminate (Steward *et al.*, 2001). However, coexistence of different classes of β-lactamases in a single bacterial isolate may pose diagnostic challenges. The ability to detect and distinguish between AmpC and ESBL-producing organisms has epidemiological significance and may have therapeutic importance as well.

Table (4-8): ESBL-producing *E. coli* and *Klebsiella* spp. isolates recovered from Merjan Teaching hospital.

Origin of isolate	No. (%) of ESBL-producers		Total
	E. coli	***Klebsiella* spp.**	
Waste containers	9(50%)	12(66.7%)	21(58.3%)
Bathrooms	2(11.1%)	6(33.3%)	8(22.2%)
Tables	3(16.7%)	0(0%)	3(8.3%)
Beds	3(16.7%)	0(0%)	3(8.3%)
Medical equipment	1(5.6%)	0(0%)	1(2.8%)
Total	18(45%)	18(42.85%)	36(43.9%)

In this investigation, amoxi-clav resistance was observed (Figure 4-2), this is likely due to the heavy selection pressure from overuse of antibiotics and seem to be losing the battle. However, these may indicate that another resistance mechanism was probably present among ESBL-positive and negative isolates, since ESBL is inhibited *in vitro* by clavulanic acid. Thus, amoxi-clav could actually be associated with the selection of isolates producing this other resistance mechanism. Resistance against β-lactamase

inhibitors occurs mainly by several mechanisms: hyperproduction of β-lactamases, production of β-lactamases resistant to inhibitors and chromosomal cephalosporinases (Espinasse *et al*., 1997).

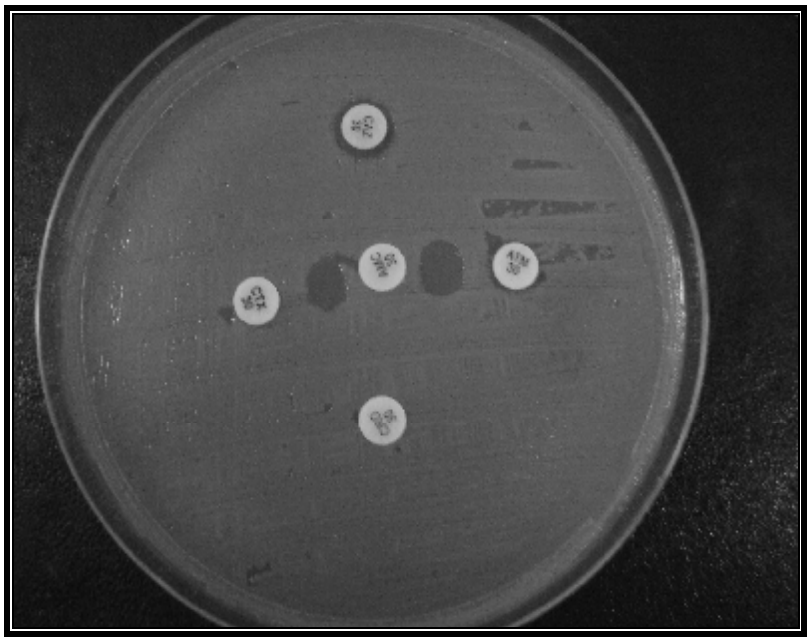

Figure (4-2): ESBL-producing *E. coli* E12 isolate exhibit resistance to AMC. AMC, Amoxi-clav; ATM, Aztreonam; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime.

Although β-lactam / β-lactamase inhibitors are usually active against organisms producing a single ESBL, their effectiveness may be reduced in organisms producing multiple ESBLs (Chanawong *et al*., 2002). The hyper-production of the parent enzymes (for example, TEM-1 or SHV- 1) in ESBL- producing organisms or the combination of β-lactamase production and porin loss can also lead to a reduction in activity of β-lactamase inhibitor (Akhan *et al*,. 2001).

The CLSI (2007) recommended that for all confirmed ESBL producing strains, the test results should be reported as resistant for all penicillins, cephalosporins and aztreonam regardless of the routine susceptibility test results. Whereas, β-lactam/β-lactamase inhibitor combinations (for example:

piperacillin/tazobactam and amoxicillin/clavulanate) are reported as susceptible.

4-6 Susceptibility of ESBL-producing isolates to antibiotics:

Organisms that express an ESBL are frequently resistant to other antibiotics, as many of these additional resistance genes are encoded on the ESBL-associated plasmids (Jacoby and Munoz-Price, 2005). However, the antibiotic susceptibility of ESBL-producing and non-producing isolates were evaluated (Table 4-9) and compared among the ESBL-producing isolates. High level of resistance was detected in ESBL-producing isolates against carbenicillin (100%) and piperacillin (91.7%), whereas the susceptibilities of non-ESBL were 95.7% and 73.9%, respectively. However, these results are in agreement with those reported by Hadi (2008) who found that all ESBL-producing and (93.5%) of non-producing isolates were resistant to carbenicillin in Najaf.

The results also indicate that ESBL-producing isolates were resistant to third, fourth cephalosporins and monobactam, including cefotaxime, ceftriaxone, cefepime (86.1% each), ceftazidime and aztreonam (83.3% each) than were ESBL-negative isolates. Such resistance in present study may be emerged because of the widespread use of these drugs in Merjan hospital. In India, ESBL producing Gram-negative isolates revealed a level of resistance 76.6% to cefepime and aztreonam, each alone (Taneja *et al*., 2008). In Brazil, it was found that resistance to ceftriaxone, ceftazidime, cefepime and aztreonam was significant and more frequently observed in ESBL producing isolates (Superti *et al*., 2009).

The results in the present study also indicated variable resistance rates for aminoglycosides among ESBL-positive isolates; ranging from high

levels 63.9%, 61.1% to gentamicin and tobramycin, respectively to low level 5.6% to amikacin, whereas ESBL-negative isolates were more resistant to amikacin (8.7%) and less resistant to gentamicin (37%) and tobramycin

Table (4-9): Susceptibility profiles of ESBL-producing and non-producing isolates to various antibiotics

Type of antibiotic	No. (%) of resistant isolates		Total (n=82)
	ESBL (n=36)	Non-ESBL (n=46)	
Carbenicillin	36 (100 %)	44 (95.7%)	80(97.6%)
Piperacillin	33 (91.7%)	34 (73.9%)	67(81.7%)
Amoxi-clav	29 (80.6%)	42 (91.3%)	71(86.6%)
Cefoxitin	13 (36.1%)	20 (43.5%)	33(40.2%)
Ceftazidim	30 (83.3%)	24 (52.2%)	54(65.9%)
Cefotaxime	31 (86.1%)	28 (60.9%)	59(72%)
Ceftriaxone	31 (86.1%)	27 (58.7%)	58(70.7%)
Cefepime	31 (86.1%)	25 (54.3%)	56(68.3%)
Imipenem	1 (2.8%)	0(0%)	1(1.2%)
Aztreonam	30 (83.3%)	23 (50%)	53(64.6%)
Amikacin	2 (5.6%)	4 (8.7%)	6(7.3%)
Gentamycin	23 (63.9%)	17 (37%)	40(48. 8%)
Tobramycin	22 (61.1%)	8 (17.4%)	30(36.6%)
Nalidixic acid	11 (30.6%)	19 (41.3%)	30(36.6%)
Ciprofloxacin	6 (16.7%)	15 (32.6%)	21(25.6%)

(17.4%). Mechanisms of resistance are not clear, but one possible mechanism is the co-transmission of ESBLs and resistance to other antimicrobials within the same conjugative plasmids. The plasmids that harbor genes encoding ESBLs frequently contain other genes encoding mechanisms of resistance to aminoglycosides (Villa *et al*., 2000). However, the activity of amikacin against these isolates was high. Aminoglycosides resistant rates in this study were much lower than those given by other authors, Babypadmini and Appalaraju (2004), found that 75% and 14% of

ESBL-producers obtained from urine samples were resistant to gentamicin and amikacin, respectively. Agrawal *et al*. (2008) reported high resistance rates 86.75% to gentamicin and 70% to amikacin, among ESBL- producers. Whereas results reported herein indicated variable resistance rates to aminoglycosides, if compared with those found by other studies, in Singapore, Chlebicki and Oh (2004), stated that 46.7% and 13.6% of ESBL-producing isolates were resistant to gentamicin and amikacin, respectively. While El-Astal and Ramadan (2004), reported that 45.45% and 27.27% of ESBL-producers were resistant to gentamicin and amikacin, respectively.

The results from Table (4-9) also indicated that 30.6% and 16.7% of ESBL-positive isolates were found to be resistant to nalidixic acid (quinilone) and ciprofloxacin (fluoroquinilone), respectively. Although fluoroquinolones resistance has almost invariably been found to be encoded on the chromosome, this trait is common in ESBL-producing organisms. For example, Paterson *et al*. (2000) performed a prospective study of *K. pneumoniae* bacteremia in 12 hospitals in seven countries, among the ESBL-producing strains, 18% were also resistant to ciprofloxacin. The close relation between ciprofloxacin resistance and ESBL production in some cases may be due to the organism exposure to antibiotic selection pressure in the environment rather than co-carriage of the resistance gene on plasmids. Moreover, recent finding of quinilone resistance from transferable plasmids is concerning (Wang *et al*., 2003). There have been increasing reports of plasmid-encoded decrease in susceptibility to quinolones, frequently in association with plasmid-mediated cephalosporin resistance (Mammeri *et al*., 2005). Even when plasmid-encoded decrease in quinolone susceptibility is not present, there is a strong association between quinolone resistance and ESBL production (Lautenbach *et al*., 2001). The reason for this association

is not well understood. Martinez-Martinez *et al*. (2002) have performed a careful analysis of mechanisms of quinolone resistance in *K. pneumoniae* isolates, these authors found that porin loss was observed only in those *K. pneumoniae* strains producing an ESBL. Lautenbach *et al*., (2001) found that long-term-care facility residence was a significant risk factor for quinolone resistance in ESBL-producing *K. pneumoniae* and *E. coli* isolates. Fam and El-Damarawy (2008) fond that all ESBL-positive isolates expressed co-resistance to two or more aminoglycosides and fluoroquinolones and were defined as multidrug resistant.

