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Occurrence and Characterization of AmpC β-Lactamases in *Klebsiella pneumoniae* Isolated from Some Medical Centers in Najaf

A Thesis

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Summary

The purpose of this study was to detect and characterize of AmpC β-lactamases in *Klebsiella pneumoniae*.

Consecutive 1015, clinical (n=770) and hospital environment (245) samples were collected during the period from April to July 2010. *K. pneumoniae* isolates were identified by traditional biochemical tests, then confirmed by API 20E system. Antibiotic susceptibility and minimum inhibition concentration tests were performed by disk diffusion and agar dilution methods, respectively. Production of β-lactamase was evaluated by iodometric and nitrocefin methods. Extended spectrum β-lactamase (ESBL), AmpC β-lactamase and carbapenemase were initially detected and confirmed by the phenotypic methods. Plasmid mediated *ampC*-gene families encoded FOX, CIT, DHA, EBC, ACC and MOX were detected by conventional and multiplex PCR. The presence of *bla*-genes encoded AmpC (for AmpC β-lactamase), CTX-M, SHV, TEM, OXA, VEB, PER and GES (for ESBLs) and KPC, IMP and VIM (for carbapenemase) were detected by conventional PCR. AmpC β-lactamase was semi-purified from cell sonicate by ammonium sulfate precipitation and gel filtration chromatography. Optimum temperature, optimum pH and kinetic parameters of AmpC β-lactamases were described.

A total of 217 *Klebsiella* spp. isolates were identified. Of these, 130 were belonged to *K. pneumoniae*. All *K. pneumoniae* were identified to the level of subspecies. Results revealed that 111 (85.4%) and 19 (14.6%) isolates were belonged to *K. pneumoniae* subsp. *pneumoniae* and *K. pneumoniae* subsp. *ozaenae,* respectively. When a 130 *K. pneumoniae* isolates were primarily screened for β-lactams resistance, 103 (79.2%) were found to be β-lactams resistant. All β-lactams resistant isolates were resistant to a minimum of three classes of antibiotics; hence, these isolates were considered as multidrug resistant (MDR). Out of β-lactams

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resistant isolates, 78 (75.7%) and 63 (61.2%) were manifested as β-lactamase producers with nitrocefin and rapid iodometric methods, respectively.

Of the 78 β-lactamase producing isolates, 76 (97.4%) were initially screened as positive for ESBL. CHROMagar technique was the most ambient method than the disk combination and disk approximation tests regarding confirmation of ESBL production.

All the 103 β-lactams resistant isolates were tested for cefoxitin susceptibility and ability to produce AmpC β-lactamase by three methods; modified three dimension, disk based inhibitor and AmpC disk methods. Results revealed that 73 (70.9%) isolates were cefoxitin resistant and 31 (30.1%), 31 (30.1%) and 30 (29.1%) isolates were confirmed as AmpC producers by three above methods, respectively. Whereas, two (1.9%) isolates were identified as inducible AmpC β-lactamase producers by the ceftazidime-imipenem antagonism test. Only 20 (19.4%) isolates gave positive results with bla_{AmpC} gene. When these isolates were tested for antibiotic susceptibility against 26 antibiotics and for MIC against selected β-lactam antibiotics, results revealed that 11 (55.0%) and 9 (45.0%) of the isolates were MDR and extensive drug resistant (XDR), respectively. The MIC_{50}/MIC_{90} values for isolates were in zone of resistance (>32/256 μg/ml) to ampicillin, piperacillin, cephalothin, cefotaxime and ceftazidime, while meropenem were the most effective antibiotics against isolates.

All bla_{AmpC} positive isolates showed diversity presence of plasmid-mediated AmpC β-lactamases. Genes that encoded FOX, CIT, DHA, EBC and ACC were found in percentage of 55.0, 40.0, 20.0, 20.0 and 5.0, respectively. Multiplex PCR assay revealed that three (bla_{FOX}, bla_{CIT}, bla_{EBC}) and two (bla_{FOX}, bla_{CIT}) *ampC* genes were detected in 2 (10%) and 4 (20%) isolates, respectively. While, the remaining 14 (70.0%)

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isolates were carried only one *ampC* gene. PCR assay revealed that, 14 (70.0%), 10 (50.0), 5 (25.0%), 2 (10.0%) and 1 (5.0%) of the bla_{AmpC} positive isolates harboured bla_{CTX-M}, bla_{SHV}, bla_{TEM}, bla_{OXA} and bla_{VEB} genes, respectively. While, bla_{PER} and bla_{GES} genes were not detected in this investigation.

Four (20.0%) of the bla_{AmpC} positive isolates were resistant to both imipenem and meropenem by disk diffusion method. All the carbapenems resistant isolates were tested for their ability to produce carbapenemase by the three tests (imipenem-EDTA disk, modified Hodge, and KPC CHROMagar). Two isolates gave positive results with the first test , while all other isolates gave positive results with second and third tests. None of the four isolates were positive for carbapenemase encoding genes (bla_{KPC}, bla_{IMP}, bla_{VIM}). After the AmpC β-lactamases of *K. pneumoniae* isolate K19 was partial-purified from cell sonicate, two peaks of homologous enzyme activities were separated in gel filtration chromatography step. The yield of the enzyme and its final purification fold were 0.6%, 0.1% and 43.3, 15 for first and second peaks, respectively, when compared with starting materials. The maximum AmpC β-lactamases activity was obtained at 37°C and pH 7.5. AmpC β-lactamases exhibited relatively high value of hydrolysis efficiency (191.32%) to cefotaxime with low K_m (3.36) and high V_{max} (0.045) values, compared with the meropenem.

Such results insure that AmpC β-lactamases producing *K. pneumoniae* were recognized in both phenotypic and molecular methods in local isolates recovered from health settings in Najaf.

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List of Abbreviations

Microorganisms

Abbreviation	*Key*
A. baumannii	*Acinetobacter baumannii*
C. freundii	*Citrobacter freundii*
E. cloacae	*Enterobacter cloacae*
E. coli	*Escherichia coli*
H. alvei	*Havenia alvei*
K. aerogenes	*Klebsiella aerogenes*
K. atlantae	*Klebsiella atlantae*
K. edwardsii	*Klebsiella edwardsii*
K. ornithinolytica	*Klebsiella ornithinolytica*
K. oxytoca	*Klebsiella oxytoca*
K. ozaenae	*Klebsiella ozaenae*
K. planticola	*Klebsiella planticola*
K. pneumoniae	*Klebsiella pneumoniae*
K. rhinoscleromatis	*Klebsiella rhinoscleromitis*
K. trrevisanii	*Klebsiella trrevisanii*
K. terrigena	*Klebsiella terrigena*
P. aeruginosa	*Pseudomonas aeruginosa*
P. mirabilis	*Proteus mirabilis*
S. enteriditis	*Salmonella enteriditis*

General Abbreviations

Abbreviation	*Key*
A	Absorbance
ACC	*H. alvei* origin AmpC β-lactamase
AmpC	Molecular class C β-lactamases
ampR	AmpC regulator gene
ANOVA	Analysis of variance
API	Analytical profile index
ATCC	American type culture collection
bla gene	β-lactamases gene

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BSA	Bovine serum albumin
C3b	Complement 3b
CAZ	Ceftazidime
CFU	Colony forming unit

List of Abbreviations

CHRMOMagar	Orientation chromogenic agar
CIT	*Citrobacter* origin AmpC β-lactamase
CMY	First discovered plasmid mediated AmpC β-lactamase
CLSI	Clinical and Laboratory Standards Institute
COG	Cluster of orthologous group
CTX	Cefotaxime
CTX-M	Cefotaximase, β-lactamase active on cefotaxime
D.W.	Distilled water
DHA	β-lactamase discovered at dhahran hospital in Saudi Arabia
DNA	Deoxyribonucleic acid
EBC	*Enterobacter* origin AmpC β-lactamase
EC	Enzymatic commession
EDTA	Ethylene diaminetetraacetic acid
ESBL	Extended-spectrum β-lactamase
FOX	AmpC enzyme act on cefoxitin
GES	Guiana extended spectrum β-lactamase
GIM	German imipenemase
Gly	Glycine
HCl	Hydrochloric acid
ICU	Intensive care unit
IMP	Imipenemase, β-lactamase active on imipenem
IS	Insertion sequence
K	Capsular antigen
K_m	Michaelis constant
KPC	*Klebsiella pneumoniae*-carbapenemase
LPS	Lipopolysaccharide
MBL	Metallo-β-lactamase
MDR	Multi –drug resistance
MHA	Mueller-Hinton agar
MHT	Modified Hodge test
MIC	Minimum inhibitory concentration
MTDT	Modified Three-dimensional test
NCCLS	National Committee for Clinical Laboratory Standards
OMP	Outer membrane protein
OXA	Oxacillinases, β-lactamase active on oxacillin
PABLs	Plasmid-mediated AmpC β-lactamases
PBP	Penicillin-binding protein
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PDR	Pan-drug resistance
PER	*Pseudomonas* extended resistant

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pH	Power of hydrogen (H^+)
PIPES	1,4- Piperazine-diethanesulphonic acid
prA	Promotor gene of MIR-1 enzyme
R	Reveres
SDS	Sodium dodecyl sulfate

SHV	Sulfhydryl variable β-lactamase
SIM	Seoul imipenemase
TBE	Tris borate-EDTA buffer
TE	Tris-EDTA buffer
TEM	β-lactamase named after first patient isolated from Temarian
tnpA	Transposase gene
Tris	Tris 2-Amino-2-Hydroxymethylpropane-1,3-Diol
Tris-OH	Tris-(Hydroxymethyl) methylamine
TSI	Triple sugar iron test
VEB	Vietnam extended-spectrum β-lactamase
VIM	Verona integron-encoded metallo-β-lactamases
V_{max}	Maximum velocity
XDR	Extensive-drug resistance

Units of Measurement

Abbreviation	*Key*
µg	Microgram
µM	Micro molar
bp	Base pair
C	Degree Celsius
g	Gram
kDa	Kilo Dalton
mg	Milligram
mm	Millimeter
mM	Mill molar
rpm	Revolutions per minute
U	Unit
µmol	Micromole
µL	Microlitre
mL	Milliliter

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Introduction:

Most notoriously, *K. pneumoniae* is a prominent nosocomial pathogen mainly responsible for bacteraemia, urinary tract, respiratory tract , and wound infections. Most *K. pneumoniae* are hospital associated with a high fatality rate if incorrectly treated. Isolates from hospitals often display antibiotic resistance phenotypes (Woodford *et al.,* 2007), while resistant isolates may also spread into the community settings (Colodner *et al.,* 2004).

The increment of irrational use of antibiotics promotes bacterial resistance to these drugs. Moreover, the resistance mechanisms in *K. pneumoniae* is developed by the production of β-lactamase, which can destroy the β-lactam antibiotics (Herewana *et al.,* 2008). Extended-spectrumβ-lactamases (ESBLs) are most prevalent in *K. pneumoniae*, subsequently have resulted in their resistance to third-generation cephalosporins. 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).

AmpC β-lactamases are one of the most important β-lactamases in Gram-negative bacteria. It was thought that the AmpC β-lactamases were mediated by chromosome, 20 years ago. However, plasmid-mediated AmpC β-lactamases have been found in recent 10 years (Li *et al.,* 2009). AmpC β-lactamases are termed class C. Thus, they provide resistance to oxyimino-7-α-methoxy-cephalosporins, monobactams and cephamycins, moreover, are not blocked by commercially available inhibitors with low affinity to carbapenems (Jacoby, 2009).

Risk factors for infections caused by AmpC-producing strains of *K. pneumoniae* include long hospital stay, and prior administration of

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antibiotics, especially broad-spectrum cephalosporins and β-lactamase inhibitor combinations, are thus similar to risk factors for infection by ESBL-producing *K. pneumoniae* strains (Pai *et al.,* 2004). Whereas, now people have realized that the AmpC β- lactamase-producing *K. pneumoniae* did not only cause fatal infection, but also led to a global dissemination of strains carrying plasmid-encoded *ampC* genes. At present, six main families of plasmid mediated AmpC β-lactamases including more than 45 varieties have been described by different countries and areas. These enzymes lead to epidemic status in geographic regions, including America, Europe, Africa, and Asia (Li *et al.,* 2009). Often, the strain with a plasmid-mediated AmpC enzyme also produced other ESBL such as TEM-1 or SHV-5. The coexistence of different classes of β-lactamases in a single bacterial isolate may pose diagnostic and therapeutic challenges, the presence of which may complicate detection of the AmpC phenotype (Jacoby, 2009). For these purposes, molecular biological techniques such as detection of enzyme hydrolysis parameter, isoelectric point, gene detection, could be used for the identification of plasmid-mediated AmpC β-lactamases. Recently, molecular analysis like multiplex PCR has been developed as the "gold standard" for family-specific plasmid-mediated AmpC β-lactamase detection (Perez-Perez and Hanson, 2002; Naas *et al*., 2004).

Nevertheless, knowledge of the AmpC β-lactamases is still limited at presence. In Iraq, little attention has been paid to the β-lactamases producing isolates. However, in the Najaf city, no information are regarding the molecular studies of the occurrence of plasmid mediated AmpC β-lactamases-producing *K. pneumoniae* recovered from clinical cases and hospital environments. Therefore, there is an increase demand to investigate the role of these isolates in hospital infections. Hence, the proposed aim of

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this study is to detect and characterize of AmpC β-lactamases in *K. pneumoniae* isolates recovered from both hospital and community settings in the Najaf city. For this aim the following steps were performed:-

1- Isolation and identification of AmpC β-lactamases-producing *K. pneumoniae* isolates from clinical and hospital environmental samples.
2- Comparing the performance of AmpC β-lactamases detection by phenotypic methods and molecular analysis.
3- Attempting to detect the genetic factor controlling ESBL and carbapenemases in AmpC producing isolates.
4- Describing the enzymatic parameters of partial purified AmpC β-lactamases.

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2. Review of Literatures

2.1.Genus *Klebsiella*

Genus *Klebsiella* is among the oldest known genera in the family *Enterobacteriaceae*. The German bacteriologist Edwin Klebs (1834–1913) was the first who named this bacterium. This genus was described for the first time by Trevisan in 1885 (Bruckner *et al.,* 1999).

Klebsiella are Gram-negative , non-spore forming, non-motile, rod-shaped, capsulated organisms with prominent polysaccharide-based capsule. *Klebsiella* are ubiquitous and may colonize the skin, pharynx, or gastrointestinal tract in humans (Ryan and Ray, 2004), and cause several diseases such as pneumonia, urinary tract infection and septicaemia (Feglo *et al.,* 2010).

This microorganism is much more frequently associated with infections being responsible for 3% of community-acquired and 9% of hospital-acquired cases of septicaemia in one hospital . *Klebsiella* is well known to most clinicians as a cause of community-acquired bacterial pneumonia (Tsai *et al.,* 2009).