The results of this study indicated that 36.1% ESBL-positive and 43.5% of ESBL-negative isolates were resistant to cefoxitin. Since ESBLs do not confer resistance to cefoxitin, such resistance might possibly be due to high selection pressure exerted on this cephamycin. Resistance to cephamycins could be due to AmpC β-lactamase production or mutations resulting in impermeability through porins (Ananthan and Subha, 2005).

It can be observed that imipenem was an active agent in both ESBL-producing (97.2%) and non- producing (100%) isolates and activity was not affected by ESBL production, indicating that imipenem is the drug of choice for treating serious infections caused by ESBL-producing isolates.

Extended-spectrum β-lactamases have become a widespread serious problem. These enzymes are becoming increasingly expressed by many strains of pathogenic bacteria with a potential for dissemination. The continued emergence of ESBLs presents diagnostic challenges to the clinical microbiology laboratories, which need to be more aware of the necessity for their detection.

In conclusion, the results of this study suggest that the importance of ESBL-producing *E. coli* and *Klebsiella* spp. as a cause of infections at

Merjan hospital. The high prevalence of ESBL-producing isolates should be taken into account when choosing therapeutic agents. Since resistance can differ according to geographical location, continuous local monitoring of resistance patterns is necessary to adequately select an empirical antimicrobial therapy.

4-7 Detection of bla_{CTX-M} and bla_{OXA} ESBL genes:

CTX-M is a recently described family of the ESBLs, these enzymes are not very closely related to TEM or SHV β-lactamases in that they show only approximately 40% identity with these two commonly isolated β-lactamases (Tzouvelekis *et al.,* 2000). In most European countries, Latin America, and East Asia, CTX-M variants have displaced TEM and SHV enzymes as the predominant β-lactamases produced by Gram-negative pathogens, especially *K. pneumoniae* and *E. coli* (Livermore *et al.*, 2007).

The results of this study revealed that of 18 ESBL-positive isolates analyzed, 7(38.9%) isolates yielded amplification products with CTX-M-PCR specific primers (Table 4-10) (Figure 4-3). In a related study from Brazil, bla_{CTX-M} were detected in 33.9% of ESBL-producing *Enterobacteriaceae* isolated from inpatients and outpatients at a public teaching hospital (Dropa *et al.*, 2009). In 2006 CTX-M enzymes were detected in 70% of the isolates in the University Health System in San Antonio, the United States (Lewis *et al.*, 2007).

The results of this study indicated that 5/10 (50%) *E. coli* isolates gave PCR products with CTX-M specific primers (Figure 4-3). This result was lower than those reported by Rodriguez-Bano *et al*. (2006) who found that 70% of the ESBL producing *E. coli* obtained from bacteremia cases in Spain had CTX-M types. In Korea, Park *et al*. (2009) found that PCR experiments

detected the bla_{CTX-M} genes in 84.7% of *E. coli* isolates with an ESBL phenotype and they concluded that CTX-M-type enzymes were the most common type of ESBL in *E. coli* isolates. However, the results obtained in the present study is much higher than that found in Malaysia (20%) (Lim *et al.*, 2009) and in the United States (7.69%) (Robberts *et al.*, 2009).

Table (4-10): Distribution of bla_{CTX-M} and bla_{OXA} genes in ESBL positive *E. coli* and *Klebsiella* spp. isolates

Type of isolate	No. of isolates	Type of β-lactamase enzyme	
		bla_{CTX-M}	bla_{OXA}
E. coli	10	5 (50%)	0
K. pneumoniae	6	2 (33.3%)	0
K. oxytoca	2	0	0
Total	18	7(38.9%)	0

In this investigation, of the 18 PCR analyzed isolates, 2(11.1%) *Klebsiella* spp. isolates gave amplification products with CTX-M specific primers. This result was much lower compared to the results reported from India, where *Klebsiella* isolates represents 50.5% of bla_{CTX-M} producing *Enterobacteriaceae* isolates (Jemima and Verghese, 2008). In Egypt, PCR amplification revealed that of the 10 ESBL-producers analyzed were positive for bla_{CTX-M} 5(50%) were *Klebsiella* isolates (Fam and El-Damarawy, 2008).

In a study reported herein all of the *Klebsiella* isolates that gave positive results with CTX-M specific primers were *K. pneumoniae*

2/8(25%). The spread of CTX-M β-lactamase in *K. pneumoniae* strains was world wide, in Shanghai, China, CTX-M enzymes were the second most frequent ESBLs after SHV enzymes in *K. pneumoniae* strains 10% (Xiong *et al*., 2002), whereas in Thailand, 65.2% of *K. pneumoniae* were able to yield amplification product with bla_{CTX-M} gene (Chanawong *et al*., 2007).

The CTX-M β-lactamases rates in the present study suggest that the horizontal transfer of bla_{CTX-M} genes, mediated by plasmids and/ or mobile elements, contributes to ease with which these enzymes are spreading in *E. coli* and *K. pneumoniae* isolates and the dissemination of CTX-M enzymes to Merjan teaching hospital. Moreover, in hospital environment, plasmids could be transferred easily between patients through health care workers due to hand carriage and antimicrobial selection pressure.

It should be noted that the same organism may harbor both CTX-M-type and SHV-type ESBLs or CTX-M-type ESBLs and AmpC-type β-lactamases, which may alter the antibiotic resistance phenotype (Yan *et al*., 2000). Thus, it indicates the need for a more detailed surveillance and epidemiological survey in Iraqi hospitals.

In this study, *E. coli* and *Klebsiella* spp. isolates were negative in OXA PCRs, which could be either due to the absence of bla_{OXA} gene or the presence of other type of genes that could not be targeted by the primers used in this study. While most ESBLs have been found in *E. coli*, *Klebsiella* spp., and other *Enterobacteriaceae*, the OXA-type ESBLs predominantly occur mainly in *P. aeruginosa* but have been detected in many other Gram-negative bacteria (Weldhagen *et al.*, 2003). In 2008, Bhattacharjee *et al.* reported that of the 361 ESBL-positive enterobacterial isolates from different clinical specimens in India, only one isolate harbored the OXA-10. Whereas in a study done in New York, Jones *et al*. (2009) found that over

40% of the Gram-negative clinical isolates collected carried genes encoding an OXA-type β-lactamase.

In this study, although the PCR results revealed that only 7(38.9%) isolates were able to yield amplification products with CTX-M-PCR specific primers, it is possible that other enzymes such as AmpC, TEM, and SHV might be present in *E. coli* and *Klebsiella* spp. isolates. The PCR analyses usually give reliable and satisfactory results (Senda *et al*., 1996), but this method is of limited practical use for daily application in clinical laboratories because it is rather laborious and expensive. However, PCR analysis serves as the gold standard in epidemiological studies on ESBL enzymes.

4-8 Plasmid profiles of ESBL-positive *E. coli* and *Klebsiella* isolates:

In the present study, CTX-M-positive *E. coli* and *Klebsiella pneumoniae* isolates were tested to determine their plasmid profiles patterns. Since standard plasmid of known molecular weight (size marker) was unavailable, the molecular weights of plasmids isolated were not determined. DNA samples were subjected to gel electrophoresis, which involved separation of small and large plasmids according to their molecular weights (Figure 4-3). In this investigation, the tested samples indicated different plasmid bands when electrophoresis was performed. This finding corresponding to that reported by Siu *et al*. (1999) who illustrated that the plasmids of *E. coli* and *K. pneumoniae* clinical strains were distributed.

Figure (4- 3): Agarose Gel Electrophoresis of DNA content of *E. coli* and *K. pneumoniae* isolates.

Lane 1: DNA content of *E. coli* E9.

Lane 2: DNA content of *E. coli* E15.

Lane 3: DNA content of *Klebsiella pneumoniae* E2.

Lane 4: DNA content of *Klebsiella pneumoniae* E18.

4-9 Determination of MICs for selected ESBL-positive *E. coli* and *Klebsiella* isolates:

In the present study, the MICs values of six ESBL-positive *E. coli* (n=3) and *K. pneumoniae* (n=3) isolates against selected β-lactam antibiotics (ampicillin, amoxicillin, amoxicillin/clavulanic acid, cefotaxime, ceftazidime, and ceftriaxone) were determined by using two-fold agar dilution susceptibility method.

The results of this study indicated that all the *E. coli* and *K. pneumoniae* isolates were highly resistant for ampicillin and amoxicillin

with concentrations reached beyond the break point values. The MICs values of ampicillin and amoxicillin for all tested ESBL-positive isolates were (>128µg/ml) (Table 4-11). This rate is in agreement with those reported in a local studies, Al-Jobouri (1997) found that all *Enterobacteriacea* isolates were resistant to ampicillin and amoxicillin. In a previous study, Al-Charrakh (2005), found that the MICs values of ampicillin and amoxicillin for 8 ESBL-producing *K. pneumoniae* isolated from different environmental and clinical samples in Hilla city ranged from 64 to >128 µg/ml and 32 to >128 µg/ml, respectively. In a study from Najaf, Hadi (2008) found that the MICs values of ampicillin and amoxicillin for 11 ESBL producing *E. coli* and *K. pneumoniae* isolates collected from patients with significant bacteriurea were >128 µg/ml.

Table (4-11): MICs of β-lactam antibiotics for selected ESBL-positive *E. coli* and *Klebsiella* isolates

Isolate	AMP (≥32 µg/ml)	AMX (≥32 µg/ml)	AMC (≥32/16 µg/ml)	CTX (≥64 µg/ml)	CRO (≥64 µg/ml)	CAZ (≥32 µg/ml)
***E. coli* E3**	>128	>128	32/16	32	64	32
***E. coli* E7**	>128	>128	32/16	64	64	32
***E. coli* E12**	>128	>128	32/16	64	64	64
***K. pneumoniae* E2**	>128	>128	16/8	32	64	32
***K. pneumoniae* E9**	>128	>128	16/8	64	32	64
***K. pneumoniae* E16**	>128	>128	32/16	64	64	16

* Number between brackets refers to break points recommended by CLSI (2007).
AMP, ampicillin; AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime.