As opportunistic pathogen, *Klebsiella* spp. primarily cause diseases in immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus and chronic pulmonary obstruction. It is infrequently found in the oropharynx of normal persons (1% to 6% carrier rate), a prevalence as high as 20% occur in hospitalized patients. Urinary tract is the most common site of infection and shows an even higher incidence in specific groups of patients at risk (Koneman *et al.,* 1994; Tsai *et al.,* 2009) .

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2.1.1.Taxonomy of the Genus *Klebsiella*

The taxonomy of genus *Klebsiella*, recognised seven named species; *K. aerogenes, K. oxytoca, K. edwardsii, K. atlantae, K. pneumoniae , K. ozaenae and K. rhinoscleromatis* (Barr, 1977). *K. ornitholytica, K. planticola* and *K. terrigena* have been reclassified as species in the new genus *Raoultella* (Drancourt *et al.,* 2001).

Originally, the medical importance of the genus *Klebsiella* was subdivided into three species corresponding to the diseases: *K. pneumoniae*, *K. ozaenae*, and *K. rhinoscleromatis*. As the taxonomy became increasingly refined due to the development of new methods such as numerical taxonomy, the species classification in this genus was continually revised. In time, three main classifications emerged, those of Cowan *et al.* (1960), Bascomb *et al.* (1971) and Ørskov, (1984). The adoption of a consistent nomenclature has been further complicated by the fact that Great Britain and the former Commonwealth countries adhere to the classification of Cowan, while the USA prefer Ørskov's classification. Recently, phylogenetic analyses of *Klebsiella* species confirmed that the genus *Klebsiella* is heterogeneous and composed of species, first: contained the type strains of *K. pneumoniae* subsp. *pneumoniae, K. pneumoniae s*ubsp. *rhinoscleromatis,* and *K. pneumoniae* subsp. *Ozaenae,* second contained *K. oxytoca*. (Drancourt *etal.,* 2001).

2.2. *Klebsiella pneumoniae*

2.2.1. General Characterization

Historically, *K. pneumoniae* named Friedlander's bacilli, exhibits mucoid growth, thick polysaccharide capsule and non-motility. They are straight Gram-negative rods, 0.3-1 µm in diameter and 0.6-6 µm in length arranged singly, in pairs or in short chains, their DNA molecule contain 53-

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58% of G+C % (Hart, 2006). *K. pneumoniae* is facultative bacteria having both a respiratory and a fermentative type of metabolism. Optimal temperature is 37°C large colonies with a mucoid consistency are recovered on primary isolation plates (Brooks *et al.,* 2001). On MacConkey agar, the colonies typically appear large, mucoid, with red pigment usually diffusing into the surrounding agar, indicating fermentation of lactose and acid reduction (Koneman *et al.,* 1994).

Klebsiella pneumoniae produces lysine decarboxylase but not ornithine decarboxylase and arginine dihydrolase. In addition, it hydrolyzes urea slowly, producing a light pink color on the slant of Christensen's urea agar. Production of indole from tryptophan is negative. It reduces nitrates and gives oxidase negative. It usually gives positive test for citrate and Voges-Proskauer reactions (Brooks *et al.,* 2001).

2.2.2. Pathogenicity

Klebsiella pneumoniae is an opportunistic pathogen found in the environment and on mammalian mucosal surfaces (Feglo *et al.,* 2010). Most of the information on transmission of *K. pneumoniae* comes from studies of outbreaks nosocomial infections, especially caused by *Klebsiella* spp. *K. pneumoniae* is medically most important species of this genus, and most frequently recovered from clinical specimens (Podschun and Ullmann, 1998). Reservoirs of *K. pneumoniae* tend to be the intestinal tract of hospitalized patients, although other sites such as the oropharynx, skin and vagina may be carriage sites (Ryan and Ray, 2004). The pathogenesis and pathogenic determinants of nosocomial and opportunistic *K. pneumoniae* infections included:

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a) Capsular Antigens

Capsules are essential to the virulence of *K. pneumoniae*. The capsular material forms thick bundles of fibrillous structures covering the bacterial surface in massive layers. This protects bacterium from phagocytosis by polymorphonuclear granulocytes and prevents killing of the bacteria by bactericidal serum factors.

There are more than 80 different capsular (K) serotypes and subtypes with similar antigenicity but different polysaccharide backbones have been described (Sikarwar and Batra, 2011). *K. pneumoniae* subsp. *pneumoniae* is predominantly serotype 3, as is *K. pneumoniae* subsp. *rhinoscleromatis. K. pneumoniae* subsp. *ozaenae* encompasses serogroups 3, 4, 5 and 6, but each of the serogroups has been associated with *K. pneumoniae* subsp. *aerogenes* (Hart, 2006).

b) Adhesins

The adhesive properties are generally mediated by different types of pili. Pili are filamentous projections on the bacterial surface demonstrated mainly on the basis of their ability to agglutinate erythrocytes of different animal species (Huang *et al.,* 2009). Of the different types of pili described in enterobacteria, these are two predominant types in *K. pneumoniae*, Type 1 binding of the bacteria to mucus or to epithelial cells of the urogenital, respiratory and intestinal tracts (Podschun and Ullmann, 1998), and type 3 pili, in the kidneys, these pili mediate bacterial adhesion to tubular basement membranes, Bowman's capsules, and renal vessels (Huang *et al.,* 2009).

c) Lipopolysaccharide (LPS)

The repertoire of O-antigens on *K. pneumoniae* LPS is limited to eight (O types 1, 3–5, 7–9 and 12), O:1 being the most common (Hart, 2006). Capsular polysaccharide may cover and mask the underlying LPS and

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exhibits a surface structure that does not activate complement. On the other hand, the O side chains of the LPS may reach through the capsule layer and be exposed to the environment in certain *Klebsiella* capsular types. Since LPS is generally able to activate complement, C3b is subsequently deposited onto the LPS molecules (Podschun and Ullmann, 1998).

d) Toxins

Klebsiella pneumoniae strains carrying genes for expressing heat-labile and heat-stable enterotoxins. Their clinical importance is unclear. A channel-forming bacteriocin (microcin E492) of *K. pneumoniae* is able to induce apoptosis in human cells (Hetz *et al*., 2002).

e) Iron Scavenging

Klebsiella pneumoniae is able to scavenge iron from its surrounding medium using either enterochelin (phenolte siderophore), which is detected more frequently in pathogenic isolates, or aerobactin (hydroxamate siderophore) (Brooks *et al.,* 2001).

2.2.3. Clinical Features

Nosocomial *Klebsiella* infections can occur at almost any site, and the clinical features do not differ from those caused by any of the *Enterobacteriaceae.* Although *K. pneumoniae* subsp. *pneumoniae* is historically associated with pneumonia (Feglo *et al.,* 2010). It can produce extensive hemorrhagic necrotizing consolidation of the lung (Brooks *et al.,* 2001).

In addition a distinctive syndrome of *K. pneumoniae* bacteraemia in association with community-acquired liver abscesses, meningitis or endophthalmitis was found. The first being found almost exclusively in

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Taiwan (Ko *et al*., 2002). Risk factors for community-acquired *K. pneumoniae* bacteraemia include alcoholic liver disease and Diabetes mellitus (Tsay *et al*., 2002).

Rhinoscleroma begins as a painless chronic inflammatory swelling which causes nasal or respiratory tract obstruction. The nasal lesions enlarge locally to produce the so-called Hebra nose, which is grossly distorted and splayed. Local spread and local metastatic foci, often with lymph node involvement, are frequently described. Ozaena is seldom seen nowadays and there are doubts over its status as a disease entity (Hart, 2006).

2.2.4. Epidemiology

Klebsiella species are important nosocomial pathogens worldwide. In a UK prevalence survey, *Klebsiella* spp. were responsible for 17% of bloodstream infections (RHAIHE, 2009). In addition, *K. pneumoniae* occasionally produces bacteremia with focal lesions in debilitated patients (Brooks *et al.,* 2001). In neonatal intensive care units (ICU) *Klebsiella* spp. appear to be the most common organisms causing neonatal sepsis both in developed and developing countries (Shitaye, 2008). *K. pneumoniae* causes significant infections. It is present in the respiratory and intestinal tract of about 5% of normal individuals. It causes a small proportion (about 1%) of bacterial pneumonia (Brooks *et al.,* 2001).

Surveillance studies in Europe during 2006 revealed that 8.5% of ICU acquired pneumonia and 5% bloodstream infections were caused by *K. pneumoniae* respectively (IPSE, 2006). Health Protection Scotland (2007), stated that *Klebsiella* spp. was the most frequent bacteria as urinary tract infection causative agent. However , in a local study, *Klebsiella* was the predominant in all pediatric blood cultures studied (Al-Charrakh *et al.,*

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2000). It was predominant isolates in clinical and local hospital environment samples (Hadi, 2008; Al-Hilli, 2010)

However, feces are probably the most significant source of patient infections. Approximately one-third of patients carry it in their stools, but rates may increase as much as threefold during hospitalization and antimicrobial usage in adults (Podschun and Ullmann, 1998). Moreover, fecal carriage rates for children may be as high as 90 to 100%, even in the absence of antimicrobial therapy (Abbott, 2003).

2.2.5. Antimicrobial Susceptibility

Most *Klebsiella* are intrinsically resistant to ampicillin, but acquisition of plasmid-encoded resistance has resulted in outbreaks of nosocomial infection. In the 1970s and 1980s outbreaks of infection due to gentamicin and tobramycin (but not amikacin) resistant *K. pneumoniae* were reported in increasing numbers (Barros *et al.,* 1999).

With the introduction of second- and third-generation cephalosporins, isolates of *K. pneumoniae* resistant to cefuroxime, ceftazidime and cefotaxime soon emerged. Initially this was chromosomally encoded, but subsequently plasmid-encoded ESBL caused large outbreaks of infection especially in adult intensive care units (Jacoby and Medeiros, 1991).

Resistance to fluoroquinolones can be high; ciprofloxacin-resistant *K. pneumoniae* has increased worldwide in recent years. In a study in Pakistan hospitals, the highest resistance was shown by *K. pneumoniae* to be 72.22%. This increased resistance showed that *K. pneumoniae* had adapted to survive in presence of ciprofloxacin (Ali *et al.,* 2010).

The increasing antimicrobial resistance among ESBL producing *K. pneumoniae* strains. Besides, they have acquired plasmid-mediated AmpC

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enzymes, unlike ESBLs, AmpCs are poorly inhibited by β-lactamase inhibitors and are less active against cefepime and cefpirome than ESBLs (Thomson, 2001), that makes therapy very difficult and leads to use of expensive broad spectrum drugs, such as carbanpenems, which are known to be the most effective antibiotics against these organisms (Livermore, 1998). However, the most common mechanism of carbapenem resistance among *Enterobacteriaceae* in the United States is the production of the *K. pneumoniae* carbapenemase (KPC) (Babouee *et al.,* 2011) .

2.3. β-lactam Antibiotics

These antibiotics have been isolated from numerous sources but principally from bacteria and fungi, the history of β-lactam antibiotics began when Alexander Fleming description of antibacterial activity of a substance produced by penicillium mold and gave it the name penicillin in 1929. However, the phenomenon of antibiotic was already well known at this time (Albert and Sussman, 1998).

β-lactam antibiotics are one of the most important groups of antimicrobial agents administered worldwide, covering as much as 50% of all prescribed drugs. This is because they are highly effective and their efficacy, broad spectra, and low toxicity (Samaha-Kfoury and Araj, 2003).

The β-lactams belongs to the group of antimicrobial agents inhibiting the cell wall synthesis (Mims *et al.,* 2008). They have a bactericidal effect on the microorganisms and are classified into several groups; penicillins, cephalosporins, cephamycins, carbapenems, monobactams, and the β-lactam/β-lactamase inhibitors. The different groups consist of both natural and synthetic compounds varying in their chemical structure with the β-

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lactam ring as the common property. Each group have different ring structures and acyl side chains attached to the β-lactam ring (Essack, 2001).

2.3.1. General Structure and Function

β-lactam is a generic name for all β-lactam antibiotics that contain a β-lactam ring, a heteroatomic ring structure, consisting of three carbon atoms and one nitrogen atom. The principal classification of β-lactams is based upon the structure (Wilke *et al.*, 2005). Penicillins are a group of either natural or semi-synthetic β-lactam antibiotics that are characterized by the presence of a β-lactam ring and a thiazolidine ring; this structure is defined as 6-amino-penicillanic acid and its presence is essential for the activity of these antimicrobial agents. Hydrolysis of the C=O union by the activity of β-lactamases gives rise to a bacteriologically inactive molecule. The differences of activity among the members of this group are due to the lateral chain found in the position 6 of the 6-amino-penicillanic acid (Samaha-Kfoury and Araj, 2003).

Cephalosporins are structurally similar to the penicillins; however, the members of this group present a dihydrothiazine ring instead of the thiazolidine ring which is present in the penicillins. As for the penicillins, the introduction of additional side chains will give rise to different cephalosporins with a different level of antimicrobial activity (Livermore and Williams, 1996; Yao and Moellering, 2003).

Carbapenems are also structurally very similar to the penicillins, but the sulphur atom in the position 1 of the structure has been replaced with a metal group. The additional side-chains and its special disposition confer an important affinity towards the penicillin binding proteins (PBPs) and make this group of antibiotics resistant to a great part of β-lactamases (Queenan and Bush, 2007; Jacoby and Bush, 2009).

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Monobactams are monocyclic compounds derived from the 3-amino monobactamic acid. Their major characteristic is the presence of the β-lactam ring alone and not fused to another ring (Livermore and Williams, 1996; Samaha-Kfoury and Araj, 2003).

Currently, the clinical use of β-lactamases inhibitors is exclusively based on clavulanic acid, tazobactam and sulbactam. The clavulanic acid has a structure similar to the penicillins but the sulphur atom has been substituted for an oxygen atom which increases the affinity of these molecules for the β-lactamases. On the other hand, the sulbactam and the tazobactam have an oxidation of the sulphur present in the β-lactam ring (Jacoby and Bush, 2009).

2.3.2. Mode of β-Lactam Antibiotics Action

Cell wall synthesis in bacteria is dependent on the PBPs enzymes transpeptidases, carboxypeptidases and transglycosylases. These enzymes complete the final stages of cross-linking the peptidoglycan polymers in the wall. β-lactams interfere during this stage directly inhibiting one or several of the enzymes (Perry and Jstaley, 1997).

The β-lactam antibiotics are analogues of the terminal amino acid (D-alanyl-D-alanine) residues on the precursor N-acetylmuramic acid and N-acetylglucose amine peptide subunits of the peptidoglycan layer. The nucleus of the β-lactam molecule irreversibly binds to the Serine 403 residue of the PBP active site. This prevents the transpeptidation of the peptidoglycan layer, and hence disrupts the synthesis of the cell wall (Bradford, 2001).

The effectiveness of β-lactam antibiotics relies on the ability to reach the PBP intact and the ability to bind to the PBP. The activity also depends on the growth rate; slow growing cells are killed more slowly than rapidly

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growing ones (Livermore and Williams, 1996; Livermore and Woodford, 2006).