Although high MIC of amoxicillin was observed in this study, but there was a reduction in the MIC value of this drug by two to three-folds when it was combined with clavulanic acid. The combination of amoxicillin/ clavulanic acid was found to be active against all the test isolates and activity was equal to or below the MIC break point of amoxicillin alone. The MICs values of amoxicillin/ clavulanic acid for all tested isolates were ranged from 16/8 to 32/16µg/ml. Despite the presence of clavulanic acid, 4(66.7%) isolates (3 *E. coli* and 1 *K. pneumoniae*) kept their high level resistance to amoxicillin. Amoxicillin/ clavulanic acid activity was two-fold below (16/8µg/ml) than the break point of amoxicillin alone for 2(33.3%) *K. pneumoniae* isolates.

The discrepancies among the results produced by various antimicrobial susceptibility testing methods have been reported for several of the β-lactam-β-lactamase inhibitor combinations (Jones and Dudley, 1997), for example, Oliver *et al*. (1999) reported that various results were obtained when a fixed ratio of β-lactam to β-lactamase inhibitor (as opposed to a fixed concentration of inhibitor) was used to test the *in vitro* activity of β-lactam-β-lactamase inhibitor combinations, such as amoxi-clav, against *E. coli*. Since disk diffusion and agar dilution methods may give discrepant results, it is often difficult to compare the results for β-lactam-β-lactamase inhibitor combinations reported by various surveillance systems, making comparisons even more complicated, the standards-setting bodies of various countries use differing susceptibility break points for amoxi-clav as well as differing susceptibility-testing methods (Simpson *et al*., 1998). Additionally, it is well known that ESBL-producing organisms may continue to harbor parent enzymes (for example, SHV-1 or TEM-1). Hyperproduction of these non-ESBLs or the combination of β-lactamase production and porin loss can

also lead to a reduction in activity of β-lactamase inhibitors (Akhan *et al*., 2001). However, the MIC results suggest that amoxicillin/clavulanic acid was active against the tested isolates.

The results in Table (4-11) also indicated that the MIC value of cefotaxime for tested isolates ranged from 32 to 64µg/ml. 4(66.7%) isolates (2 *E. coli* and 2 *K. pneumoniae*) were able to grow in concentration equal to the break point (64µg/ml) and 2(33.3%) isolates (one *E. coli* and one *K. pneumoniae*) were able to grow in concentration less than the break point value (32µg/ml). These results were lower than that reported by Wachino *et al*. (2004) who found that MICs of cefotaxime for six ESBL-producing *K. pneumoniae* clinical isolates from neonates in Japan were variable and ranged from (16 to 128 µg/ml). In Egypt, the MIC values of 10 ESBL-producing (5 *E. coli* and 5 *K. pneumoniae*) isolates were 64µg/ml (Fam and El-Damarawy, 2008). Other studies reported much higher MIC values than in this study, in the United States, Jett *et al*. (1995) found that of 83 *Klebsiella* spp. and 25 *E. coli* ESBL-producing isolates, the MIC values for 90% of the isolates ranged from 64 µg/ml for *Klebsiella* and 1024 µg/ml for *E. coli* isolates.

The results presented in Table (4-11) indicated that MIC values of ceftriaxone were ranged from 32 to 64µg/ml. 5(83.3%) isolates (all *E. coli* and 2 *K. pneumoniae*) were able to grow in concentration equal to the break point value (64 µg/ml), while only 1(16.7%) *K. pneumoniae* isolate was able to grow in concentration one-fold below the break point value (32µg/ml). The level of resistance to ceftriaxone in the present study is lower than that reported by other studies, Chanawong *et al*. (2007) found that MIC for 90% of 19 *E. coli* and 23 *K. pneumoniae* isolates tested >128 µg/ml. In Najaf, Hadi (2008) reported that 100% of ESBL-producing *E. coli* and *K.*

pneumoniae isolates were resistant to ceftriaxone with high MIC values ranged from 64 to 128µg/ml. On the other hand, the results of the present study are higher than those given in the United States, it was found that MICs of ceftriaxone for ESBL-positive *E. coli* clinical isolates ranged from 16 to>32 µg/ml (Robberts *et al*., 2009).

For ceftazidime, the MIC values ranged from 16 to 64µg/ml. MIC value of 3(50%) isolates (2 *E. coli* and 1 *K. pneumoniae*) were equal to the break point value (≥ 32µg/ml), whereas 2(33.3%) isolates (one *E. coli* and one *K. pneumoniae*) were highly resistant to ceftazidime with concentration of two-fold the break point value (64 µg/ml), while only one *K. pneumoniae* isolate was able to grow at concentration less than the break point value (16 µg/ml) which may be due to low level of ESBL activity. These values are in agreement with those given by Fam and El-Damarawy (2008), who found that the MICs of ceftazidime for ESBL-positive *E. coli* and *K. pneumoniae* isolated from ICU patients ranged between 16 to 64 µg/ml. While they are lower than that found in the United States by Paterson *et al*. (2001), who found that MIC for 66.5% of ESBL-producing *K. pneumoniae* bloodstream isolates was ≥ 64 µg/ml.

4-10 AmpC β-lactamase production:

4-10-1 Cefoxitin susceptibility:

The first bacterial enzyme reported to destroy penicillin was the AmpC β-lactamase of *E. coli*, although it had not been so named in 1940 (Abraham and Chain, 1940). AmpC β-lactamases are cephalosporinases that are poorly inhibited by clavulanic acid. They are clinically significant because they may confer resistance to a wide variety of β-lactam drugs, including α-methoxy- β-lactams (cephamycins) such as cefoxitin, narrow-, broad-, and

expanded-spectrum cephalosporins, β-lactam-β-lactamases inhibitor combinations, and aztreonam (Rodriguez-Martinez *et al*., 2003).

In this study, cefoxitin susceptibility of 82 *E. coli* and *Klebsiella* spp. isolates was tested by the standard Kirby-Bauer disk diffusion method (Bauer *et al*., 1966). The results indicated that 33 (40.2%) isolates yielded cefoxitin zone diameter less than 18 mm, these isolates may be AmpC producers (Table 4-12). However, the prevalence of cefoxitin resistance among clinical isolates varies from country to country and from institution to institution. In a study from India, Arora and Bal (2005), reported that of 284 Gram-negative isolates collected from Kolkata hospital, 27(10.5%) isolates were found to be resistant to cefoxitin. In China, Li *et al*. (2008), found that of 1935 *E. coli* and *Klebsiella* spp. isolates collected from medical center, 327(16.9%) were detected as cefoxitin resistant.

Table (4-12): Frequency of cefoxitin resistance among *E. coli* and *Klebsiella* spp. isolates

Type of isolate	Total No.	No. (%) of cefoxitin resistance isolate
E. coli	40	22(55%)
K. pneumoniae	25	7(28%)
K. oxytoca	17	4(23.5%)
Total	82	33(40.2%)

Although cephamycin-resistant *E. coli* and *Klebsiellae* are relatively uncommon, widespread use of β-lactam antibiotics may contribute to the development and spread of these strains. However, the phenotypic data generated in this study indicated that 22(55%) of the 40 *E. coli* isolates were

phenotypically confirmed to be cefoxitin resistant (Table 4-12) which were more than that reported by Subha *et al.* (2003) who found that 37.5% of *E. coli* isolates were cefoxitin resistant in Chennai, India. The screening of 29,323 clinical isolates of *E. coli* collected in 1999 to 2000 from 12 hospitals in Canada identified 232 (0.79%) strains that were resistant to cefoxitin (Mulvey *et al.*, 2005).

In the present study, among 40 *Klebsiella* spp. isolates, cefoxitin resistance was detected in 7/25(28%) *K. pneumoniae,* and 4/17(23.5%) *K. oxytoca* (Table 4-12). In a study reported during the SENTRY Antimicrobial Surveillance Programme, cefoxitin was the most successful cephalosporin against European *K. pneumoniae* (95.6% susceptible) and *K. oxytoca* (98.6% susceptible) (Nijssen *et al.*, 2004).

Through the study, of the 36 ESBL-positive isolates only 13(36.1%) were resistant to cefoxitin, of which 8/18(44.4%) *E. coli* and 4/18(22.2%) *Klebsiella* spp. Results also revealed that 20(43.5%) of the 46 ESBL-negative isolates were resistant to cefoxitin, of which 13/22(59.1%) *E. coli* and 7/24(29.2%) *Klebsiella* spp. (Table 4-13). According to such observation, cefoxitin resistance has an elevated levels among both ESBL-positive and ESBL-negative isolates, and it may be concluded that such resistance is suggestive of an AmpC enzyme, but it is not specific since cefoxitin resistance can also be produced by decreased levels of production of outer membrane porins in both *K. pneumoniae* and *E. coli* (Hernandez-Alles *et al.*, 2000). However, in this study the coexistence of different classes of β-lactamases or mixed type of drug resistance mechanisms in the bacterial isolates is possible and may indicate the spread of multidrug resistant isolates in Merjan hospital which may pose a diagnostic challenges and treatment failure, in other studies, a range of cefoxitin resistance values

has been reported for examples, in 2001, Paterson *et al.* found that from 85 ESBL-producing *K. pneumoniae* isolates obtained from patients with bacteremia in the United States, Taiwan, Australia, South Africa, Turkey, Belgium, and Argentina, 1.4% were cefoxitin resistant. In a laboratory surveillance study in Canada, Mulvey *et al*. (2005) reported that a total of 411 ESBL producing *E. coli* isolates, 232(56.4%) isolates were identified as resistant to cefoxitin.

Table (4-13): Frequency of cefoxitin resistance among ESBL-positive and ESBL-negative isolates

Type of isolate	No. (%) of ESBL-positive		No. (%) of ESBL-negative	
	Total No.	Cefoxitin resistance	Total No.	Cefoxitin resistance
E. coli	18	8(44.4%)	22	13(59.1%)
K. pneumoniae	12	3(25%)	13	5(38.5%)
K. oxytoca	6	2(33.3%)	11	2(18.2%)
Total	36	13(36.1%)	46	20(43.5%)

The cefoxitin resistance phenotype can be a result of overexpression of the chromosomal *ampC* gene , acquisition of a plasmidic *ampC*, alternation in the permeability of the cell to cefoxitin, or a combination of these factors (Mulvey *et al*., 2005). The fact that a standardized phenotypic method for screening and detection of these types of resistance does not exist makes the surveillance and characterization of strains problematic. Screening with cefoxitin disk is recommended for initial detection. However, it does not reliably indicate AmpC production. Some of the phenotypic tests include the

three dimensional test and AmpC disk test could be used as confirmatory tests.