Inhibition leads to bacteriolysis caused by autolysins activation of the cells autolytic system. This happens when precursors of the cell wall accumulate and the wall is unable to resist osmotic pressure (Perry and Jstaley, 1997; Ryan and Ray, 2004).

2.3.3. Mechanisms of β-Lactam Antibiotics Resistance

There are several mechanisms of resistance to β-lactam antibiotics and they are generally due to point mutations on the chromosome or to the acquisition of mobile elements such as plasmids or transposons (Lawley *et al.,*2004). The resistance to β-lactam antibiotics can be due to the expression of a single mechanism of resistance or to the additive effect of several mechanisms, resistance to β-lactam antibiotics in bacteria could be due to four mechanisms.

a) Resistance by Increased Efflux Pump

Genes encoding efflux pumps may be on the chromosome or on transmissible elements, such as plasmids, and are present in antibiotic-susceptible and antibiotic-resistant bacteria, efflux systems are now recognized as an important contributor to antimicrobial resistance, and are more commonly found in Gram-negative bacteria, with resistance mediated by increased expression of the efflux pump protein or a mutation in the protein increasing the efficiency of export (Piddock, 2006).

b) Resistance by Decreased Antibiotics Uptake

Before a β-lactam reaches bacterial PBP targets which are on the outer surface of the cell membrane it must diffuse across the outer membrane of the cell, by using the pores that are formed by porins, and then cross the periplasm. The porins, which represent one family of outer membrane

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proteins (Omps), form channels to permit diffusion of small hydrophilic solutes through the outer membrane (Yildirim *et al.,* 2005) .

Usually, *K. pneumoniae* strains express OmpK35 and OmpK36, while the ESBL producing strains commonly express only one of these, normally OmpK36, or no porin at all (Martinez-Martinez, 2008). In some instances, porin loss in ESBL-producing isolates increases resistance to fourth generation cephalosporins and /or carbapenems (Poole, 2004).

c) Resistance by Alteration of the Target Site

Resistance caused by alterations in PBPs can occur by acquisition of an increased target PBP number and reduced affinity of this target. PBPs with reduced affinity are an important mechanism of resistance to β-lactamse specially in Gram-positive when β-lactamases are absent (Livermore, 2002).

d) Resistance by Enzymatic Inactivation

Antibiotic-inactivation enzymes, like β-lactamses, are the most important single cause of resistance to β-lactams. Over 700 unique enzymes have been identified and they are either chromosomally or plasmid mediated. These enzymes make biologically inactive products of the antibiotic by efficient hydrolysis of the amide bond in the β-lactam ring (Livermore, 2003). β-lactamases were initially noted in *Staphylococcus aureus* and are now common in Gram-negative bacteria such as *E. coli, K. pneumoniae* and *Proteus mirabilis*, but have also been found in other *Enterobacteriaceae* (Ali Shah *et al.,* 2004).

2.4. β-Lactamases

2.4.1. Classification and Nomenclature of β-Lactamases

β-lactamases are large, heterogeneous group enzymes. Many attempts have been made to categorize and classify them (Livermore, 2003). Different classifications based on phenotype, gene or amino acid protein

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sequences and function have been attempted since the beginning of the 1970s (Hall and Barlow, 2005).

The β-lactamases are divided into two classes; serine and metallo β-lactamases that do not share sequence or structural homology. One of the most used classification schemes is Ambler's based upon amino acid sequences. By this classification the β-lactamases are divided into four molecular classes, A, B, C and D. Originally, only the serine and metallo β-lactamase classes were designated, hence, class A, C and D have serine moieties in their active site, while class B enzymes contain the metallo enzymes that have zinc molecules in the active site (Ambler *et al*., 1991). However, both β-lactamase classes hydrolyse the amide bond of the four-membered β-lactam ring. The three classes of serine β-lactamases, A, C and D share similarity on the protein structure level, which proves that they descended from a common ancestor (Hall and Barlow, 2005). The metallo β-lactamases need a bivalent cation, usually zinc, to be able to hydrolyse the β-lactam ring by activate a water molecule and catalyse its direct addition to the β-lactam ring, but serine β-lactamases open the β-lactam bond via a covalent acyl enzyme intermediate (Garau *et al.*, 2005).

In addition to the Ambler classification, Bush, Jacoby and Medeiros divide the enzymes into four groups based on their substrate and inhibitor profiles. Penicillinases, ESBLs, AmpC β-lactamases and carbapenemases. This classification does not follow the phylogeny of these enzymes. The nomenclature of β-lactamases is not logical. They are named in different ways, some after their preferred substrate, like IMP (active on imipenem) and OXA (oxacillinase), others according to biochemical properties, while others are named according to bacteria, patient or hospitals (Bush *et al.,* 1995).

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2.4.2. Molecular Aspects of β-Lactamases

The β-lactamases can be both chromosomal and plasmid-encoded. Most of the *Enterobacteriaceae* species have at least one chromosomal β-lactamase (Bradford, 2001).

2.4.2.1. Chromosomal β-Lactamase

The chromosomally encoded enzymes can be inducible or constitutively expressed. An inducible gene is a gene whose expression is responsive to an environmental change, while a constitutively expressed gene is transcribed continually. The β-lactamase production, which can be induced by the presence of certain antibiotics, is usually encoded by the bacterial chromosome (Jacoby, 2009).

2.4.2.2. Transferable and Plasmid Encoded β-Lactamase

Transferable β-lactamase genes can be spread on plasmids, transposons, insertion sequences and integrons, by conjugation, transduction or transformation. Plasmid is an extra-chromosomal double-stranded DNA molecule that occurs naturally in bacteria. Plasmids are capable of autonomous replication within a suitable host (Lawley *et al.*, 2004).

Transposons are mobile genetic elements that can move around to different positions within the genome of a single cell. Hence, class II transposons allow transfer and permanent addition of genes encoding antibiotic resistance. When the transposable elements lack additional genes, they are known as insertion sequences. So, insertion sequences are small, around 700 to 2500 base pair in length, which participate in rearrangement of chromosomes and plasmid integration (Hancock and Pallister, 1999) .

Integrons are genetic elements that contain gene cassettes that can be mobilized to other integrons or to secondary sites in the bacterial genome

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(Fluit and Schmitz, 1999). Most of the β-lactamases are integrated within plasmids and transposons that enable the rapid transfer of these resistance genes between microbes. The association of insertion sequences with these β-lactamase genes are also involved in their dissemination and expression (Bradford, 2001). The β-lactamase genes are often found within integrons as part of multi-drug resistance cassettes that confer resistance to several other antibiotic classes such as aminoglycosides, macrolides, sulphonamides and chloramphenicol (Wilke *et al*., 2005).

2.5. The clinically Most Important β-Lactamases

2.5.1. Extended Spectrum β-Lactamases (ESBLs)

Over the last 20 years many new β-lactam antibiotics, specifically designed to resist known β-lactamases, have been developed . However, almost invariably new β-lactamases have emerged to combat each new class of β-lactams. Plasmid-mediated ESBLs emerged in Gram-negative bacilli in Europe in the 1980s (Zeba, 2005).

ESBLs, so named because of their increased spectrum of activity (Bradford, 2001). ESBL-producing bacteria are typically resistant to penicillins, first-and second-generation cephaloporins as well as the third-generation oxyimino cephalosporins (cefotaxime, ceftazidime, ceftriaxone) and monobactams (aztreonam), retaining susceptibility only to cephamycins, fourth-generation cephalosporins (cefepime, cefpirome) and carbapenems, ESBLs are inhibited by β-lactamase inhibitors such as clavulanic acid and tazobactam, which confer an extended spectrum of activity (Poole, 2004).

ESBLs have been reported nationwide in many different genera of *Enterobacteriaceae* and in *Pseudomonas aeruginosa* (Hadi, 2008; Al Hilali, 2010; Al Hilli, 2010; Al-Muhannak, 2010; Shamkhi, 2011). However, they are most common in *K. pneumoniae* and *E. coli*. ESBL-producing

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organisms are often multiresistant to several other classes of antibiotics, as the plasmid with the genes encoding ESBLs often carry other resistance determinants (Bradford, 2001). Initially, ESBL-producing organisms were usually isolated from nosocomial infections, but these organisms are now also being isolated from community and rest home patients (Pitout *et al.,* 2005) .

Typically, ESBLs are plasmid encoded but also present on chromosomes, often in association with integrons. These enzymes are derivatives, predominantly, of class A and D β-lactamases. Classical ESBLs evolved from class A, TEM (from TEM-1 or TEM-2) and SHV (from SHV-1) enzymes, and these remain the most prevalent types of ESBLs, though class D ESBLs (OXA family) have also been known for some time (Bradford, 2001).

a) CTX-M β-lactamases

In 1986, a non-TEM and a non-SHV ESBL cephalosporinase was discovered in a cefotaxime-resistant *E. coli*. A few years later in 1989, similar cefotaxime-resistant strains were found in Germany, France and Argentina (Bauernfeind *et al.*, 1992). This cefotaxime resistance was due to enzymes named cefotaximases (CTX-M), and these CTX-Ms showed a much higher degree of activity to cefotaxime than to ceftazidime (Chia *et al*., 2005).

The CTX-Ms represent plasmid acquisition of β-lactamase genes that can normally be found on the chromosome of *Kluyvera* species (Humeniuk *et al*., 2002). It was believed that CTX-M-24 in *K. pneumoniae* was conferred by plasmids of other enteric bacilli (Chen *et al.,* 2005). The bla_{CTX-M} gene variants show less than 40% identity to bla_{SHV} and bla_{TEM}, and form a relatively heterogeneous family of 50 members, which are sub classified into

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five major lineages: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 groups. The predominant CTX-M types were occurring widely in UK, Canada and Russia (CTX-M-15), in Argentina and Palestine (CTX-M-2), in some parts of China (CTX-M-14) and in Spain (CTX-M-9 and -12) and (CTX-M-14, -15, and -27) in Egypt (Al-Agamy *et al.,* 2006). In Taiwan, the prevalence rate of CTX-M-14-producing *K. pneumoniae* and *E. coli* isolates increased significantly (Chia *et al*., 2005), and then in Iraq studies stated the widely predominant of CTX-M type (Al-Hilali, 2010; Al-Muhannak, 2010; Shamkhi, 2011).

b) TEM β-Lactamases

The TEM-1 (named after a Greek patient Temoneira) was first reported in 1965 from an *E. coli* strain and represents today one of the most prevalent β-lactamase in *Enterobacteriaceae* (Datta and Kontomichalou, 1965). Isolates harbouring TEM-1 β-lactamases are resistant to ampicillin. Although TEM-type β-lactamases are most often found in *E. coli* and *K. pneumoniae*, they are also found with increasing frequency in other Gram-negative species (Bradford, 2001). The TEM-1 β-lactamase encoded by the bla_{TEM-1} gene is present on Tn2 and Tn3 transposons (Datta and Kontomichalou, 1965).

TEM-3 reported in 1987 was the first TEM variant with increased activity against extended-spectrum cephalosporins. The TEM ESBL phenotype is produced by single amino acid substitutions at positions 104, 164, 238, and 240, but ESBLs usually have more than a single amino acid substitution (Bradford, 2001). Currently, more than 165 bla_{TEM} gene variants exist including the gene variants that are resistant to inhibitors like clavulanic acid (Jacoby and Bush, 2009).

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c) SHV β-Lactamases

The SHV enzymes, named after the "sulfhydryl variable" active site are commonly associated with *K. pneumoniae* (Tzouvelekis and Bonomo, 1999). SHV-1 was first described in 1972 as Pit-2, while SHV-2 was detected in Germany in the beginning of the 1980s (Kliebe *et al.*, 1985). The SHV β-lactamases have a similar structure to TEM, with which they share 68% of their amino acids (Tzouvelekis and Bonomo, 1999). Both chromosomal, bla_{SHV-1} and bla_{SHV-11}, and plasmid-mediated bla_{SHV} genes are found (Ford and Avison, 2004).

The amino acid changes at positions 238 and 240 according to the Ambler classification (Ambler *et al.*, 1991) are present in the SHV variant with ESBL activity. Presently, 120 SHV varieties have been detected and they are found worldwide. Moreover, the SHV-2, SHV-5 and SHV-12 variants are the most common ones (Jacoby and Bush, 2009). The SHV ESBLs were the predominant ESBL type in Europe, and in the United States during the beginning of the 21st century (Paterson *et al.*, 2003), and were predominant in Iraqi hospitals (Hadi, 2008; Al- Hilali, 2010; Al-Hilli, 2010; Belal, 2010; Shamkhi, 2011).

d) OXA β-Lactamases

Extended spectrum β-lactamase members of the OXA family (OXA-18 and derivatives of OXA-2 and -10) are comparatively rare, and found mostly in *P. aeruginosa* (Sevillanoa *et al.,* 2006). OXA-type ESBLs typically carry multiple mutations, with OXA-10-derived ESBLs often carrying substitutions at Gly167 that are responsible for resistance to ceftazidime. OXA-type ESBLs can be plasmid or chromosomal but apparently are not associated with classical integrons, in contrast to OXA determinants for

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narrow-spectrum enzymes. This enzyme confers resistance to ampicillin and cephalothin (Poole, 2004).

e) VEB β-Lactamases

This enzyme is class A β-lactamase and was named VEB-1 (for Vietnamase extended-spectrum beta-lactamase). The latter confers high-level resistance to amoxicillin, ticarcillin, piperacillin, cefotaxime, ceftazidime, and aztreonam which is inhibited by clavulanate (Poirel *et al.,* 1999). Unlike most ESBL genes, bla_{VEB-1} is part of a gene cassette located in class 1 integrons and/or transposon. The VEB-1 enzyme is typically plasmid-borne in *Enterobacteriaceae* but chromosomal in *P. aeruginosa* and *Acinetobacter baumannii* (Poole, 2004).

f) PER and GES β-Lactamases

PER and GES β-lactamases represent a distinct class A cephalosporinase phenotype so PER β-lactamases only restricted to South America and Europe . Although possessing only 26% identity to the TEM-type ESBLs. PER and GES β-lactamases also confer resistance to oxyimino-β-lactams, such as cefotaxime, ceftazidime, and aztreonam (Paterson *et al.,* 2003). In addition, a novel GES-type variant as part of a class 1 integron, as emergence of GES type ESBLs. The substitution of the amino acids sequence that results in increased hydrolysis of imipenem as in GES-2, GES-4, GES-5, and GES-6 and substitution of the glycine at position 243 in GES-11 was associated with increased activity toward aztreonam (Moubareck *et al.,* 2009).

2.5.2. Carbapenemases

Carbapenemases represent the most versatile family of β-lactamases, with a breadth of spectrum unrivaled by other β-lactam-hydrolyzing enzymes, some investigators have preferred the nomenclature "carbapenem-

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hydrolyzing enzymes" to the term "carbapenemases," suggesting that carbapenems are their substrate spectrum (Queenan and Bush, 2007).

Carbapenemases belong to two major molecular families, distinguished by the hydrolytic mechanism at the active site. The first carbapenemases described were from Gram-positive bacilli. These enzymes were inhibited by EDTA. In the mid to late 1980s, another set of carbapenem-hydrolyzing enzymes emerged among the *Enterobacteriaceae*, but EDTA did not inhibit their activity (Medeiros and Hare, 1986).

2.5.2.1. Class A and D Carbapenemases (Serin β-Lactamases)

Class A serine carbapenemases of functional group have appeared sporadically in clinical isolates since their first discovery over 20 years ago (Munoz-Price and Quinn, 2009). These β-lactamases have been detected in *Enterobacter cloacae*, *Serratia marcescens*, and *Klebsiella* spp. as single isolates or in small outbreaks (Medeiros and Hare, 1986). Bacteria expressing these enzymes are characterized by reduced susceptibility to imipenem, but MICs can range from mildly elevated (Babouee *et al.,* 2011). Three major families of class A serine carbapenemases include the IMI, SME, are chromosomal encoded, and can be induced in response to imipenem and cefoxitin and KPC enzymes are plasmid encoded. Their hydrolytic mechanism requires an active-site serine at position 70 in the Ambler numbering system for class A β-lactamases (Queenan and Bush, 2007).

2.5.2.2. Class B Carbapenemases (Metallo β-Lactamase)

This class of β-lactamases is characterized by the ability to hydrolyze carbapenems and by its resistance to the commercially available β-lactamase inhibitors but susceptibility to inhibition by metal ion chelators (Sekowska *et al.,* 2010). The substrate spectrum is quite broad in addition to the

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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 in the active site of the enzyme, resulting in the distinctive trait of their inhibition by EDTA (Queenan and Bush, 2007).

The most common metallo-β-lactamase families include the VIM, IMP, GIM, and SIM enzymes, which are located within a variety of integron structures, where they have been incorporated as gene cassettes. When these integrons become associated with plasmids or transposons, transfer between bacteria is readily facilitated (Mounz-Price and Quinn, 2009).

2.5.3. AmpC β-Lactamases

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 (Jacoby, 2009). Swedish investigators began a systematic study of the genetics of penicillin resistance in *E. coli* in 1965. Mutations with stepwise-enhanced resistance were termed *ampA* and *ampB* (Eriksson- Grennberg, 1968). A mutation in an *ampA* strain that resulted in resistance and overproduced β-lactamase was then designated *ampC*. *ampC* was the structural gene for the enzyme (Linstrom *et al.,* 1970). Most of the *amp* nomenclature has changed over the years, but the designation *ampC* has persisted. The sequence of the *ampC* gene from *E. coli* was reported in 1981 (Jaurin and Grundstrom, 1981). It differed from the sequence of penicillinase type β-lactamases such as TEM-1 but, like them, had serine at its active site (Jacoby, 2009).

In the Ambler structural classification of β-lactamases AmpC enzymes belong to class C, while in the functional classification scheme of Bush *et al.* they were assigned to group 1. When the functional classification scheme was published in 1995, chromosomally determined AmpC β-lactamases in

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Enterobacteriaceae and also in a few other families were known (Bush *et al.,* 1995).

In GenBank, *ampC* genes are included in cluster of orthologous groups (COG) 1680, which comprises other penicillin binding proteins as well as class C β-lactamases and includes proteins from archaea as well as bacteria. Sequence alone is insufficient to differentiate an AmpC β-lactamase from ubiquitous low-molecular-weight penicillin binding proteins involved in cell wall biosynthesis, such as D-peptidase (D-alanyl-D-alanine carboxypeptidase/transpeptidase). Both have the same general structure and share conserved sequence motifs near an active-site serine (Knox *et al.,* 1996).

Class C β-lactamases (AmpC) are an important group of proteins that are broadly distributed. This is the second most common β-lactamase group (Bush *et al.,* 1995). AmpC is typically encoded on the chromosome of Gram-negative bacteria including *Citrobacter, Serratia* and *Enterobacter* species where its expression is usually inducible. About 20 years ago, the inducible chromosomal genes were detected on plasmids and were transferred to organisms, typically not expressing these types of β-lactamase, like *Klebsiella* spp., *E. coli*, or *Salmonella* spp. (Li *et al.*, 2009).

AmpC β-lactamases, in contrast to ESBLs, hydrolyse broad and extended-spectrum cephalosporins but are resistant to inhibition by β-lactamase inhibitors like clavulanic acid, sulbactam and tazobactam have little effect towards these enzymes (some are inhibited by tazobactam and sulbactam). They are poorly inhibited by *p*-chloromercuribenzoate and not at all by EDTA. Good inhibitors are substances like cloxacillin, oxacillin and aztreonam, and boronic acid reversibly inhibiting AmpC (Majiduddin *et al.,* 2002; Jacoby, 2009).

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Strains only producing AmpC can be detected but organisms producing plasmid-encoded AmpC and ESBLs are difficult to distinguish by phenotypical testing. Cefoxitin (cephamycin) resistance may indicate the possibility of AmpC-mediated resistance but it can also be an indication of reduced outer membrane permeability (Philippon *et al.*, 2002).

2.5.3.1. Chromosomal AmpC β-Lactamase

AmpC β-lactamase are encoded and found in all *Enterobacteriaceae*, except from *Salmonella, E. coli, Klebsiella, Proteus* and *Shigella* spp. produce low basal levels of the intrinsic AmpC β-lactamase (Jaun *et al.,* 2005). This occurs in species expressing chromosomal *ampC* like in *Pseudomonas, Acinetobacter, Citrobacter* and *Enterobacter, Serratia, Morganella* and *Providencia*. These bacteria are intrinsic resistant to ampicillin, first generation cephalosporins and are not inhibited by β-lactamase inhibitor (Parveen *et al.,* 2010).

Organisms that constitutively overexpress the chromosomal genes are collectively called derepressed mutants (Hanson and Sandres, 1999).

In *E. coli* no obvious regulators are present, and therefore the AmpC expression is uninducible. *E. cloacae* is inherently resistant to the first and second generation cephalosporins and to aminopenicillins, a mechanism mediated by the production of chromosomal AmpC (Jacoby, 2009).

The inducible *ampC* expression in enteric bacteria, involves a signalling system where several regulators such as *ampG, ampR* and *ampD* are involved. When one or several PBPs are inactivated by a β-lactam, a higher amount of peptidoglycan degradation products enter the cell (Chahboune *et al.,* 2005).

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2.5.3.2. Plasmid-Mediated AmpC β-Lactamase

The majority of plasmid-mediated *ampC* genes are found in nosocomial isolates of *E. coli* and *K. pneumoniae* (Perez–Perez and Hanson, 2002). Like the chromosomally determined AmpC β-lactamases, the plasmid-mediated enzymes confer resistance to a broad spectrum of β-lactams including penicillins, oxyimino cephalosporins, cephamycins, and (variably) aztreonam. Susceptibility to cefepime, cefpirome, and carbapenems (Bauernfeind *et al.,* 1998).

Minor differences in amino acid sequence have given rise to families. Forty-three CMY alleles are currently known in GenBank. Sequence data can be found for seven varieties of FOX; four varieties of ACC, LAT, and MIR; three varieties of ACT and MOX; and two varieties of DHA. Some of these varieties are determined by chromosomal genes and represent possible progenitors for the plasmid-determined enzymes. (Jacoby, 2009).

The genes for ACT-1, DHA-1, DHA-2, and CMY-13 are linked to *ampR* genes and are inducible, while other plasmid-mediated *ampC* genes are not (Miriagou *et al*., 2004). There are six main families of plasmid mediated AmpC genes (Figure 2-1).

The genes encoding plasmid-mediated AmpC β-lactamases are of chromosomal origin, derived from members of the family *Enterobacteriaceae.* To last decade, 29 different genes encoding 28 different plasmid-mediated AmpC β-lactamases have been identified . They can be grouped based on their chromosomal origins (Perez-Perez and Hanson, 2002).

The genes encoding the AmpC β-lactamases LAT-1, CMY-2, and BIL-1 are 90.4% similar to the chromosomal *ampC* gene of *C. freundii* strain OS60. The ability to group different *ampC* genes allows the evaluation of

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similarity clusters. A high degree of similarity within these clusters can result in a primer design capable of amplifying family-specific genes (Li *et al*., 2009). Twenty-nine plasmid-mediated gene sequences and one chromosomal gene (ACC-2). Six different groups were identified depending on percent similarities. These groups include ACC (origin *H. alvei*), FOX (origin unknown), MOX (origin unknown), DHA (origin *Morganilla morganii*), CIT (origin *C. freundii*), and EBC (origin *E. cloacae*). The percent similarities among the family members within these clustered groups were 94.3, 94.2, 89.4, 95.7, 98.6, and 84.2% for the ACC, FOX, MOX, DHA, CIT, and EBC groups, respectively (Perez–Perez and Hanson, 2002).

2.5.3.3. Physical Properties of AmpC β-Lactamases

AmpC enzymes typically have molecular masses of 34 to 40 kDa and isoelectric points (6.6- >8.0) (Jacoby, 2009). If benzylpenicillin as the substrate, typical pH-activity and temperature-activity curves obtained with β-lactamases from Gram-positive bacteria shows maxima in the range of pH 6.0-7.0 and 30-40 °C, respectively (Eda, 2003). The optimal activity of β-lactamase for Gram-negative bacteria is at temperature ranging between 35-37 oC, and pH between 6.5-7.5 (Flayeh, 2005).

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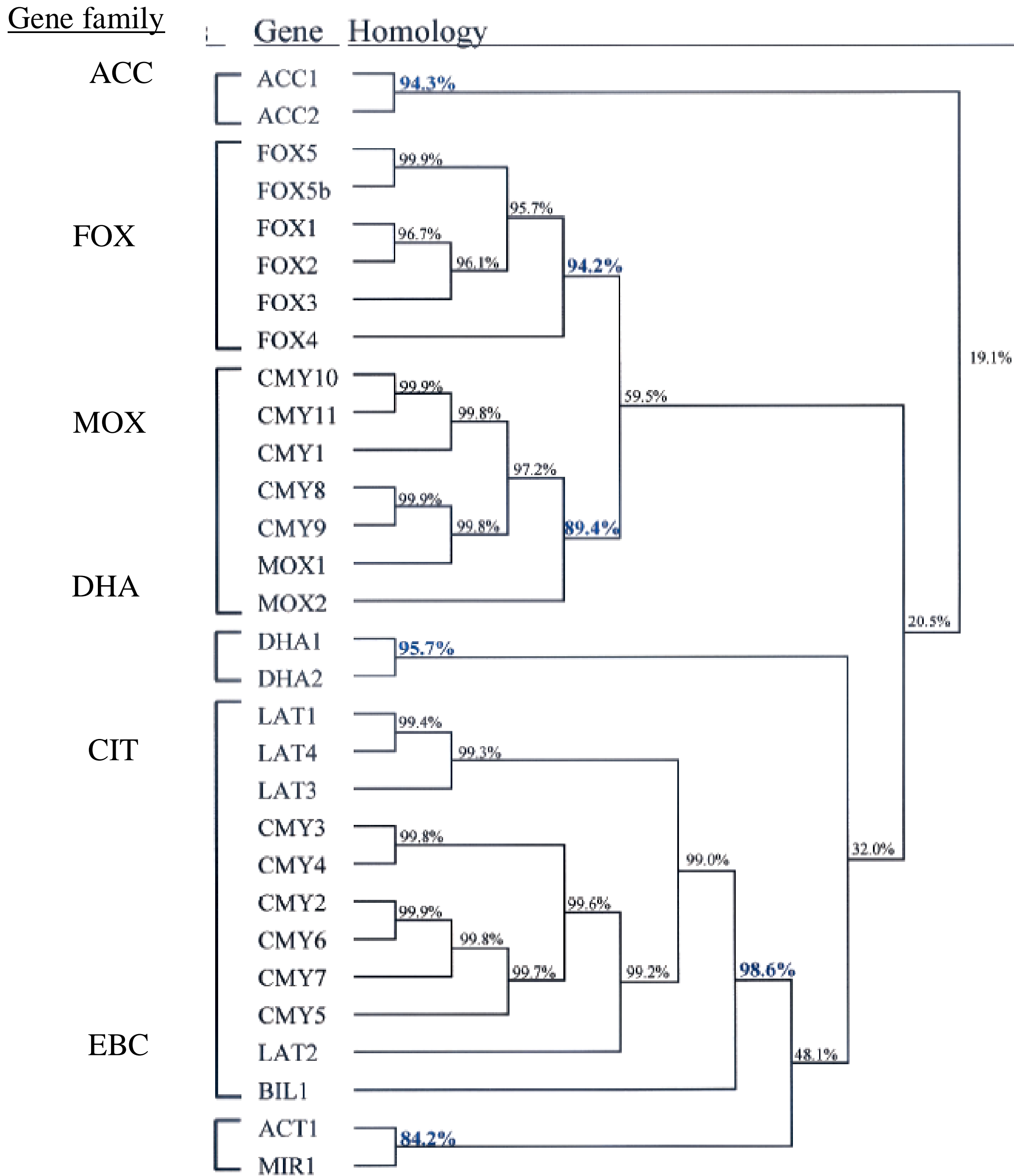

Figure (2-1): AmpC dendrogram showing gene families and values correspond to the percent similarity between the most distinct member of each cluster and the other members within that cluster. Sequence were downloaded from the GenBank database, and percent similarities were analyzed by using DNAsis for Windows, version 2.6 (Hitachi Software) (Perez-Perez and Hanson, 2002).

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2.5.3.4. Epdemiology of AmpC β-Lactamases

Since the first description ESBL production by *K. pneumoniae* in 1983 (Pai *et al.,* 2004), antibiotic- resistant strains that produce ESBLs have emerged among the members of the family *Enterobacteriaceae*. In the past decade, a new problem has emerged in enteric bacteria is plasmid-mediated AmpC enzymes.

They are derived from chromosomal *ampC* genes of Gram-negative organisms. Plasmid-encoded *ampC* genes have been known since 1989. They have been found around the world in nosocomial and non nosocomial isolates (Philippon *et al.,* 2002).

DHA-1 from a clinical isolate of *S. enterica* serovar Enteritidis from Saudi Arabia is the first identified plasmid-encoded inducible cephalosporinase (Yan *et al.,* 2002), DHA-1-related β-lactamase, named DHA-2, was identified more recently from a *K. pneumoniae* isolate in France (Jacoby, 2009). CIT-type (and, to a lesser extent, DHA type) enzymes are prevalent in Asia and Canada, while a minority of ACC-type enzymes were reported in the United Kingdom(Li *et al.,* 2009).

There are fewer published data in the United States, but one study reported that FOX type enzymes were predominantly present in *Enterobacteriaceae* with plasmid-mediated enzymes (Tan *et al.,* 2009). Further validation of the study methods in different geographic regions may be warranted. *K. pneumoniae* strains producing plasmid-mediated AmpC β-lactamases such as MIR-1, ACT-1, and more recently ACC-1 have been implicated in numerous nosocomial outbreaks. AmpC ACC-1 is a plasmid-encoded class C β-lactamase originating from *Hafnia alvei* and now found in

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various members of the family *Enterobacteriaceae* in North Africa and Europe (Bidet *et al.,* 2005; Li *et al.,* 2009).