4-10-2 AmpC β-lactamases: modified three dimensional test (MTDT):

In a former study, Coudron and Moland (2000) reported that the TDT (three-dimensional test) did not reveal false negative results and only 1(3.6%) of the 28 AmpC harboring *E. coli* and *Klebsiella* spp. isolates was false positive. This suggests that this technique can be used for routine screening of the AmpC enzyme in a clinical laboratory. But the sensitivity of the test has not been confirmed for organisms other than *E. coli* and *Klebsiella* spp. (Manchanda and Singh, 2003).

In a study reported herein, out of 82 β-lactam resistant *E. coli* and *Klebsiella* spp. isolates, AmpC β-lactamase production was confirmed by MTDT in 7(8.5%) isolates, of which 5(12.5%) *E. coli*, 1(4%) *K. pneumoniae*, and 1(5.9%) *K oxytoca* (Table 4-14) (Figure 4-4). The incidence of AmpC β-lactamase in isolates tested may reflect two modes of production: hyper production of chromosome-mediated and plasmid-mediated AmpC β-lactamase.

During the entire study period, all AmpC-positive isolates were resistant to cefoxitin, whereas the remaining 26/33(78.8%) cefoxitin resistant isolates were non-AmpC β-lactamase producers (Table 4-14). This result indicates that even though the screening methods that use the cefoxitin for the detection of AmpC-producing isolates are easily performed, but they are not accurate. Cefoxitin resistance in non-AmpC β-lactamase producers may be due to some other resistance mechanisms such as lack of permeation of porins (Pangon *et al.*, 1989). Other study has demonstrated that the interruption of a porin gene by insertion sequences is a common type of mutation that causes loss or decrease of outer membrane porin expression

and increased cefoxitin resistance in *E. coli* and *Klebsiella* spp. (Hernandez-Alles *et al*., 2000).

Table (4-14): AmpC β-lactamases production in *E. coli* and *Klebsiella* spp. isolates

Type of isolate	No. (%) of cefoxitin resistance isolates	No. (%) of AmpC-positive isolates		No. (%) of AmpC-negative isolates
		MTDT	AmpC Disk Test	
***E. coli* (n = 40)**	22 (55%)	5(12.5%)	5(12.5%)	17(42.5%)
***K. pneumoniae* (n=25)**	7(28%)	1(4%)	1(4%)	6 (24%)
***K. oxytoca* (n=17)**	4(23.5%)	1(5.9%)	1(5.9%)	3 (17.6%)
Total (n=82)	33 (40.2%)	7 (8.5%)	7(8.5%)	26(31.7%)

A positive MTDT result in the present study differentiates between AmpC production and reduced outer membrane permeability. However, in a previous study in Greek, Gazouli *et al*. (1998) tested 2133 *E. coli* isolates from 10 hospitals and found that 55(2.6%) contained AmpC β-lactamases. In another study Coudron *et al*. (2000) estimated that 1.6% of *E. coli* isolates and 1.1% of *K. pneumoniae* isolates were cefoxitin-resistant AmpC β-lactamase producers, mostly via transmissible plasmids. In a previous study conducted in India, AmpC β-lactamases were produced by 47.8% *E. coli* and 13% *K. pneumoniae* isolates (Arora and Bal, 2005). The detection of AmpC β-lactamases by MTDT method is still not satisfactory. As suggested earlier was sensitive but it is more technically challenging and labor intensive than other methods (Thomson and Sanders, 1992).

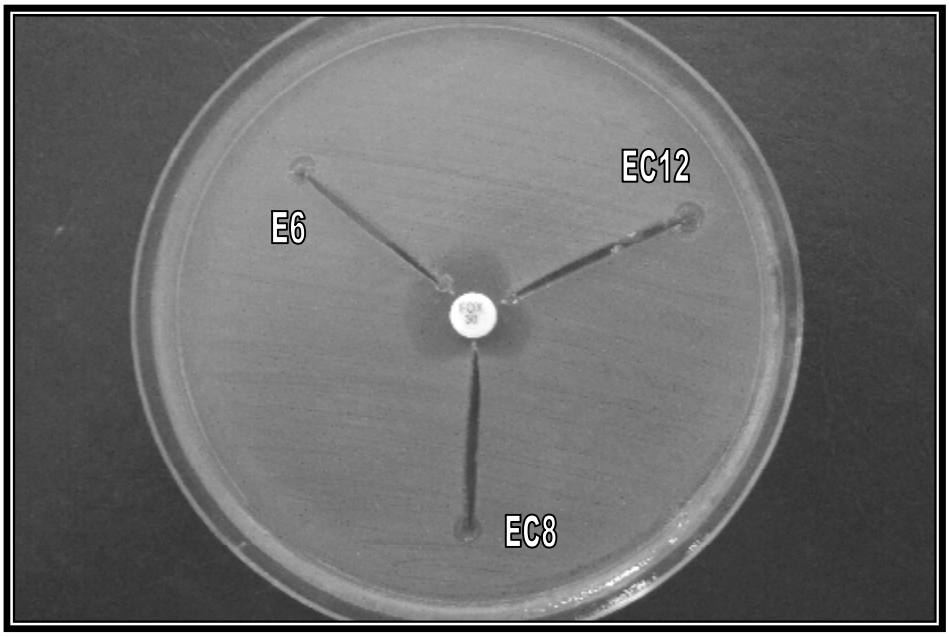

Figure (4-4): MTDT to detect AmpC β-lactamase. Growth of *E. coli* ATCC 25922 strain around slits containing AmpC enzyme extractions of *E. coli* EC12 and EC8 test isolates, exhibit clear distortion of the zone of inhibition of cefoxitin. *E. coli* E6 isolate exhibit negative result.

4-10-3 AmpC β-lactamases: The AmpC disk test:

The AmpC disk test provided a simple, convenient, and accurate method of detection of isolates that harbor AmpC β-lactamases. The test accurately distinguished between cefoxitin insusceptibility caused by AmpC production and non-β-lactamases mechanisms, such as reduced outer membrane permeability (porin mutations). Distinguishing between these types of mechanisms is a current diagnostic problem for laboratories wanting to detect AmpC β-lactamases.

Of the 82 β-lactam resistant *E. coli* and *Klebsiella* spp. isolates, 7 isolates, which were positive by MTDT, also revealed positive results by

AmpC disk test (Table 4-14). Indentation indicating strong AmpC producer was observed in 5 isolates (Figure 4-5), whereas flattening (weak AmpC) was observed in 2 *E. coli* isolates. In a similar study Singhal *et al*. (2005) reported that AmpC β- lactamases was confirmed in 36% (22/61) of *E. coli* and *Klebsiella* spp. isolates from tertiary care hospitals by the MTDT, and all the 22 AmpC producer isolates were positive by AmpC disk test. Strong AmpC production observed in 15 isolates whereas weak AmpC was observed in 7 isolates.

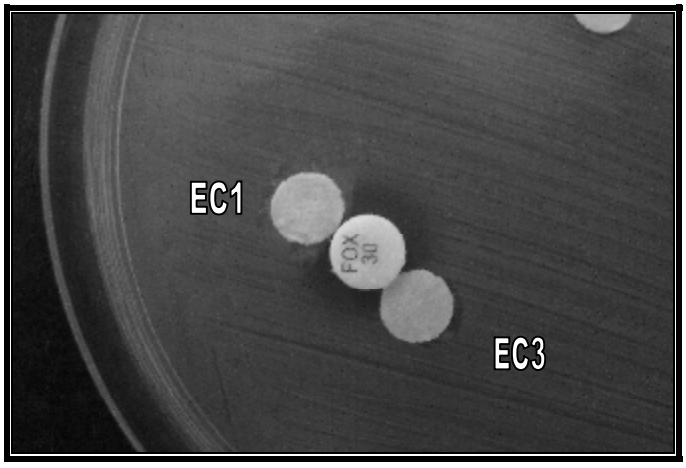

Figure (4-5): AmpC disk test to detect AmpC β-lactamase production in *E. coli* EC1 (strong positive result) and EC3 (positive result).

In the present study, 7(8.5%) of 82 isolates found to harbor an AmpC enzyme by the MTDT and AmpC disk test. This low occurrence (if compared with cefoxitin survey results) may be due to a lack of permeation of porin or that some of the isolates may have *ampC* genes, but might not be expressed in all the isolates. This means they might have 'silent genes' or there might be low level expression of *ampC* genes that was not detected. Since only genes that are expressed cause resistance, phenotypic tests may be more valuable (Jacoby, 2009). The AmpC disk test does not discriminate

between positive results due to upregulated chromosomally mediated AmpC β-lactamases and those due to plasmid-mediated AmpC β-lactamases. Additional studies reported that the AmpC disk test also reliably detected plasmid-mediated AmpC β-lactamases and also high- level production of chromosomally mediated AmpC β-lactamases in *Enterobacteriaceae* (Black *et al*., 2005).

The antibiotic susceptibility profiles of AmpC-positive isolates are shown in (Figure 4-6). All the 7 isolates were resistant to amoxi-clav, cefoxitin and carbenicillin. Concerning third generation cephalosporins and aztreonam, 6(85.7%) isolates revealed resistance to ceftriaxone; 5(71.4%) were resistant to ceftazidime and cefotaxime; and 4(57.1%) were aztreonam resistant. Results also revealed that 4 (57.1%) isolates were resistance to cefepime, piperacillin, and nalidixic acid each; 3 (42.9%) isolates were resistant to gentamicin and tobramycin, this suggests the absence of outer membrane porin defect frequently reported in such species (Coudron, 2005). The results of this study suggested that imipenem and ciprofloxacin (sensitivity 100% each) were the most active agents against AmpC-producing isolates followed by amikacin (sensitivity 80%). The present study also revealed that one *E. coli* C5 isolate indicated a significant ceftazidime-imipenem synergism (Figure 4-7), this isolate detected as AmpC β-lactamase-producer in MTDT, and as weak producer in AmpC disk test, this synergism may indicate that imipenem can not induce AmpC β-lactamases in this isolate although it is a potent inducer (i.e. AmpC non-inducible producer). But imipenem act together with ceftazidime to increase the sensitivity of this isolate for these two drugs. The available data suggest that carbapenems are more effective than cefepime in treating serious infections caused by AmpC producing organisms (Zanetti *et al*., 2003).