2.5.3.5. Detection of AmpC β-Lactamase

The National Committee for Clinical Laboratory Standards has issued recommendations for ESBL screening and confirmation for isolates of *E. coli* and *Klebsiella* spp. (NCCLS, 2007). There are presently no Clinical Laboratories Standard Institute or other approved criteria for AmpC detection (Jacoby, 2009). Organisms producing enough AmpC β-lactamase will typically give a positive ESBL screening test but fail the confirmatory test involving increased sensitivity with clavulanic acid (Bell *et al.,* 2007).

The differentiation of organisms expressing ESBLs from organisms expressing plasmid-mediated AmpC β-lactamases is necessary in order to address surveillance and epidemiology as well as hospital infection control issues associated with these resistance mechanisms. Several phenotypic tests can distinguish these two resistance mechanisms, but such a test cannot differentiate between an AmpC enzyme encoded on a plasmid or on the chromosome,and unable to differentiate the different types or families of plasmid-mediated AmpC β-lactamases. In addition, the use of automated systems, while adequate for less complicated organisms, is not adequate for the newer generation of antibiotic-resistant pathogens that express multiple resistance mechanisms and produce multiple β-lactamases (Perez-Perez and Hanson, 2002). For these purposes, and as the current gold standard for plasmid-mediated AmpC β-lactamase detection, multiplex PCR has been developed by utilizing six primer pairs (Hanson *et al.,* 1999).

2.5.3.6. Treatment of AmpC β-Lactamase Producer *K. pneumoniae*

Strains with *ampC* genes are often resistant to multiple agents, making the selection of an effective antibiotic difficult. β–lactam/β-lactamase

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inhibitor combinations and most cephalosporins and penicillins should be avoided because of in vitro resistance (Pai *et al.,*2004). Temocillin, a 6-α-methoxy derivative of ticarcillin, is active in vitro against many AmpC-producing *Enterobacteriaceae* whether the enzyme is determined by chromosomal or plasmid genes and is also active against ESBL producer (Glupczynski *et al*., 2007).

Carbapenem therapy has usually been successful, but has also been followed by the emergence of carbapenem-resistant *K. pneumoniae* associated with ACT-1 β-lactamase production and outer membrane porin loss (Pai *et al.,* 2004; Bidet *et al.,* 2005). If the isolate is susceptible, fluoroquinolone therapy is an option especially for non-life-threatening infections such as urinary tract infection. Tigecycline is another option, it had good activity *in vitro* against 88% of AmpC-hyperproducing isolates of *K. pneumoniae* in the United Kingdom (Hope *et al.,* 2006).

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3. Materials and Methods

3.1. Materials

3.1.1. Instruments and Equipment

Type of equipment	Manufacturing company (Origin)
Autoclave	Hiclave- Hirayama (Japan)
Bench centrifuge	Hettich (Germany)
Cold centrifuge	Hettich
Compound light microscope	Olympus (Japan)
Distillator (Water distiller)	GFL (Germany)
Digital camera	Sony (Japan)
Deep freezer	GFL (Germany)
Dialysis tubes	Spectrum (USA)
Electric oven	Memmert (Germany)
Electrophoresis unit	Labner (Taiwan)
Glass separation column	Quickfit (England)
Gel documentation system	Biometra (Germany)
High speed cold centrifuge	Hettich
Incubator	Memmert
Laminar flow	Cruma (Spain)
Magnetic stirrer	Labtech (Germany)
Micropipette set (1-1000µl)	Eppendorf (Germany)
Millipore filter (0.22µm)	Difco (USA)
PCR system	GeneAmp (Singapore)
pH-meter	LKB (Sweden)
Shaker water bath	Memmert
Standard loop 0.01 ml	Himedia (India)
Sensitive balance	Memmert
Sonicator	Vibra cell (USA)
UV-transilluminator	Clinex (Taiwan)
UV spectrophotometer	Shimadzu (Japan)
Vortex	Thermolyne (USA)
Visible spectrophotometer	Apel (USA)

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3.1.2. Biological and Chemical Materials

Materials	Manufacturing company (Origin)
Agar-agar	Himedia (India)
Agarose	Promega (USA)
Ammonium sulfate	BDH(England)
Beef extract	Difco (USA)
Bromocresol purple	BDH
Barium chloride ($BaCl_2 .2H_2O$)	Fluka (Switzerland)
Boric acid	Ficher (USA)
Comassie brilliant blue	Thomas Baker (India)
Chloroform	BDH
Cresol red	BDH
Disodium hydrogen phosphate (Na_2HPO_4)	BDH
DNA loading buffer	Promega
D- Mannitol	BDH
D-Xylose	BDH
D-Mannose ($C_6H_{12}O_6$)	Difco
D- Sorbitol	Difco
DL-Phenylalanine	BDH
Ethyenediamine tetra-acetic acid (EDTA)	AppliChem (Germany)
Ethidium bromide	Sigma (USA)
Ethanol (96%)	BDH
ESBL supplement	CHROMagar (France)
Ferric chloride ($FeCl_3$)	Sigma
Glucose ($C_6 H_{12} O_6$)	Difco
Glycerol ($C_3H_8O_3$)	Fluka
Gram stain	Himedia
Gelatin	BBL (USA)
$HgCl_2$	Sigma
Hydrochloric acid (HCl)	BDH
Hydrogen peroxide (H_2O_2) 3%	Himedia
Isopropyl alcohol	Mast Diagnostic (USA)
Iodine	Mast Diagnostic
Kovac's reagent	Himedia

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KPC supplement	CHROMagar (France)
Lactose	BDH
L-Lysine	BDH
L-Arginine	BDH
L-Arabinose	BDH
L-Ornithine	BDH
Maltose	Difco
Methyl red	BDH
Molecular grad water	Promega
α-Naphthol ($C_{10}H_8O$)	BDH
Peptone	Difco
1,4-Piperazine-diethanesulphonic acid (PIPES)	Sigma
Phosphoric acid (H_3PO_4)	Fisher scientific co.
Phenol red	BDH
Potassium iodide (KI)	Mast Diagnostic
Pyridoxal phosphate	BDH
Serum bovine albumin (SBA)	BDH
Sephadex G 75	Pharmacia fine chemicals (Sweden)
Sulfuric acid (H_2SO_4)	BDH
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
Soluble starch	Difco
Sodium dodecyl sulfate (SDS)	AppliChem (UK)
Tetramethyl *p*-phenyl diamine-dihydrochloride	BDH
Tris-(hydroxymethyl) methylamine $NH_2.(CH_2OH)_3$ (Tis-OH)	BDH
Tris- EDTA (TE) buffer molecular grad	Promega (USA)
Tris-Borate-EDTA Buffer (TBE buffer)	Promega
Urea solution	Mast Diagnostic

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3.1.3. Culture Media

Medium	Manufacturer (Origin)
Brain heart infusion broth	Himedia (India)
Blood agar base	Himedia
CHROMagar orientation	ChroMagar (France)
Eosin methylen blue agar	Himedia
MacConkey agar	Himedia
Muller-Hinton agar	Himedia
MR-VP broth	Oxoid (UK)
Nutrient broth	Himedia
Nutrient agar	Himedia
Peptone water	Himedia
Simmon's citrate agar	Mast Diagnostic (UK)
Tryptic soy broth	Alpha (USA)
Triple sugar iron agar	Himedia
Urea agar base	Biolife (Italy)

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3.1.4. Antibiotics

3.1.4.1. Antibiotic Disks

Antibiotic disks					
Class	**Subclass**	**Antibiotic**	**Symbol**	**Content**	**Origin**
Penicillins	Carboxypenicillin	Carbenicillin	PY	100 µg	Bioanalyse (Turkey)
	Ureidopenicillin	Piperacillin Ticarcillin	PRL TIC	100 µg 75 µg	Bioanalyse Bioanalyse
β-Lactams /β-lactamase inhibitor combinations		Amoxi-clav	AC	30 µg	Himedia (India)
		Cefotaxime-Clavulanat	CTC	30 µg	Bioanalyse
Cephems (Oral)	Cephalosporin	Cefixime	CFX	5 µg	Himedia
Cephems(parenteral)	Cephalosporin I	Cefazolin	CZ	30 µg	Himedia
	Cephamycin	Cefoxitin	FOX	30 µg	Bioanalyse
	Cephalosporin III	Ceftazidime	CAZ	30 µg	Bioanalyse
		Cefotaxime	CTX	30 µg	Bioanalyse
		Ceftriaxone	CRO	30 µg	Bioanalyse
	Cephalosporin IV	Cefepime	FEP	30 µg	Bioanalyse
Penems	Carbapeneme	Imipenem Meropenem	IMP MEM	10 µg 10 µg	Bioanalyse Bioanalyse
Monobactams		Aztreonam	ATM	30 µg	Bioanalyse
Aminoglycosides		Amikacin	AK	30 µg	Bioanalyse
		Gentamicin	CN	10 µg	Bioanalyse
		Tobramycin	TOB	10 µg	Bioanalyse
Quinolones	Quinolones	Nalidixic acid	NA	30 µg	Bioanalyse
	Fluoroquinolones	Ciprofloxacin	CIP	5 µg	Bioanalyse
		Levofloxacin	LE	5 µg	Bioanalyse
		Gatifloxacin	GT	5 µg	Bioanalyse
		Norfloxacin	NX	5 µg	Bioanalyse
Tetracyclines		Tetracycline	TE	30 µg	Bioanalyse
Folate pathway Inhibitors		Trimethoprim	TMP	5 µg	Bioanalyse
Phenicols		Chloramphenicol	C	30 µg	Himedia
Nitrofurantoins		Nitrofurantoin	NI	300 µg	Bioanalyse

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3.1.4.2. Antibiotic Powders

Antibiotic	Manufacturer (Origin)
Penicillin G	Troge medical GmbH (Germany)
Ampicillin	MAH Import Export GmbH (Germany)
Piperacilline	Panpharma (France)
Amoxicillin	Panpharma S.A. (France)
Cloxacillin	PanpharmaS.A.
Cephalothin	Panpharma S.A.
Cefotaxime	Kon Tam pharma (China)
Ceftazidime	Julphar (Ras Al-Khaimah)
Meropenem	Dainippon sumitomo pharma (UK)

3.1.5. Prepared Kits

Kit type	Manufacturer (Origin)
API 20 E test	BioMerieux (France)
β-lactamase assay	BDH (England)
Nitrocefin diagnostic disk	Fluka (Switzerland)

3.1.6. Standard Bacterial Strain

Standard Strain	Key characteristics	Source
Escherichia coli ATCC 25922	Susceptible to ampicillin, cephalosporins and gentamicin	American Type Culture Collection (ATCC)

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3.1.7. Polymerase Chain Reaction Materials
3.1.7.1. Master Mix

Type	Description	Purpose	Origin
Go Tag Green Master mix	2X Green Taq Reaction buffer pH 8.5, 400μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP, and 3mM $MgCl_2$.	Monoplex PCR	Promega (USA)
Taq Ready Mix	2X Ready Mix containing Taq DNA polymerase (0.05U/μl), reaction buffer with Mg^{2+} and 0.4mM each dNTP.	Monoplex PCR	Kapa Biosystem (USA)
2G Fast Multiplex Mix	2X cocktail containing 2G fast HotStart DNA polymerase (1U/ 25 l) reaction, Kaoa2G buffer A (1.5X at 1X), dNTPs (0.2mM each dNTP at 1X), $MgCl_2$(3.0mM at 1X) and stabilizer	Multiplex PCR	Kapa Biosystem

3.1.7.2. Molecular Weight DNA Marker

DNA marker	Description	Origin
100 bp Ladder with Loading dye	100-1500 base pairs (bp). The ladder consists of 11 double strand DNA fragment ladder with size of (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500 bp). The 500bp present at triple the intensity of other fragments and serve as a reference. All other fragments appear with equal intensity on gel.	Promega (USA)
10000 bp Ladder	The ladder is composed of 21 chromatographically purified DNA fragments ranged from (100, 200, 300,400,500,600,700,800,900,1000,1200,1500,2000 ,2500,3000, 3500, 4000, 5000, 6000,8000,10000 bp), the reference bright bands are 500, 1000 and 3000.	Kapa Biosystem (USA)

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3.1.7.3. Primers

a) Primers of Monoplex PCR

β-lactamase	Primer name	Oligo sequence (3'-5')	Product size (bp)	Reference	Origen
AmpC BL*	bla_{AmpC}	**F**: ATCAAAACTGGCAGCCG **R**: GAGCCCGTTTTATGCACCCA	550	Kaczmarek *et al.*, 2006 Paterson *et al.*,2003	Biocorp, (Canada)
ESBL*	bla_{CTX-M}	**F**:CGCTTTGCGATGTGCAG **R**:ACCGCGATATCGTTGGT	550	Bhattacharjee *et al.*, 2007	Biocorp
	bla_{TEM}	**F**:AAACGCTGGTGAAAGTA **R**:AGCGATCTGTCTAT	822	Hujer *et al.*, 2006 Paterson *et al.*,2003	Biocorp
	bla_{SHV}	**F**:ATGCGTTATATTCGCCTGTG TGCTTTGTTATTCGGGCCAA	750	Hujer *et al.*, 2006 Paterson *et al.*,2003	Biocorp
	bla_{OXA}	**F**:ATATCTCTACTGTTGCATCTC **R**:AAACCCTTCAAACCATCC	616	Karami and Hannoun, 2008	Biocorp
	bla_{VEB}	**F**:GCGGTAATTTAACCAGA **R**:GCCTATGAGCCAGTGTT	961	Wang *et al.*, 2006	Alpha, (Montrial)
	bla_{GES}	**F**:ATGCGCTTCATTCACGCAC **R**:CTATTTGTCCGTGCTCAGG	846	Wang *et al.*, 2006	Alpha
	bla_{PER}	**F**:AGTCAGCGGCTTAGATA **R**:CGTATGAAAAGGACAATC	978	Wang *et al.*, 2006	Alpha
Carbapenemases	bla_{IMP}	**F**:CGGCCKCAGGAGMGKCTTT **R**:AACCAGTTTTGCYTTACYA	587	Yin *et al.*, 2008	Alpha
	bla_{VIM}	**F**:ATTCCGGTCGGRGAGGTCCG **R**:GAGCAAGTCTAGACCGCCCG	633	Yin *et al.*, 2008	Alpha
	bla_{KPC}	**F**:ATGTCACTGTATCGCCGTCT **R**:TTTTCAGAGCCTTACTGCCC	882	Schechner *et al.*, 2009	Biocorp

***BL: β-lactamase ; ESBL: Eextended spectrum β-lactamase**

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b) Primers used in Monoplex and Multiplex PCR (Biocorp, Canada)

β-lactamase type	Primer name	Oligo sequence (3'-5')	Product size (bp)	Reference
Plasmid mediated AmpC β-lactamase	*bla*$_{FOX}$	**F**: AAC ATG GGG TAT CAG GGA GAT G **R**: CAA AGC GCG TAA CCG GAT TGG	190	Perez-Perez and Hnson, 2002
	bla$_{CIT}$	**F**: TGG CCA GAA CTG ACA GGC AAA **R**: TTT CTC CTG AAC GTG GCT GGC	462	
	bla$_{DHA}$	**F**: AAC TTT CAC AGG TGT GCT GGG T **R**: CCG TAC GCA TAC TGG CTT TGC	405	
	bla$_{EBC}$	**F**: TCG GTA AAG CCG ATG TTG CGG **R**: CTT CCA CTG CGG CTG CCA GTT	302	
	bla$_{ACC}$	**F**: AAC AGC CTC AGC AGC CGG TTA **R**: TTC GCC GCA ATC ATC CCT AGC	346	
	bla$_{MOX}$	**F**: GCT GCT CAA GGA GCA CAG GAT **R**: CAC ATT GAC ATA GGT GTG GTG C	520	

3.2. Methods

3.2.1. Preparation of Reagents

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

3.2.1.1. Catalase Reagent

Hydrogen peroxide (3%) was prepared and used for detection the ability of bacteria to produce catalase enzyme.

3.2.1.2. Oxidase Reagent

This reagent was prepared freshly in a dark bottle by dissolving 0.1 gm of tetramethyl *p*-phenyl diamine- dihydrochloride in 10 ml D.W.

3.2.1.3. Methyl Red Stain

It was prepared by dissolving 0.1 gm of 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.

3.2.1.4. Voges–Proskauer Reagent

The reagents were prepared as follows:

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Solution A (α-naphthol) : prepared by dissolving 5 gm of α-naphthol in 100 ml of ethanol.

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

Two drops of 40% KOH solution and 6 drops of 5% solution of α-naphthol were added to each test tube

3.2.1.5. Bromocresol Purple Stock Solution

This solution was prepared by dissolving 1 gm of the dye in 500 ml D.W.

3.2.1.6. Cresol Red Stock Solution

The solution was prepared by dissolving 1 gm of the cresol red in 500 ml D.W.

3.2.1.7. Gelatin Liquefaction Reagent

It was prepared by dissolving 5 gm of $HgCl_2$ in 20 ml of concentrated HCl, then the mixture was completed to 100 ml with D.W (Collee *et al*., 1996).

3.2.1.8. Aqueous Ferric Chloride Reagent

This solution was prepared by dissolving 12 gm of $FeCl_3$ in 2.5 ml of concentrated HCl and completed to 100 ml with D.W (MacFaddin, 2000).

3.2.2. Preparation of Culture Media

3.2.2.1. Ready-Made Culture 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 min. After sterilization, blood agar base was supplemented with 5% human blood after cooling the medium to 45°C, then poured into sterile Petri dishes, and urea agar base was supplemented with 20% sterile urea solution (sterilized by Millipore filters 0.22µm).

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3.2.2.2. Laboratory- Prepared Culture Media

3.2.2.2.1. Carbon Source Fermentation Medium

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

a) Basal Medium

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

The pH was adjusted to 7.4 and Durham tube was located at the bottom of each tube, then 5ml of media distributed into test tubes. The tubes were then autoclaved at 121˚C for 15 minutes.

b) Carbon Source Solutions

One percent of each of the following sugars was used; glucose, lactose, sucrose, L-arabinose, maltose, D-mannitol, D-mannose, D-sorbitol, D-xylose . All sugar solutions were sterilized by millipore filters (0.22µm), 0.1 ml of each sterile sugar solution was added to each tube containing medium base.

3.2.2.2.2. Moller's Decarboxylase Medium (MacFaddin, 2000)

The base of this medium was prepared by dissolving the following ingredients in 1000 ml of D.W, and the pH was adjusted to 6.0 before the addition of the indicators.

Peptone	5 gm
Beef extract	5 gm
Glucose	0.5 gm
Pyridoxal phosphate	5 gm
Bromocresol purple (3.2.1.5)	5 ml

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Cresol red (3.2.1.6)	2.5 ml

One percent of each L-ornithine, L-lysine and L-arginine hydrochloride was added separately to the base medium. The medium were distributed into test tubes (5ml each one) and autoclaved at 121°C for 15 min.

3.2.2.2.3. Phenylalanine Medium (MacFaddin, 2000)

The base of this medium was prepared by dissolving the following ingredients in 1000 ml of D.W.

DL- Phenylalanine	2gm
Yeast extract	3gm
NaCl	5gm
Na_2HPO_4	1gm
Agar-agar	12gm

The ingredients were dispensed (4 ml) into test tubes, autoclaved at 121°C for 15 min, and solidified in slanted position.

3.2.2.2.4. Motility Medium

It was prepared by dissolving 0.5 gm of agar-agar in 100 ml of brain-heart infusion broth, then contents were dispensed into test tubes (5ml each) and autoclaved at 121˚C for 15 min (MacFaddin, 2000).

3.2.2.2.5. Gelatin Agar Medium

It prepared by adding 4.4 % gelatin to the nutrient agar medium, autoclaved at 121˚C for 15 min and poured into sterile Petri dishes (Collee *et al*., 1996).

3.2.2.2.6. Maintenance Medium:

This medium consisted of nutrient broth as a basal medium, supplemented with 15% glycerol. After autoclaving at 121˚C for 15 min, and cooling to 56˚C in water bath, 5 ml aliquots were distributed in sterile tubes,

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and 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).

3.2.3. Preparation of Buffers and Solutions

Buffers and solutions which require sterilization, were autoclaved at 121 °C for 15 minutes. Millipore filters (0.22 µm) were used for sterilization of heat-sensitive solutions like antibiotics. pH of the solutions were adjusted using 1M NaOH or 1M HCl.

3.2.3.1. McFarland (0.5) Turbidity Standard

It was prepared by adding 0.5 ml of a 1.175% (w/v) $BaCl_2.2H_2O$ solution to 99.5 ml of 1 % (v/v) H_2SO_4. The McFarland standard tube were sealed with parafilm to prevent evaporation and stored for up to 6 months in the dark at room temperature. Accuracy of the density of a prepared 0.5 McFarland standard was checked by using a spectrophotometer. The absorbance of the wavelength of 625 nm should be between 0.08 and 0.1 (CLSI, 2010).

3.2.3.2. Normal Saline Solution

This solution was prepared by dissolving 0.85 gm of NaCl in 90 ml D.W. and further completed to 100 ml with D.W. Autoclaved at 121 °C for 15 minutes (Collee *et al*., 1996).

3.2.3.3. 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.

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Then 87.7 ml of solution A was added to 12.3 ml of solution B, and the pH was adjusted to 6 (Collee *et al.*, 1996).

3.2.3.4. β-lactam Antibiotic Solutions

β-lactam solutions were prepared as stock solutions with concentration of 10 mg/ml by dissolving 1gm of the following antibiotics: Amoxicillin, Ampicillin, Cloxacillin and Cephalothin in a small volume of sterile PBS (pH 6.0), or by dissolving in sterile D.W for cefotaxime, piperacillin and meropenem, while ceftazidime was solubilized in Na_2HCO_3 . Each of these solutions was further diluted with sterile D.W to volume 100 ml, and stored at 4˚C until use (CLSI, 2010).

3.2.3.5. Solutions of β-lactamase Detection

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

a) Pencillin G Solution: it was prepared by dissolving 0.569 gm of penicillin G in PBS. The solution was sterilized, dispensed in small vials and stored at -20˚C.

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

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

3.2.3.6. CHROMagar and Supplement Solution

The supplements were prepared according to the manufacturer recommendations by dissolving 57mg/ml of ESBL supplement and 40 mg/ml of KPC supplemen, separately, in sterile D.W., vortexed,

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homogenized and added in proportions of 10 ml/l of final melted orientation CHROMagar after cooled at 45°C, then poured in plates and used freshly.

3.2.3.7. EDTA Solution for Disks Preparation

This solution was prepared by complete dissolving of 190 mg of EDTA in 1ml of D.W. after pH was adjusted to 8, it was sterilized by autoclaving. EDTA solution was added to a 6-mm Whatmann filter No.1 disks (n=100) and allowed to dry. Each disk contained approximately 1900 µg of EDTA and used for detection of metallo-β-lactamases producing isolates (Lee *et al*., 2003).

3.2.3.8. Cloxacillin Disks (500µg) Preparation

These disks were prepared according to the method described by NCCLs (2007) with some modifications, as follows:

A Whattman No.1 filter paper was cut into 6 mm-diameter disks using paper clipper (n=100). These disks were autoclaved for 15 min at 121°C.

The stock solution of the antibiotic (50 mg/ml) was prepared by dissolving 500 mg of the antibiotic completely into 10 ml sterile D.W. Using micropipette, 10 µl of antibiotic stock solution was added to the blank disks. The disks were allowed to absorb the antibiotic for 30 min, then dried by placing them in an incubator at 40- 50°C for further 30 min. They were used immediately after preparation in AmpC β-lactamase detection.

3.2.3.9. DNA Extraction Solutions

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

a) 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.

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b) SDS Solution (25%)

Sodium dodecyl sulfate (25 gm) was dissolved in 100 ml of D.W., autoclaved and stored at 4˚C.

c) NaCl Solution (5 M)

Sodium chloride (14.625 gm) was dissolved in 50 ml D.W., autoclaved at 121°C for 15 min and stored at 4˚C.

d) Phenol: Chloroform: Isoamyl Alcohol (25:24:1)

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

3.2.3.10. Gel Electrophoresis Solutions

The solutions were prepared as described by Bartlett and Stirling (1998) as follows:

a) 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 at 121°C for 15 min, and stored at 4˚C.

b) Ethidium Bromide Solution

Stock solution was prepared by dissolving 0.005 gm of ethidium bromide in 10 ml of D.W., and stored in dark reagent bottle.

3.2.3.11. AmpC β-lactamase Extraction and Purification Buffers

a) PIPES Buffer (25mM)

This buffer was prepared by dissolving 0.025 M PIPES in 800 ml D.W., completed to one litter by D.W., and the pH was adjusted to 7.0 then sterilized by autoclaving at 121°C for 15 min before storing at 4˚C. This buffer was used for crud enzyme extraction and partially purification.

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b) Sodium Phosphate Buffer (0.05M)

This buffer was prepared by dissolving 7.2 gm of $NaH_2PO_4.2H_2O$ in 1000 ml of D.W. and the pH was adjusted to 7.0 .

c) Sodium Acetate 0.05M Buffer (pH 4-6)

This buffer was prepared by mixing 1.44 ml of glacial acetic acid with an amount of D.W., then volume was completed to 100 ml with D.W. and the pH was adjusted.

d) Tris Hydrochloride 0.05M Buffer (pH 6.5- 10)

This buffer was prepared by dissolving 0.6057 gm of tris-HCl in an amount of D.W. then completed to 100 ml of D.W., and the pH was adjusted.

3.2.3.12. Protein Assay Solutions

These solutions were prepared as described by Bradford (1976).

a) Comassie Brilliant Blue G-250 Solution

It was prepared by dissolving 0.1 gm of Comassie Brilliant blue G-250 in a mixture of 100 ml of phosphoric acid (85%) and 50 ml of ethanol (95%) in stirrer ice path. The volume was completed to one litter by D.W. and the solution was filtered through out the Whattman filter paper No.1.

b) NaOH Solution (1M)

This solution was prepared by dissolving 4 gm of NaOH in 50 ml of D.W. then volume was completed to 100 ml with D.W.

c) Serum Bovine Albumin (SBA) Stock Solution (2mg/ml)

This solution was prepared by dissolving 0.2 gm of SBA in a small volume of D.W., and completed to 100 ml. It was used as a stock solution.

3.2.4. Samples Collection

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A total of 1015 samples were collected during the period from April to July 2010. The samples were divided into two groups as following :

a) Clinical Samples

Seven hundreds and seventy clinical samples of sputum , urine and burn wounds were collected from patients visited/or admitted to different hospitals and laboratories in the Holly Najaf province included: Al-Sader Medical City , Al-Hakeem General Hospital, Al-Furat Teaching Hospital, Respiratory and Chest Diseases Center and Public Health Laboratory. Patients were tabulated according to their age, gender, hospitalization, antibiotic receiving and immunity state. Hence, the patients were considered immunsuppresed if they have certain underlying diseases such as malignancy, cirrhosis, renal failure, diabetes mellitus and burns (Tsai *et al.*, 2009).

b) Hospital Environmental Samples

A total 245 hospital environmental samples were screened randomly from different hospitals in the Holly Najaf province.

Types and numbers of clinical and hospital environmental samples are listed in Table (3-1).

Table (3-1): Types and numbers of clinical and hospital environmental samples were collected in the present study

Sample type	Source	No.
Clinical	Sputum	450
	Urine	210
	Burns wounds	110
	Total	770
Hospital environment e	Operation hall	30
	Intensive care unit (ICU)	20
	Birth auditorium	25
	Burns unit	30
	Wards	50

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	Kitchen	45
	Bathrooms	45
	Total	245

All samples were collected using sterile containers and transport swabs damped with normal saline.

3.2.5. Isolation and Identification of Bacterial Isolates

Suspected *Klebsiella* spp. isolates were recovered from clinical and hospital environmental samples after culturing on MacConkey agar and incubated for overnight at 37°C. This medium is specially made to distinguish lactose-fermenting (pink to red colonies) from non lactose fermenting bacteria (colorless or slightly beige). All lactose-fermenting isolates were subcultured and incubated for additional overnights. Suspected bacterial isolates which their cells are Gram negative and negative to oxidase which further identified by the following biochemical test according to Holt *et al*. (1994) and Baron and Finegold (1994), then reidentified by API system.

3.2.5.1. Biochemical Tests

3.2.5.1.1. Indole Production

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

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3.2.5.1.2. Methyl Red

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 and the result was read immediately. Changing the color to red indicates a positive test (MacFaddin, 2000).

3.2.5.1.3.Voges-Proskauer

Methyl red-Voges-Proskauer broth was inoculated with a young agar culture and incubated at 37˚C for 48 hr.. A positive reaction was indicated by the development of a pink color in 20 minutes (MacFaddin, 2000).

3.2.5.1.4. Simmons Citrate

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).

3.2.5.1.5. Triple Sugar Iron (TSI)

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 uniformatted 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

3.2.5.1.6. Urease

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

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days. Changing the color of medium to purple-pink indicate a positive result (MacFaddin, 2000).

3.2.5.1.7. Carbon Source Fermentation

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

3.2.5.1.8. Amino acid Decarboxylase

Moller's decarboxylase tubes containing L-lysine, L-ornithine and L-arginine (3.2.2.2.2) were inoculated with a straight wire, incubated and read daily for 4 days. The color change of the medium from yellow to violet indicated that decarboxylation occurred.