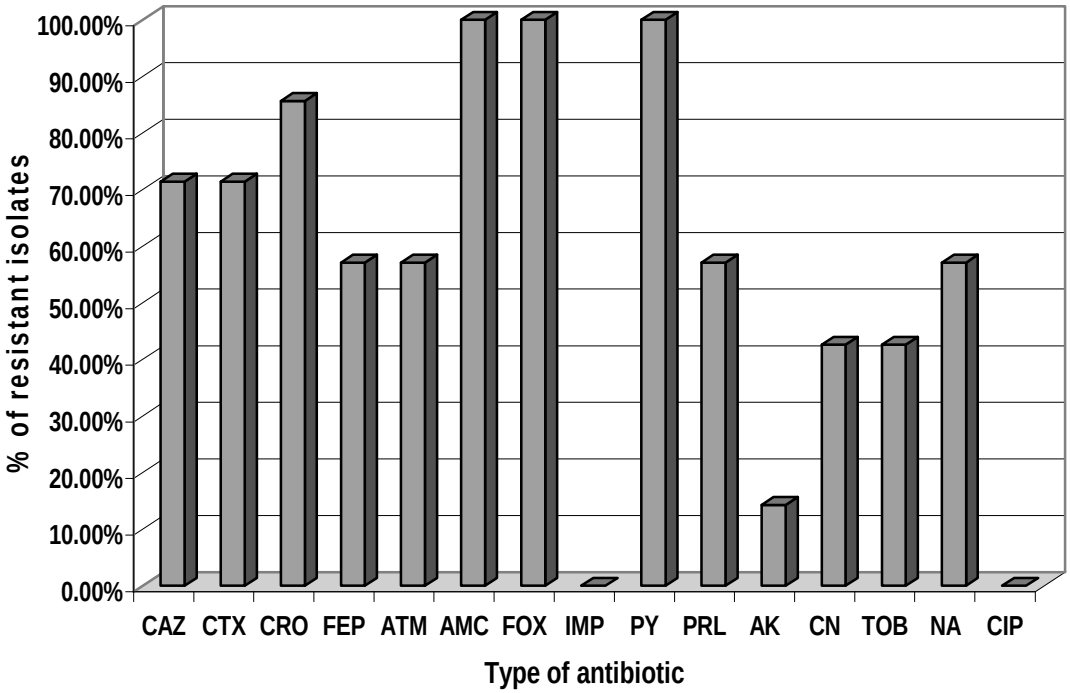

Figure (4-6): Resistance of AmpC-positive *E. coli* and *Klebsiella* spp. isolates (n=7) to various antibiotics

AMC, Amoxi-clav; FOX, Cefoxitin; PY, Carbenicillin; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime; PRL, Piperacillin; NA, Nalidixic acid; FEP, Cefepime; CN, Gantamycin; TOB, Tobramycin; ATM,Aztreonam; AK, Amikacin; IMP, Imipenem; CIP, Ciprofloxacin

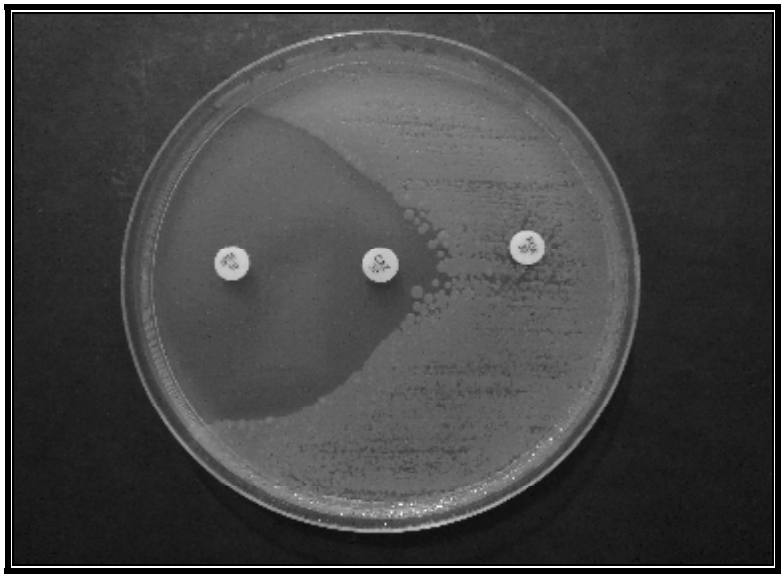

Figure (4-7): *E. coli* C5 isolate exhibit a significant ceftazidime-imipenem synergism. CAZ, Ceftazidime; IMP, Imipenem; FOX, Cefoxitin.

4-10-4 Presence of inducible AmpC β-lactamases:

Chromosomal AmpC β-lactamases are usually inducible, while plasmid-mediated AmpC enzymes are not (Philippon *et al*., 2002). In many Gram-negative species, inducible AmpC β-lactamases are normally produced at very low levels but are induced to several hundred fold higher by the presence of β-lactams (e.g., cefoxitin, ceftazidime etc.) and certain β-lactamases inhibitors (e.g., clavulanic acid) (Jacoby, 2009). As some bacterial species may produce inducible AmpC β-lactamases that can be easily overlooked by routine susceptibility tests, Cantarelli *et al.* (2007), reported a new test to detect and confirm the presence of inducible AmpCs among enterobacterial strains, the ceftazidime-imipenem antagonism test (CIAT), based on the strong inducing effect of imipenem on these enzymes and the consequent antagonism with ceftazidime.

Table (4-15) indicate that only 2 (2.4%) of β-lactams resistant isolates

were positive for inducible AmpC β-lactamases, one was *E. coli* C4 and other was *K. oxytoca* C7 isolates. While the remaining 5(6.1%) AmpC-positive isolates were negative for inducible AmpC β-lactamase.

Table (4-15): Frequency of inducible and PABLs producers in *E. coli* and *Klebsiella* spp. isolates

Type of isolate	Total No.	No.(%) of :		
		Inducible AmpC-producers	PABLs producers	Both ES and PABLs producers
E. coli	40	1(2.5%)	4(10%)	3(7.5%)
K. pneumoniae	25	0	1(4%)	0
K. oxytoca	17	1(5.9%)	0	0
Total	82	2(2.4%)	5(6.1%)	3(3.7%)

The results of the present study suggested that the chromosomally encoded AmpC enzymes were rare among the *E. coli* and *Klebsiella* isolates collected from Merjan hospital environments. These two isolates were detected as non-ESBLs and confirmed as AmpC-producers by MTDT and AmpC disk tests. *E. coli* C4 isolate revealed blunting of ceftazidime zone of inhibition by imipenem-induced enzyme, while *K. oxytoca* C7 isolate revealed blunting of ceftazidime zone of inhibition by cefoxitin-induced enzyme Figure (4-8). The blunting of ceftazidime zone by imipenem-induced enzyme is

more pronounced, compared with the cefoxitin-induced enzyme (Cantarelli *et al.*, 2007). In a recent study, Taneja *et al*. (2008), found that among 205 highly drug resistant uropathogenic isolates, only one isolate was positive for inducible AmpC β -lactamases. In 2008, Chaudhary *et al.* reported that out of 126 *E. coli* and 42 *Klebsiella* spp., inducible AmpC β-lactamase-production was detected in 24(19.1%) and 5(11.9%) isolates, respectively.

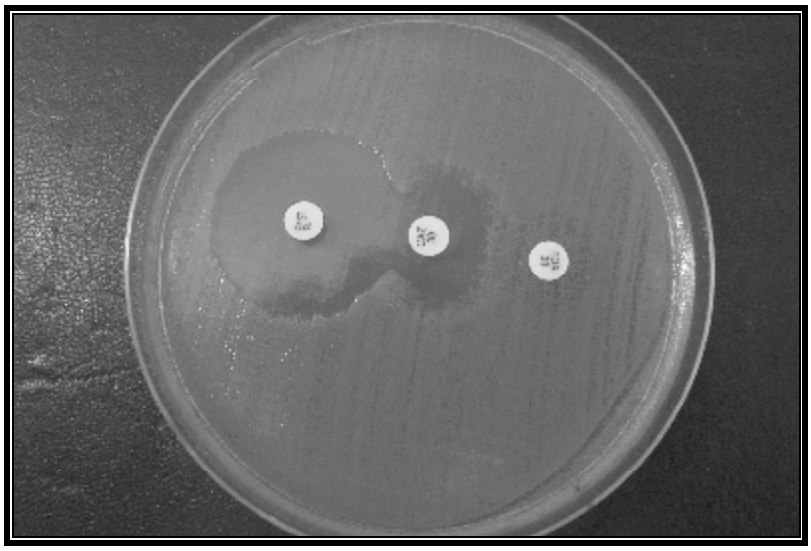

Figure (4-8): Disk antagonism for inducible AmpC β-lactamase in *K. oxytoca* C7 test isolate exhibit blunting of ceftazidime zone of inhibition adjacent to cefoxitin disk by cefoxitin-induced enzyme.

In many *Enterobacteriaceae*, AmpC expression is low but inducible in response to β-lactam exposure. β-lactams differ in their inducing abilities. Benzylpenicillin, ampicillin, amoxicillin, and cephalosporins such as cefazolin and cephalothin are strong inducers and good substrates for AmpC β-lactamase. Imipenem is also strong inducer but is much more stable for hydrolysis. Cefotaxime,

ceftriaxone, cefuroxime, cefepime, piperacillin, and aztreonam are weak inducers and weak substrates but can be hydrolyzed if enough enzyme is made (Pai *et al.*, 2004). β-lactamase inhibitors are also inducers, especially clavulanate, which has little inhibitory effect on AmpC β-lactamase activity (Weber and Sanders, 1990), but in case of inducible AmpC β-lactamases can paradoxically appear to increase AmpC mediated resistance in an inducible organism, thus this type of drug can cause more harm than help (Arora and Bal, 2005). DHA-1 type of inducible AmpC β-lactamases was first reported from Saudi Arabia in 1998 (Barnaud *et al*., 1998), and later on from Taiwan in 2002 (Yan *et al*., 2002). The prevalence rate of DHA-1-producing *K. pneumoniae* significantly increased in Korea, from 0.6% in 2002 to 2.8% in 2003 and to 4.3% in 2004 (Song *et al*., 2006).

The present study found that CIAT is a simple test that can be used to confirm the presence of the known, as well as the new, inducible AmpC enzymes, among *E. coli* and *Klebsiella* isolates. The test can be done directly on the initial susceptibility test or in combination with other disks used to detect ESBL production.

4-10-5 Presence of Plasmid-mediated AmpC β-lactamase (PABLs):

Plasmid-mediated AmpC β-lactamases have arisen through the transfer of chromosomal genes for the inducible AmpC enzymes of the family *Enterobacteriaceae* onto plasmids. This transfer has resulted in PABLs in isolates of *E. coli, Klebsiella* spp. and other Gram-negative bacilli (Hanson, 2003). To date, all PABLs have similar substrate profiles to the parental enzymes from which they

appear to be derived (Muratani *et al*., 2006). Recently, over 40 types of PABLs have been reported worldwide (Li *et al*., 2008).

Of the 82 isolates of *E. coli* and *Klebsiella* spp. included in the study, 5(6.1%) were confirmed as non-inducible AmpC-producers by CIAT, suggesting the presence of plasmid-mediated resistance mechanism, of which 4/40(10%) were *E. coli* and 1/42(2.4%) were *K. pneumonia* (Table 4-15). However, the prevalence of PABLs among clinical isolates differs depending on the countries and institutions. The percentages of PABLs production in this study was higher when compared to studies done in other countries. Isolates producing PABLs have been found to be now wide spread. For example, In the United States, occurrence rates of PABLs for *E. coli* were 1.6% (Coudron *et al*., 2000). In China, Li *et al*. (2008) found that 4.29% of *K. pneumoniae*, 3.03% of *K. oxytoca* isolates, were PABLs producers, whereas only 1.91% *E. coli* isolates were detected as PABLs.

The results in this study revealed that imipenem and ciprofloxacin were the only antibiotics which were found to be effective against all the PABLs producing isolates (Figure 4-9) which may be attributed to the absence of outer membrane porin defect frequently reported in AmpC β-lactamases producing species. Thus, these two drugs are the most successful in treating nosocomial infections caused by such isolates.

The results also revealed that all the PABLs isolates were resistant to amoxi-clav, cefoxitin, and carbenicillin; whereas 4(80%) were resistant to ceftazidime, cefotaxime, ceftriaxone, piperacillin, and nalidixic acid each; 3(60%) were resistant to cefepime, gentamicin and tobramycin each; 2(40%) were resistant to aztreonam;

and 1(20%) was resistant to amikacin. This could be explained by the fact that PABLs have a broad substrate profile. Plasmids carrying genes for AmpC β-lactamases often carry multiple other resistances including genes for resistance to aminoglycosides, quinolones (nalidixic acid), and other antibiotic classes, therefore these enzymes are typically associated with multiple antibiotic resistance, leaving few therapeutic options (Jacoby, 2009). On the other hand, these plasmid-mediated genes are of special interest because their mobility allows them to emerge in one genus or species and spread to different organisms (Rodriguez-Martinez *et al*., 2003).

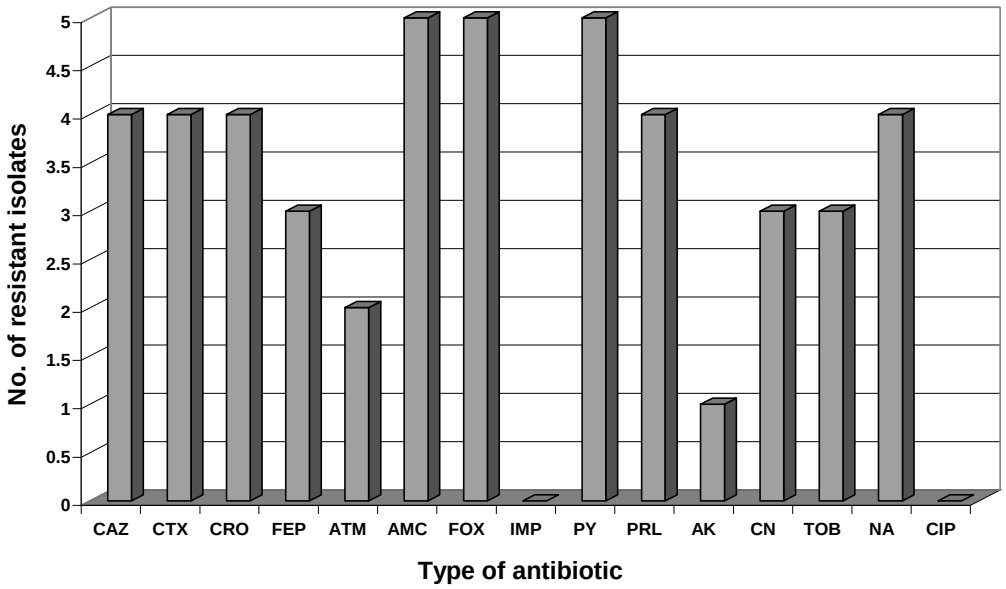

Figure (4-9): Resistance patterns of PABLs producing isolates (n=5) to various antibiotics

AMC, Amoxi-clav; FOX, Cefoxitin; PY, Carbenicillin; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime; PRL, Piperacillin; NA, Nalidixic acid; FEP, Cefepime; CN, Gantamycin; TOB,Tobramycin;ATM,Aztreonam;AK,Amikacin;IMP,Imipenem;CIP, Ciprofloxacin

4-10-6 Extended-spectrum and AmpC β-lactamases producing isolates:

Both ESBLs and AmpC β-lactamases genes are usually located on large multidrug resistance plasmids. Therefore, both types of enzymes are typically associated with resistance to multiple antibiotics, thus leaving few therapeutic options (Moland, 2002). However, coexistence of different classes of β-lactamases in a single bacterial isolate may pose diagnostic and therapeutic challenges.

In this study, coexistence of AmpC and ESBLs was detected in 3(3.7%) of the 82 *E. coli* and *Klebsiella* isolates, all of which were PABLs-producing *E. coli* (3/40,7.5%) (Table 4-15) (Figure 4-10), this could be explained by the dissemination of plasmid-mediated AmpC β-lactamases among *Enterobacteriaceae*, sometimes in combination with ESBLs, suggesting a clonal spread of multidrug-resistant *E. coli* at Merjan hospital. *Enterobacteriaceae* producing both AmpC β-lactamases and ESBLs have been increasingly reported worldwide (Chanawong *et al*., 2002; Spanu *et al*., 2002). However, Data from the SENTRY antimicrobial surveillance program for North America reported that 19/65 ESBL screen-positive *E. coli* isolates harbored PABL (Deshpande *et al*., 2006). In addition, out of 36 *E. coli* and *K. pneumoniae* AmpC β-lactamase producing isolates, the majority 31 (86.1%) occurred in combination with ESBLs (Hemalatha *et al*., 2007).

Table (4-16) reveals that all the 3 ESBL and PABLs co-producing *E. coli* isolates were resistant to cephalosporins, amoxi-clav, cefoxitin, carbenicillin and piperacillin; while 2(66.7%) isolates were resistant to aztreonam, nalidixic acid, gentamicin, and tobramycin. The resistance to cephalosporins is often mediated by the production of ESBLs, however, it may be attributed to AmpC cephalosporinases, which in *E. coli* generally arises by acquisition of a PABLs (Baudry *et al*., 2009). Both ESBLs and

plasmid-mediated AmpC β-lactamases are typically associated with broad multidrug resistance and may be co-transferred with plasmids mediating aminoglycosides resistance (usually a consequence of genes for other antibiotic resistance mechanisms residing on the same plasmids as the ESBL and AmpC genes) and confer a selective advantage to strains harboring these enzymes in a hospital setting. Thus, antibacterial choice is often complicated by multi-resistance (Rupp and Fey, 2003).

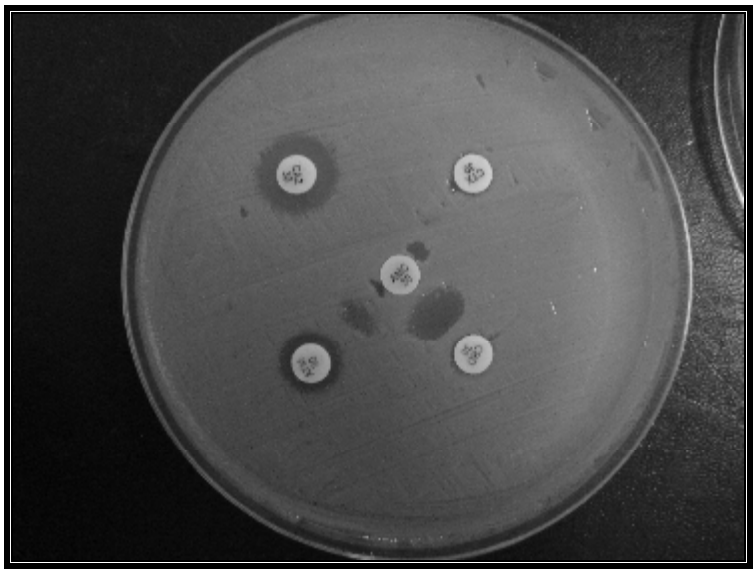

Figure (4-10): AmpC and ESBL-production in *E. coli* E8 isolate. The zone of inhibition between aztreonam/ ceftriaxone and amoxi-clav disk indicating positive ESBL. The resistance to amoxi-clav due to the presence of AmpC β-lactamase. AMC, Amoxi-clav; ATM, Aztreonam; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime

The results in this study also indicated that all the 3 *E. coli* isolates were susceptible to imipenem, amikacin and ciprofloxacin, suggesting that these drugs are the most active agents in AmpCs and ESBLs co-producing isolates. The effect of concurrent ESBL and plasmid-mediated AmpC gene expression may adversely affect the performance of current ESBL screening

and confirmatory testing, as the two enzyme groups have overlapping hydrolysis spectra. Based on SENTRY Asia-Pacific data; up to 75% of non confirming isolates were found to harbor plasmid-mediated AmpC genes (Bell *et al*., 2007).