3.2.5.1.9. Phenylalanine Deaminase

Slants phenylalanine medium (3.2.2.2.3) were inoculated with a young agar culture by a straight wire, incubatedat 37°C for 24hr and added the reagent directly over the slant . The color change of the slant to light or deep green indicates a positive result.

3.2.5.1.10. Motility Test

Tubes containing motility medium (3.2.2.2.4) 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 bacterial motility (MacFaddin, 2000).

3.2.5.1.11. Gelatin Liquefaction

Gelatin agar (3.2.2.2.5) was streaked with bacterial culture and incubated at 37°C for 3-5 days. The plate was flooded with reagent (3.2.1.7) for 5-10 min. Appearance of clear zones around the growing colonies indicates production of gelatinase enzyme (Collee *et al.,* 1996).

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3.2.5.2. API 20E Multi Test System

The identified *K. pneumoniae* isolates were confirmed by API 20E system. This test was used according to manufacturer's protocol for *Enterobacteriaceae*. Wells of biochemical test were inoculated with overnight 0.5 McFarland bacterial suspension and incubated at 37°C for 24hr. The results were read after addition of reagents, as 7 digit number that identify by API 20E analytical index.

3.2.6. Preservation and Maintenance of Bacterial Isolates

The bacterial isolates were preserved on nutrient agar slant at 4˚C. The isolates were maintained monthly by reculturing on new medium. Nutrient broth supplemented with 15% glycerol was used for long preservation and the isolates were maintained frozen at -70 ºC (deep freeze) for several months (long term maintenance) (Collee *et al*., 1996).

3.2.7. Subculture of Preserved and Frozen Stock Cultures

Frozen stock cultures were sub-cultured on fresh blood agar plates, and then incubated in aerobic condition at 37°e for 24 hr (Thomas, 2007).

3.2.8. Screening Test for β-latam Resistance

Ampicillin and amoxicillin (β-latams) were added, separately, from the stock solution to the cooled Muller-Hinton agar at final concentrations of 100 and 50 µg/ml, respectively. The medium was poured into sterilized Petri dishes, then stored at 4˚C. Preliminary screening of *K. pneumoniae* isolates resistante to both antibiotics was carried out using pick and patch method on the above plates (NCCLs, 2003_b). Results were compared with *E.coli* ATCC 25922 as a negative control.

3.2.9. Production of β-lactamase

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All bacterial isolates that resisted the two β-lactam antibiotics (3.2.8) were tested for β-lactamase production by the two methods:

3.2.9.1. Rapid Iodometric Method

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 *et al.,* 1996).

3.2.9.2. Detection by Nitrocefin Disk

Number of required nitrocefin disks were placed into sterile empty Petri dish and these disks were moistened with one drop of sterile D.W.(not over moisten), then the disk was holed by sterile forceps and wiped a cross a young colony on agar plate. The development of a red color in the area of the disk where the culture was applied indicated positive result.

3.2.10. Antibiotic Susceptibility Testing

Antimicrobial susceptibility testing of β-lactam resistant *K.pneumoniae* isolates was carried out against the antibiotics shown in (3.1.4.1) using the disk diffusion method on Muller-Hinton agar medium. The cultures were incubated at 37°C for 18 hr under aerobic conditions based on the method of Barry (1976), and bacterial growth inhibition zones around the disks were measured and interpreted as recommended by the National Committee for Clinical Laboratories Standard guidelines. *E. coli* ATCC 25922 was used as the reference strain for antimicrobial susceptibility testing (CLSI, 2010).

3.2.11. Extended-Spectrum β-lactamase Production

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3.2.11.1. Initial Screening for ESBL Production

All bacterial isolates that were β-lactamase producing were tested for ESBL production by initial screen test. The isolate would be considered potential ESBL producer, if the inhibition zone of ceftazidime (30µg) disks was ≤ 22 mm (CLSI, 2010).

3.2.11.2. Confirmatory Test

All the β-lactamase producing isolates were tested also for confirmatory ESBL production by three methods, these tests were included:

a) Detection of ESBL by CHROMagar Technique

Extended spectrum β-lactamase CHROMgar plates were streaked in the same day of preparation by overnight growth of *K. pneumoniae*. The plates were incubated at 37°C for 24 hr according to manufacturer procedure. Growth of blue colonies indicated to ESBL producer. The reference strain of *E. coli* ATCC 25922 was inhibited and used as negative control.

b) Disk Combination Test (Recommended by CLSI, 2010)

The phenotypic confirmation of potential ESBL-producing isolates was performed by using disk diffusion method (3.2.10). Cefotaxime alone and in combination with clavulanic acid were tested. Inhibition zone of ≥ 5 mm increase in diameter for antibiotic tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL producing isolate (Cantarelli *et al*., 2007).

c) Disk Approximation Test

All β-lactamase producing isolates tested according to Batchoun *et al*. (2009). Antibiotic disks of cefotaxime (30µg), ceftazidime (30µg), ceftriaxione (30µg), and azetreonam (30µg) were placed 15 mm (edge to edge) around a central disk of amoxi-clav (20µg amoxicillin plus 10 µg clavulanate) on Muller-Hinton agar plates seeded with organism being tested

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for ESBL production. Plates were incubated aerobically at 37°C for 24 hr . 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. Detection of AmpC β-Lactamase

3.2.12.1. Initial Screening AmpC β-Lactamase (Cefoxitin Susceptibility)

All β-lactam resistant isolates were tested for cefoxitin susceptibility by using standard disk diffusion method (CLSI, 2010). The resistant isolates (≤18mm inhibition zone diameter) were consider as initially AmpC β-lactamase producers (Coudron *et al.,* 2003).

3.2.12.2. Confirmatory Tests of AmpC β-Lactamase

Bacterial isolates of β-lactam resistant were confirmatory tested for AmpC β-lactamase production by four methods. These tests included:

a) Modified Three Dimensional Test (MTDT)

This test was carried out according to Manchanda and Singh (2003) and Parveen *et al.*(2010) 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 min. Bacterial growth washed with normal saline 2 to 3 times. Crude enzyme extract was prepared by repeated freeze-thawing (approximately 15 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

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(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 min until the solution dried, and 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 isolates.

b) 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 37 ºC. 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 (Basak *et al.,* 2009; Parveen *et al.,* 2010).

c) Disk-Based Inhibitor Test

This test was carried out for detection of plasmid medited AmpC lactamases according to Rosco diagnostca company/ Denmark (2009) and applied by Ruppe *et al*. (2006) as follows:

Applied ceftazidime disk (30µg) and cefoxitin disk (30µg) each alone and cloxacillin (500µg) prepared disk (3.2.3.8) was placed between them at

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a distance of 5-10 mm edge to edge, on Muller-Hinton agar plate previously inoculated with a 0.5 McFarland bacterial suspension, and incubated for 24 hr at 37ºC. An isolate that demonstrated a defined increase in inhibition zone diameter between the cloxacillin disk with ceftazidime disk and/or cefoxitin disk was considered to be an plasmid medited AmpC producer.

d) Cetazidime-Imipenem Antagonism Test (CIAT)

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

Screening for the inducible AmpC β-lactamase was done by cetazidime-imipenem antagonism test (CIAT), which was consisted of an imipenem disk (10µg) 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.

3.2.13. Detection of Carbapenemases

a) Imipenem-EDTA Double Disk Synergy Test

Metallo-β-lactamase detection was performed by double disk synergy method according to Lee *et al*. (2003). A 10µg 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 (1900 µg) was placed at a distance of 15 mm center to center from the imipenem disk. The plate was incubated

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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.

b) Modified Hodge Test (MHT)

It was done as described by Lee *et al.* (2001). A 0.5 McFarland dilution (3.2.3.1) 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) min, imipenem disk (10µg) 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. Four organisms were tested on the same plate with one disk. MHT positive test has a clover leaf-like indentation of *E. coli* ATCC 25922 growing along the test organism growth streak within the disk inhibition zone. MHT negative test has no growth of *E. coli* ATCC 25922 along the test organism(CLSI, 2010).

c) Detection of KPC by CHROMagar Technique

Klebsiella pneumoniae carbapenemase CHROMagar plates were streaked in the same day of preparation by overnight growth of *K. pneumoniae*, and incubated at 37 ^{o}C for 24 hr according to manufacturer procedure. Growth of blue colonies indicated to suspected KPC producer. The reference strain of *E. coli* ATCC 25922 was inhibited and used as negative control in this test.

3.2.14. DNA extraction

Extraction of DNA from bacterial cells was performed by salting out method (Pospiech and Neumann, 1995) with some modification as follows: A loopfull of *K. pneumoniae* overnight growth were inoculated in 5 ml nutrient broth and incubated at 37°C for 24hr. The bacterial growth was centrifuged at 6000 rpm for 5min and the precipitate was rewashed twice in 5ml of TE buffer. Then the pellet was resuspended in 2.5 ml TE buffer. A

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volume of 300 µl of freshly made 25% SDS was added, mixed by inversion to the cell suspension, and incubated for 5 minutes at 55˚C. Then 1 ml of 5 M NaCl solution was added to the lysate, mixed thoroughly by inversion, and let to be cooled to 37˚C. A mixture of equal volume (3.8 ml) of [phenol: chloroform: isoamyl alcohol (25:24:1)] was added to the lysate and mixed by inversion for 30 min at room temperature. It was spun by centrifuge 4500 rpm for 15 min. Then the aqueous phase was transferred to a fresh tube, which contains nucleic acid. Isopropanol (0.6 of total volume) was added to the extract and mixed by inversion, after 3 min DNA spooled on to a sealed pasture pipette. DNA rinsed in 5 ml of 70% ethanol, air dried, and dissolved in 500µl TE buffer at 4 ^{o}C overnight, and the DNA extract was stored in freezer at -20°C until used.

3.2.15. Detection of *bla* Genes by Polymerase Chain Reaction

3.2.15.1. Preparing the Primers Suspension

The DNA primers were resuspended by dissolving the lyophilized product after spinning down briefly with TE buffer molecular grad depending on manufacturer instruction as stock suspension. Working primer tube was prepared by diluted with TE buffer molecular grad. The final picomoles depended on the procedure of each primer.

3.2.15.2. Polymerase Chain Reaction Protocols

a) Monoplex PCR Mixture

The DNA extract of *K. pneumoniae* isolates were subjected to *bla* genes listed in (3.1.7.3a) by monoplex PCR. There are two protocols used depending on manufacturer's instruction. All PCR components were assembled in PCR tube and mixed on ice bag under sterile condition as in Table (3-2).

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Table (3-2): Protocols of PCR reaction mixture volumes

b) Mutiplex PCR Mixture

All *bla*$_{AmpC}$ gene positive isolates were subjected to six genes family of plasmid mediated AmpC β-lactamase (PABL), by using Kappa 2G Fast multiplex PCR protocol. Single reaction (final reaction volume 25 µl) consisted of multiplex mix 2X 12.5 µl , plasmid mediated AmpC β-lactamase genes primers10µM (each one consist primer forward 0.5 µl and reverse 0.5 µl) , DNA template 5 µl and the reaction volume completed with 1.5µl PCR grade water. All materials were mixed in same PCR tube on ice bag under sterile condition.

3.2.15.3. PCR Thermocycling Conditions

The PCR tubes were placed on the PCR machine and the right PCR cycling program parameters conditions were installed as in Table (3-3).

PCR reaction mixture	Promega protocol (final volume 25µl)	Kappa protocol (final volume 20µl)
Master mix 2X	12.5 µl	10 µl
Primer forward (10µM)	2.5 µl	1 µl
Primer reverse (10µM)	2.5 µl	1 µl
DNA template	5 µl	5 µl
PCR grade water	2.5 µl	3 µl

3.2.16. Agarose Gel Electrophoresis

All requirements, technical and preparations of agarose gel electrophoresis for DNA detection and analysis were performed by Bartlett and Stirling (1998).

3.2.16.1. Preparation of Agarose Gel and DNA Loading

Agarose gel was prepared by adding 1.5 gm of agarose powder to 100 ml of TBE buffer previously prepared (90 ml D.W. were added to 10 ml

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TBE buffer l0X, the final concentration was 1 X and pH 8). The mixture was placed in boiling water bath until it become clear, then allowed to cool to 50°C, and ethidium bromide at concentration of 0.5 mg/ml was added.

The agarose poured kindly in equilibrated gel tray earlier set with two combs fixed in the end and in the middle, and the two ends of gel tray were sealed. The agarose allowed solidifying at room temperature for 30 min. The combs and the seal were removed gently from the tray. The comb made wells used for loading DNA samples.

Table (3-3): Programs of PCR thermocycling conditions

Monoplex gene	Temperature (^{o}C)/ Time					Cycle number
	Initial denaturation	Cycling condition			Final extension	
		denaturation	annealing	extension		
***Bla*$_{AmpC}$**	94/30 sec	94/30 sec	60/1 min	72/1 min	72/10 min	35
***bla*$_{CTX-M}$**	94/30 sec	94/30 sec	60/1 min	72/1 min	72/10 min	35
***bla*$_{TEM}$**	94/30 sec	94/30 sec	45/1 min	72/1 min	72/10 min	35
***bla*$_{SHV}$**	94/30 sec	94/30 sec	60/1 min	72/1 min	72/10 min	35
***bla*$_{OXA}$**	94/5 min	94/50 sec	55/50 sec	72/1 min	72/5 min	30
***bla*$_{VEB}$**	93/3 min	93/1 min	55/1 min	72/1 min	72/7	40
***bla*$_{GES}$**	93/3 min	93/1 min	55/1 min	72/1 min	72/7	40
***bla*$_{PER}$**	93/3 min	93/1 min	55/1 min	72/1 min	72/7	40
***bla*$_{IMP}$**	93/3 min	93/1 min	55/1 min	72/1 min	72/7	40
***bla*$_{VIM}$**	93/3 min	93/1 min	55/1 min	72/1 min	72/7	40
***bla*$_{KPC}$**	95/15 min	94/1 min	62/1 min	72/1 min	72/10 min	38
Multiplex gene						
***bla*$_{FOX}$** ***bla*$_{CIT}$**	94/3 min	94/30 sec	64/30 sec	72/1 min	72/7 min	25

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bla_{DHA} bla_{EBC} bla_{ACC} bla_{MOX}						

Five microliters of amplified PCR product were loaded to the agarose gel wells followed by DNA marker (ladder) to one of the wells. The gel tray was fixed in electrophoresis chamber and IX TBE buffer was added to the chamber until covered the surface of the gel. The electric current was performed at 70 volt for 1.5-2 hr.

3.2.16.2. Agarose Gel Documentation

The amplified PCR products were detected by agarose gel electrophoresis was visualized by staining with ethidium bromide. The electrophoresis result was detected by using gel documentation system. The positive results were distinguished when the DNA band base pairs of sample equal to the target product size. Finally, the gel was photographed using Biometra gel documentation system.