Table (4-16): Susceptibility of ESBLs and PABL-producing *E. coli* isolates to the selected antibiotics

Type of antibiotic	Susceptibility		
	***E. coli* EC12**	***E. coli* EC7**	***E. coli* EC8**
Ceftazidime	R	R	R
Cefotaxime	R	R	R
Ceftriaxone	R	R	R
Cefepime	R	R	R
Aztreonam	R	R	S
Amoxi-clav	R	R	R
Cefoxitin	R	R	R
Imipenem	S	S	S
Carbenicillin	R	R	R
Piperacillin	R	R	R
Amikacin	S	S	S
Gantamycin	S	R	R
Tobramycin	S	R	R
Nalidixic acid	R	R	S
Ciprofloxacin	S	S	S

EC, ESBL+ AmpC β-lactamase- producer; R, resistant; S, sensitive

However, in developing countries included Iraq, many clinical laboratories (as well as the wider medical community) are not fully aware of the importance of ESBLs and PABLs and how to detect them; laboratories may also lack the resources to curb the spread of these resistance mechanisms. This lack of understanding or resources is responsible for a

continuing failure to respond appropriately to prevent the rapid worldwide dissemination of pathogens possessing these β-lactamases.

4-11 Detection of carbapenemases production:

Carbapenems (imipenem, meropenem, and ertapenem) have the broadest spectra of activity of all the β-lactam antibiotics and since the 1980s, have provided a highly effective treatment option for serious infections with *P. aeruginosa* and ESBL-positive *Enterobacteriaceae*. They are active against most of the common β-lactamase enzymes (AmpC and ESBLs). The use of carbapenems in the treatment of infection has been compromised by the emergence of carbapenems-hydrolyzing β-lactamases (Queen and Bush, 2007).

There are no data on the prevalence of carbapenemases producing Gram-negative bacilli spp. in Hilla. Hence, the present study was undertaken to find out the prevalence of carbapenemases producing *E. coli* and *Klebsiella* spp. isolated at Merjan hospital. During the period of study imipenem susceptibility of 82 *E. coli* (n=40) and *Klebsiella* spp. (n=42) isolates was determined.

The result of the present study revealed that 81(98.8%) isolates were susceptible to imipenem, whereas 1(1.2%) isolate was carbapenamase positive (Table 4-17). This isolate was *K. oxytoca* EK recovered from waste container in men ward. Several studies reported that resistance to carbapenems in *Klebsiella* spp. remains infrequent (Poirel *et al.*, 2004; Ikonomidis *et al.*, 2005). The prevalence of carbapenems resistant among Gram-negative isolates vary greatly worldwide in geographical areas, and are rapidly changing over time. In a previous study, Hadi (2008) found that all ESBL-producing *E. coli* and *Klebsiella pneumoniae* isolates obtained from patients

with significant bacteriurea in Najaf were susceptible to imipenem. In India Akram *et al*. (2007) reported that among the β-lactam antibiotics, imipenem had the widest coverage against Gram-negative isolates (sensitivity 100%).

Table (4-17): Carbapenemases production in *E .coli* and *Klebsiella* spp. isolates

Type of isolate	No. of organisms	No. (%) of positive isolates	
		IMP-EDTA DDST	MHT
***Klebsiella* spp.**	42	0	1 (2.4 %)*
E. coli	40	0	0
Total	82	0	1 (1.2 %)

MHT, Modified Hodge Test; DDST, Double Disk Synergy Test; *, *K. oxytoca* EK

In this study, 35(97.2%) ESBL-producers and all 46(100%) non-ESBL isolates were susceptible to imipenem. About 10% of ESBL-producing GNB isolated from hospital in Thailand were resistant to carbapenems and is mainly caused by carbapenems-hydrolyzing β-lactamases, included IMP-, VIM-, KPC-, and OXA-48 type enzymes. The present data in conjugation with other studies suggested that, imipenem was active agent in both ESBL-producing and non-producing isolates and its activity was not affected by ESBL production.

The resistance to carbapenems in members of the Enterobacteriaceae can be caused by a variety of mechanisms, included serine-based- or metallo-β-lactamases alone or in combination with porin protein reduction (Livermore and Woodford, 2006). In a study reported herein, imipenem resistance was determined in *K. oxytoca* EK isolate by disk diffusion test,

suggesting that the isolate either serine β-lactamase (KPC or OXA type carbapenemase) or MBL producer. As this isolate was negative by PCR analysis using *bla*$_{OXA}$- specific primers, the resistance to carbapenem could be attributed to KPC or MBL, therefore the isolate was selected for confirmatory tests.

In this study, although *K. oxytoca* EK isolate indicated resistant to imipenem in screening test, the presence of EDTA did not inhibit the activity of β-lactamase (the isolate was negative by the EDTA disk synergy test), suggesting the absence of a class B enzyme. However, Lee *et al*. (2004) found that of 623 nosocomial bacteremic Gram-negative isolates, only 8(1.3%) were found to carry MBLs. In a study from Spain, VIM-1MBL, was detected in two strains of *K. pneumoniae* and *E. coli* (Lavilla *et al*., 2005). In Portugal, Conceica *et al*. (2005) reported that a chromosomal VIM-2 MBL in a clinical isolate of *P. aeroginosae* was detected in *K. oxytoca* isolate, demonstrating that the problem of MBL-producing pathogens no longer involves Gram-negative non fermenters alone but also involves *Enterobacteriaceae*. In a recent study from Italy, out of 209 *Enterobacteriaceae* isolates collected around wards of the Bolzano Regional Hospital, 24 isolates were detected as MBL producers. They compromised 7 *E. coli*, 12 *K. pneumoniae*, 3 *K. oxytoca* and 2 *C. freundii* (Aschbacher *et al*., 2008).

In 1996, a novel carbapenemase was reported from North Carolina, USA, in *K. pneumoniae*, designated KPC-1. The enzyme hydrolyzed all β-lactam antibiotics, was inhibited by clavulanic acid, and was located on a non conjugative plasmid (Yigit *et al*., 2001). However, KPC production is detected by the Modified Hodge Test when the test isolate produces the enzyme and allows growth of a carbapenem susceptible strain (*E. coli*

ATCC 25922) towards a carbapenem disk (Lee *et al*., 2001). In the present study, an indentation of the standard strain along the test isolate growth within the imipenem disk diffusion zone, this finding confirmed the production of KPC. The identification of KPC enzymes is worrisome, since their spectrum of activity could jeopardize therapy for serious infections caused by major nosocomial pathogens.

Carbapenems resistance among *Klebsiellae* isolates had been distinctly unusual. Recently, carbapenems resistance mediated by the KPC β-lactamases has been documented in isolates of *Klebsiella* spp., KPC found in New England and the mid- Atlantic region of the United States (Yigit *et al*., 2003; Bradford *et al*., 2004). Two studies from China and Greece reported on the detection of a KPC-2 producing *K. pneumoniae* isolate from a university hospitals (Wei, *et al*.; 2007; Cuzon *et al*.; 2008). The KPC epidemic now seems to be accelerating (Nordmann *et al*., 2009).

One of the major observations in this study that *K. oxytoca* EK isolate was ESBL producer as previously detected, (Table 4-18) reveals that the isolate was resistant to vast majority of antibiotics tested, included: penicillins, imipenem, third and fourth generations cephalosporins, aztreonam, cefoxitin, aminoglycosides and ciprofloxacin, while revealed sensitivity to amoxi-clav, amikacin and nalidixic acid, this finding may be explained as the organisms with KPC resistance are susceptible only to a few antibiotic agents. The only study available on the incidence of KPC in *K. oxytoca* was that reported by Yigit *et al*. (2003), who identified a *K. oxytoca* strain from urine of hospitalized patient in New York, manifesting imipenem, extended-spectrum cephalosporins and aztreonam resistance, the data they presented indicated that KPC-2 is responsible for the carbapenems resistance of this strain. In studies from New York, (Bratu *et al*., 2005a; and

Bratu *et al*., 2005b) reported that a problem began to appear with KPC expressing *K. pneumoniae* as carbapenems were considered to be one of the few treatment options during large outbreaks of ESBL-producing *Klebsiellae*. It is likely that the circulation of carbapenemases genes proceeds in two directions: environmental sources may provide genetic materials as a source of the enzymes, and clinical strains may disperse these informations both within the hospital settings and into the surrounding environment (Queen and Bush, 2007).

In conclusion imipenem should be kept on reserve, and its use should be controlled. Controlled use together with an effective control program to prevent horizontal transfer of imipenem resistant bacteria will provide a relatively resistance-free future.

Table (4-18): Antibiotic susceptibility profiles of *K. oxytoca* EK isolate obtained from Merjan Teaching Hospital

Type of antibiotic	Susceptibility
PY, PRL, FOX, CAZ, CTX, CRO, FEP, IMP, ATM, CN, TOB, CIP	R
AMC, AK, NA	S

R: resistant; S: sensitive; AMC, Amoxi-clav; FOX, Cefoxitin; PY, Carbenicillin; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime; PRL, Piperacillin; NA, Nalidixic acid; FEP, Cefepime; CN, Gantamycin; TOB, Tobramycin; ATM, Aztreonam; AK, Amikacin; IMP, Imipenem; CIP, Ciprofloxacin

Conclusions and Recommendations

Conclusions:

From the results obtained in this work, the following observations are deduced:

1- Merjan Hospital environment, as a representative of Iraqi hospital, is contaminated with coliform bacteria, which may be either of human or animal sources.

2- All of the tested *E. coli* and *Klebsiellae* isolates were multidrug resistants, therefore, such organisms represent a serious therapeutic challenge.

3- The prevalence of ESBL-producing isolates is higher than that has been found in The Middle Euphrates Region.

4- The frequency of AmpC enzymes encoded by both chromosomal and plasmid genes are also evolving and this resistance mechanism can cause nosocomial outbreaks.

5- Although for the time being, imipenem is the most effective antimicrobial agent against nosocomial infections due to *E. coli* and *Klebsiella* spp., but the identification of KPC-enzyme in *K. oxytoca* isolate is worrisome, since carbapenemases-producing isolates are difficult to treat.

Recommendations:

1- This study emphasizes the need for continued surveillance of β-lactamases- producing species of Gram-negative and Gram-positive bacteria in other hospitals in Iraq, which will be helpful in monitoring antimicrobial resistance and to guide intervention to minimize its occurrence.

2- Extended-spectrum β-lactamases detection should be carried out in all routinely tests in all Iraqi hospital laboratories.

3- The judicious use of all antibiotics to avoid misuse and overuse of these drugs to prevent the ever increasing problem of the emergence of

multidrug β-lactamases- producing *E. coli* and *Klebsiella* spp. and further to control outbreaks.

4- An imipenem resistance surveillance program with registration of its consumption in Iraq is necessary to promote an optimal use of imipenem and to encourage its rational prescribing.

5- It is recommended to use the recent techniques like PCR/*NheI*, pulsed-field gel electrophoresis, and randomly amplified DNA polymorphism analysis for identification and studying the epidemiology of β-lactamases-producing isolates.

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Appendix (1): Susceptibility profiles of β-lactam resistant *E. coli* and *Klebsiella* spp. isolates

Bacterial isolate	Resistance (+) to tested antibiotic													
	PY	PRL	CAZ	CTX	CRO	ATM	FEP	FOX	IMP	AK	CN	TOB	NA	CIP
***E. coli* E1**	+	+	+	+	-	+	+	+	-	-	+	+	-	+
***E. coli* 2**	+	+	+	+	+	+	+	+	-	-	-	+	+	-
***E. coli* 3**	+	+	+	+	+	+	+	-	-	-	+	+	+	+
***E. coli* 4**	+	-	-	+	+	-	+	+	-	-	-	-	+	+
***E. coli* 5**	+	+	-	-	-	+	-	-	-	-	-	+	-	+
***E. coli* 6**	+	+	+	+	+	+	+	-	-	-	+	+	+	+
***E. coli* E2**	+	+	+	+	+	+	+	-	-	-	+	+	+	+
***E. coli* 8**	+	-	-	-	+	-	+	+	-	-	-	-	-	-
***E. coli* 9**	+	+	+	+	-	+	+	-	-	-	+	+	+	+
***E. coli* E3**	+	+	+	+	+	+	+	+	-	-	+	+	+	-
***E. coli* E4**	+	+	+	+	+	+	+	-	-	-	-	-	+	-
***E. coli* E9**	+	+	-	+	+	-	+	-	-	-	-	-	+	-

Appendix (1): (continued)

Bacterial isolate	Resistance (+) to tested antibiotic													
	PY	PRL	CAZ	CTX	CRO	ATM	FEP	FOX	IMP	AK	CN	TOB	NA	CIP
***E. coli* E5**	+	-	+	+	+	+	+	-	-	-	-	-	-	-
***E. coli* 14**	+	+	-	-	+	+	-	-	-	-	+	-	-	+
***E. coli* 15**	+	+	-	+	+	+	+	+	-	-	+	+	+	+
***E. coli* E6**	+	-	-	+	+	+	-	+	-	-	-	-	+	-
***E. coli* 17**	-	+	-	-	-	+	-	+	-	-	-	-	-	+
***E. coli* EC8**	+	+	+	+	+	-	+	+	-	-	+	+	-	-
***E. coli* E10**	+	+	+	-	+	+	+	+	-	-	+	-	+	-
***E. coli* 20**	+	+	+	-	-	-	-	-	-	-	-	-	-	+
***E. coli* 21**	+	+	+	+	+	+	+	+	-	-	-	-	+	+
***E. coli* 22**	+	+	+	+	+	+	+	+	-	-	+	+	-	+
***E. coli* EC7**	+	+	+	+	+	+	+	+	-	-	+	+	+	-
***E. coli* 24**	+	+	-	-	-	-	-	+	-	-	+	+	+	+

Appendix (1): (continued)

Bacterial isolate	Resistance (+) to tested antibiotic													
	PY	PRL	CAZ	CTX	CRO	ATM	FEP	FOX	IMP	AK	CN	TOB	NA	CIP
***E. coli* 25**	+	+	+	+	+	+	+	+	-	-	+	+	-	-
***E. coli* 26**	+	-	+	-	-	+	-	-	-	-	-	-	+	+
***E. coli* 27**	+	+	+	+	+	+	+	-	-	-	-	-	-	-
***E. coli* 28**	-	-	+	-	-	+	-	+	-	-	-	-	+	-
***E. coli* 29**	+	+	-	+	+	+	+	+	-	-	-	-	+	+
***E. coli* E11**	+	+	+	+	+	+	-	+	-	-	-	+	-	-
***E. coli* EC12**	+	+	+	+	+	+	+	+	-	-	-	-	+	-
***E. coli* 32**	+	+	+	+	+	+	+	+	-	-	+	+	+	+
***E. coli* E13**	+	+	-	+	+	+	+	+	-	-	+	+	-	-
***E. coli* 34**	+	+	+	+	-	+	+	-	-	-	+	+	-	+
***E. coli* E14**	+	+	+	+	+	+	-	+	-	-	+	+	+	-
***E. coli* E15**	+	+	+	+	+	-	+	-	-	-	+	+	-	-

Appendix (1): (continued)

Bacterial isolate	Resistance (+) to tested antibiotic													
	PY	PRL	CAZ	CTX	CRO	ATM	FEP	FOX	IMP	AK	CN	TOB	NA	CIP
***E. coli* E16**	+	+	+	+	+	+	+	-	-	-	-	-	-	+
***E. coli* E17**	+	+	+	+	+	+	-	+	-	-	-	+	-	-
***E. coli* E18**	+	+	+	+	+	-	+	-	-	+	-	-	+	-
***E. coli* 40**	+	+	-	+	-	-	-	-	-	+	-	+	+	-
***K. pneumoniae* E1**	+	+	-	+	-	-	-	-	-	-	-	-	+	+
***K. pneumoniae* 2**	+	-	-	+	-	-	-	-	-	-	-	-	+	+
***K. pneumoniae* E2**	+	+	+	+	+	+	+	-	-	-	-	-	-	-
***K. pneumoniae* E3**	+	+	+	+	+	+	+	-	-	-	+	-	-	-
***K. pneumoniae* E4**	+	+	+	-	+	+	+	-	-	-	+	+	-	-
***K. pneumoniae* 6**	+	+	+	+	+	+	+	+	-	-	+	+	+	-

***K. pneumoniae* 7**	+	+	+	+	+	+	+	+	-	-	+	+	+	+
***K. pneumoniae* E5**	+	+	-	+	+	+	+	-	-	-	-	+	-	-

Appendix (1): (continued)

Bacterial isolate	**Resistance (+) to tested antibiotic**													
	PY	PRL	CAZ	CTX	CRO	ATM	FEP	FOX	IMP	AK	CN	TOB	NA	CIP
***K. pneumoniae* E6**	+	+	+	+	+	-	+	-	-	-	+	+	-	-
***K. pneumoniae* 10**	+	+	+	+	+	+	+	-	-	-	-	-	-	-
***K. pneumoniae* 11**	+	+	+	+	+	+	+	-	-	+	-	-	+	-
***K. pneumoniae* 12**	+	+	-	-	+	-	+	-	-	-	-	-	+	+
***K. pneumoniae* 13**	+	+	-	+	+	-	-	+	-	-	-	+	+	-

***K. pneumoniae* 14**	+	-	-	-	+	+	-	+	-	-	-	+	+	-
***K. pneumoniae* E10**	+	+	+	-	+	+	+	-	-	+	+	+	-	-
***K. pneumoniae* 16**	+	+	-	+	+	+	+	+	-	-	+	+	+	+
***K. pneumoniae* 17**	+	+	+	+	+	+	-	+	-	+	+	+	+	+
***K. pneumoniae* E11**	+	+	+	+	+	+	+	-	-	-	-	-	-	-
***K. pneumoniae* 19**	+	+	+	+	+	+	+	+	-	+	+	+	+	+
***K. pneumoniae* E12**	+	+	+	+	+	+	+	-	-	-	+	+	-	-

Appendix (1): (continued)

Bacterial isolate	Resistance (+) to tested antibiotic													
	PY	PRL	CAZ	CTX	CRO	ATM	FEP	FOX	IMP	AK	CN	TOB	NA	CIP
***K. pneumoniae* E13**	+	+	+	+	-	+	+	-	-	-	+	-	-	-
***K. pneumoniae* 22**	+	+	+	+	+	+	+	-	-	-	+	-	+	-
***K. pneumoniae* E15**	+	+	+	+	-	+	-	-	-	-	+	-	-	-
***K. pneumoniae* E18**	+	+	+	+	+	+	+	-	-	-	+	+	-	+
***K. pneumoniae* E9**	+	+	+	+	+	+	+	+	-	-	+	+	-	-
***K. oxytoca* E1**	+	+	+	-	+	+	+	-	-	-	+	+	-	+
***K. oxytoca* 2**	+	+	+	-	-	-	+	-	-	-	-	-	-	+
***K. oxytoca* 3**	+	-	-	-	-	-	+	-	-	-	-	-	+	-
***K. oxytoca* 4**	+	-	+	-	+	-	-	-	-	-	-	-	+	-

***K. oxytoca* 5**	+	-	-	-	+	-	-	-	-	-	-	-	-	-
***K. oxytoca* E7**	+	+	-	+	+	-	-	-	+	-	+	+	-	-
***K. oxytoca* 7**	+	+	+	+	-	+	-	-	-	-	-	+	+	+

Appendix (1): (continued)

Bacterial isolate	Resistance (+) to tested antibiotic													
	PY	PRL	CAZ	CTX	CRO	ATM	FEP	FOX	IMP	AK	CN	TOB	NA	CIP
***K. oxytoca* 8**	+	+	-	+	+	-	-	-	-	-	-	-	+	-
***K. oxytoca* 9**	+	-	-	-	-	-	-	-	-	-	+	-	+	-
***K. oxytoca* 10**	+	+	-	+	-	-	-	-	-	-	-	-	+	-
***K. oxytoca* 11**	+	+	+	+	+	-	+	-	-	+	+	+	+	-
***K. oxytoca* 12**	+	-	+	+	+	+	-	+	-	-	-	-	-	-
***K. oxytoca* E14**	+	+	+	+	-	+	+	-	-	-	+	+	-	-
***K. oxytoca* 14**	+	+	-	+	-	-	-	-	-	-	-	-	+	-
***K. oxytoca* E16**	+	-	+	+	+	+	+	+	-	-	-	-	+	-
***K. oxytoca* E17**	+	+	-	+	+	+	+	-	-	-	+	+	-	+
***K. oxytoca* E8**	+	+	+	+	-	+	+	+	-	-	+	+	-	-