3.2.17. Determination of MICs of bla_{AmpC} Positive Isolates

The two-fold agar dilution susceptibility method was used for determination of MICs of β-lactam antibiotics (3.1.4.2). Preparation of β-lactam antibiotic stock solutions, dilutions and suggested ranges of MICs were prepared according to Andrews (2001), in which one part of the antimicrobial solution was added to nine parts of liquid Muller-Hinton agar (Table 3-4). 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 Petri dishes. The agar was

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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. One microlitter aliquot of each inoculum was applied to the agar surface with standardized loop. Antibiotic free media were used as negative controls . The inoculated plates were allowed to stand at room temperature (for no more than 30 min) until the moisture in the inoculum spots was absorbed by the agar. The plates were inverted and incubated at 37 °C for 20 hr. To determine MICs, 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 (the growth of one or two colonies or a fine film growth should be disregarded) or that concentration (µg/ml) at which no more than two colonies were detected.

Table (3-4): Schematic table for preparing dilutions of antimicrobial agents to be used in agar dilution susceptibility test (CLSI, 2010)

Antimicrobial solution						
Step	**Concen-tration µg/ml**	**Source**	**Volume +**	***D.W =**	**Intermediate Concentration =**	**Final concentration at 1:10 dilution in agar**
1	5120	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

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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

***D.W: Distilled water**

The MIC were compared with the break points recommended by CLSI (2010).

3.2.18. Enzymatic Description of AmpC β-Lactamase

3.2.18.1. Assay of AmpC β-Lactamase Activity

AmpC β-lactamase activity was measured depending on β-lactamase assay kit (nitrocefin substituted kit) procedure which based on spectro-iodometric method according to Sawai *et al.* (1978) as follows:

The kit reagents consist of starch solution, starch iodine solution, buffer solution and penicillin G solution. After mixing all reagents in fresh tube, the sample of enzyme was added, incubated at 37°C for 5 min. Finally the mixture absorbance was read at 620 nm. The calculation of enzyme activity as follows:

From definition of activity, one unit of enzyme is the amount of enzyme that catalysis the reaction of 1 μmol of substrate (penicillin G) per min. The activity (unit/ml) is given by the following equation: Activity (U/ml) = A/ (t) x 1.2 x 0.3

Where:-

A= absorbance

1.2= absorbance without enzyme

0.3= weight of iodine equivalent in assay 30 nmol of substrate

t= time (5 min)

3.2.18.2. Assay of Protein Concentration

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Bradford (1976) method was used in protein assay in crude and purified enzyme extract as following:

a) Preparing of Standard SBA Protein Curve

Stock solution of SBA (2 mg/ml) was prepared as in (3.2.3.12.c). Then gradient concentrations of SBA stock solution were prepared (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 µg/ml).

In fresh tubes, distributed 10 µl from each concentration, and added 250 µl NaOH (1M) with 5 ml of Comassie brilliant blue G-250 solution (prepared previously in 3.2.3.12.1), mixed well and let 5 min in room temperature. Then the absorbance was read at 595 nm (assayed in replicate for each concentration). The blank tube consisted of the same component excepted SBA solution. The standard curve was drawn between SBA concentrations (µg/ml) and absorbance.

b) Protein assay in Enzyme Extractions

To assay the protein concentration in enzyme extractions, Bradford method was followed, but SBA was replaced by enzyme extraction sample.

3.2.18.3. Extraction and Purification of AmpC β-Lactamase

3.2.18.3.1. Extraction of Crude Enzyme

The AmpC crude enzyme extraction was performed according to Payne and Farmer (1998) as following:

Overnight growth of selected isolate was inculcated with 1% inoculums in 100 ml of 2X nutrient broth and incubated at 37°C until the culture had an optical density approximately 0.7 at absorbance wave length 500 nm. Then meropenem antibiotic was added to culture as enzyme inducer

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(Yang *et al.,* 1988; Jacoby, 2009) at final concentration of 1/4 the MIC of the particular isolate. The culture was further incubated at 37°C for 3 hr. Bacterial cells were harvested by centrifuged the culture at 6000 rpm for 15min. at 4°C, then the pellet was resuspended and washed in 10 ml of previously prepared PIPES buffer pH 7.0 (3.2.3.11.a). The suspension was recentrifuged at 6000 rpm for 15 min at 4°C, for the best result. The pellet was resuspended 5 ml of PIPES buffer and maintained on ice. The last suspension was sonicated on ice to disrupt bacterial cell walls (intermittent exposure of 5 x 30 seconds, separated by 1min cooling period). The cell free lysate was subjected to high speed cooled centrifugation at 14000 rpm for 2 min at 4 °C to precipitate cell debris, and the supernatant containing AmpC β-lactamases were transferred to fresh tube. The crude enzyme was estimated by added 10µl of extract to nitrocefin disk (3.2.9.2) and the color changing from yellow to red indicated to presence of enzyme within few seconds.

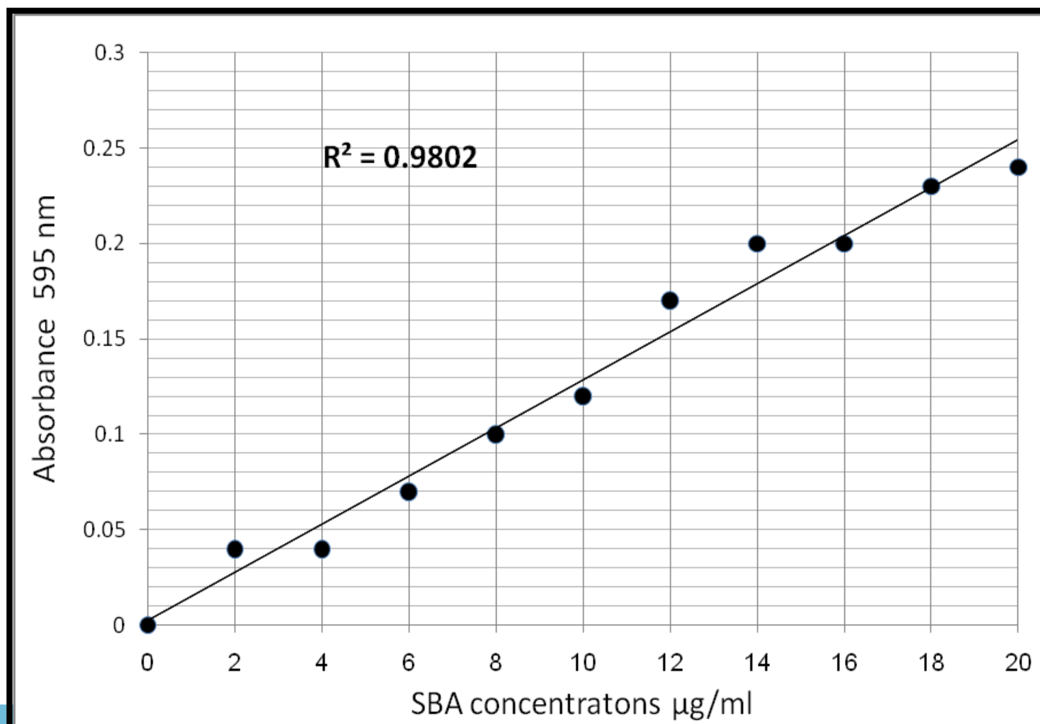

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Figure (3-1): Standard curve of SBA concentrations.

Then the enzyme activity and protein concentration were measured and the crude enzyme was stored at -20 until use.

3.2.18.3.2. Partially Purification of Enzyme

The extracted crude enzyme was partially purified in the following steps according to Yang and Livermore (1988); Conzalez Leiza *et al.* (1994) and Castillo *et al.* (2001) as follows:

3.2.18.3.2.1. Precipitation by Ammonium Sulfate

The solid ammonium sulphate was added (6.5 gm) gradually with 30% saturation to the extracted crude enzyme with continued stirred in flask that placed on ice container for 1hr, and centrifuged at 6000 rpm for 20 min at 4°C. The precipitate was neglected and the solid ammonium sulphate (7.1 gm) was added to the supernatant with 75% saturation under the same condition and centrifuged at 6000 rpm for 20 min at 4°C. After storage at 4 °C overnight, the pellet was resuspended in small volume of sodium phosphate buffer, pH 7.0 and dialysed against the same buffer for several times at 4°C for 24 hr. The enzyme activity and protein concentration were measured.

3.2.18.3.2.2. Gel Filtration Chromatography

The dialyzed extract was fractionated on a Sephadex G75 gel. The equilibration and elution were carried out with sodium phosphate buffer pH 7.0 as prepared in (3.2.3.11.b).

a) Setting of Sephadex G75 Gel Column

The sephadex G75 gel was prepared depending on manufacturer instruction. Sephadex gel was suspended in 0.05M sodium phosphate buffer

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pH 7.0 on stirrer hot plate to swell beads, cooled and poured kindly for degassing in separation column with dimension (1.5 x 45 cm). Then the column was equilibrated by the same buffer at a flow rate of 12 ml/hr.

b) Separation of Preparing Sample

The dialyzed enzyme extract that prepared in (3.2.18.3.2.1)**,** was added gradually on the gel surface. The elution was carried out with 0.05M sodium phosphate buffer pH 7.0 at a flow rate of 12 ml/hr. The eluted fractionated size was 3 ml for each fraction, and the collected fractions were scanned by measurement of absorbance at (280) nm. The fractions of highest enzyme activity in the certain pike were collected and measured their size, protein concentration and enzyme activity were assayed .

3.2.18.4. Description of AmpC β-Lactamase

a) Determination of Optimum pH for Enzyme Activity

Prepared 0.05M sodium acetate buffer for pH 4-6, and 0.05M tris hydrochloride buffer for pH 6.5- 10 as in (3.2.3.11.c) and (3.2.3.11.d), the activity of partial purified AmpC β-lactamase was measured by the same procedure in (3.2.18.1). The relationship between the pH values and activity was drawn to determine the optimum pH of enzyme activity.

b) Determination of Optimum Temperature for Enzyme Activity

Tris hydrochloride buffer 0.05M pH 7.5 was used in measurement of partial purified AmpC β-lactamase activity under different temperatures conditions. The same procedure of activity was used (3.2.18.1), but the tubes were incubated in different temperatures 25-80 ^{o}C for 10 min. The enzyme was added and incubated in same temperatures for 5 min. The relationship between the temperatures and activity was drawn to determine the optimum temperatures of enzyme activity.

c). Determination of Kinetic Properties

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Serial twofold concentrations (0.25-32 mM) of penicillin G, cefotaxime (Sawai *et al.,* 1978) and meropenem were prepared as different substrates. Tris-HCl buffer pH 7.5 was used in measurement of AmpC β-lactamase activity as in (3.2.18.1). The Lineweaver-Burk reciprocal plot method was depended to determine the kinetic parameters in which comprise K_m , V_{max} relative V_{max} and hydrolysis efficiency (relative). However, the kinetic experiments were performed at 37°C and pH 7.5. Each test were repeated three times and using the following equations (Conzalez Leiza *et al.,* 1994):

$$\frac{1}{V} = \frac{K_m}{V_{max}}\left(\frac{1}{[S]}\right) + \frac{1}{V_{max}}$$

$$\text{Relative } V_{max} = \frac{V_{max\,(Antibiotic)}}{V_{max\,(Penicillin)}} \times 100$$

$$\textbf{Hydrolysis effeciency} = \frac{V_{max}/K_m\,(\text{Antibiotic})}{V_{max}/K_m\,(\text{Penicillin})} \text{x}100$$

3.2.19. Statistical Analysis

The Chi-square test and ANOVA analysis for calculating the least significant difference (L.S.D.) test were applied to determine the statistical significance of the data. *P* value of <0.05 was considered significant. L.S.D. values were compared with values of means difference (Al-Rawi and Khalaf Allah, 2000)

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4.1. Isolation and Identification of Bacterial Isolates

The bacterial isolates obtained as a pure and predominant growth from the 770 clinical samples were only considered for the present study. Sputa were considered acceptable for culturing if they contained more than 25 polymorphonuclear cells and less than 25 epithelial cells per low-power field of the compound light microscope. Positive bacterial urine cultures was detected with the presence $\geq 10^5$ cfu/ml. Initially, isolates were examined for colony characterization after culturing on MacConkey agar and incubated for 24 hr at 37°C. Then microscopically by Gram's staining. Based on the morphological and staining behaviors of *Klebsiella* spp. (eg. isolates those exhibiting mucoid colonies and Gram-negative rods), and biochemical characterization revealed that only 217 (21.4%) isolates were belonged to *Klebsiella* spp. Of these 182 (23.6%) isolates were detected in 770 clinical samples and 35 (14.3%) isolates were recovered from hospital environmental samples. However, the frequency of the *Klebsiella* spp. isolates and their sites of isolation are listed in Table (4-1). It was found that 130 (12.8%) isolates were specified as *K. pneumoniae*.

The results of Table (4-1) showed that out of 770 clinical samples collected during the study period, 108 (14.0%) *K. pneumoniae* isolates were recovered. While from the 245 hospital environmental samples, 22 (9.0%) isolates were found to be *K. pneumoniae*. However, out of 327 Gram-negative isolates identified, 286 (37.1%) and 41 (16.7%) were recovered from clinical and hospital environment samples, respectively.

A total of 471(46.4%) samples (302 clinical samples and from 169 hospital environmental) showed no existence any bacterial growth . The subspecies level identification of *K. pneumoniae* isolates was then carried out by the standard biochemical test according to the Bergey's Manual of

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Determinative Bacteriology (Holt *et al.,* 1994) and confirmed by API 20E system (Appendix 1 and 2).

Table (4-1): Types and numbers of bacterial isolates obtained from samples of three Najaf hospitals

Source of sample	No.	No.(%) of *Klebsiella* spp. isolates	No. (%) of *K. pneumoniae* isolates	No. (%) of other Gram (-) isolates	No. (%) of No growth cultures
Clinical sample	770	182(23.6%)	108 (14.0%)	286(37.1%)	302(39.2%)
Hospital environment sample	245	35(14.3%)	22 (9.0%)	41(16.7%)	169(69.0%)
Total	1015	217 (21.4%)	130 (12.8%)	327(32.2%)	471(46.4%)

Out of 130 *K. pneumoniae* recovered during the study period, 111 (85.4%) isolates were belonged to *K. pneumoniae* subsp. *pneumoniae* and 19 (14.6%) isolates were found to belong to *K. pneumoniae* subsp. *ozaenae* (Figure 4-1). *K. pneumoniae* subsp. *rhinoscleromatis* was not detected in this study.

Table (4-2) shows that *K. pneumoniae* subsp. *pneumoniae* was the most frequent subspecies of *K. pneumoniae* isolated from the 108 clinical samples 96 (88.9%).

However, 65 (14.4%) were obtained from sputum, 17 (8.1%) from urine and 14 (12.7%) from burn wound samples. Out of the 108 *K. pneumoniae* isolates, 12 (11.1%) were identified as *K. pneumoniae* subsp. *ozaenae*. (7 from sputum, 3 from burn wound and 2 from urine).

In the same manner, table (4-3) shows the distribution of 22 *K. pneumoniae* subspecies among hospital environmental samples. It was

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found that 15 (68.2%) isolates were belonged to *K. pneumoniae* subsp. *pneumoniae*.

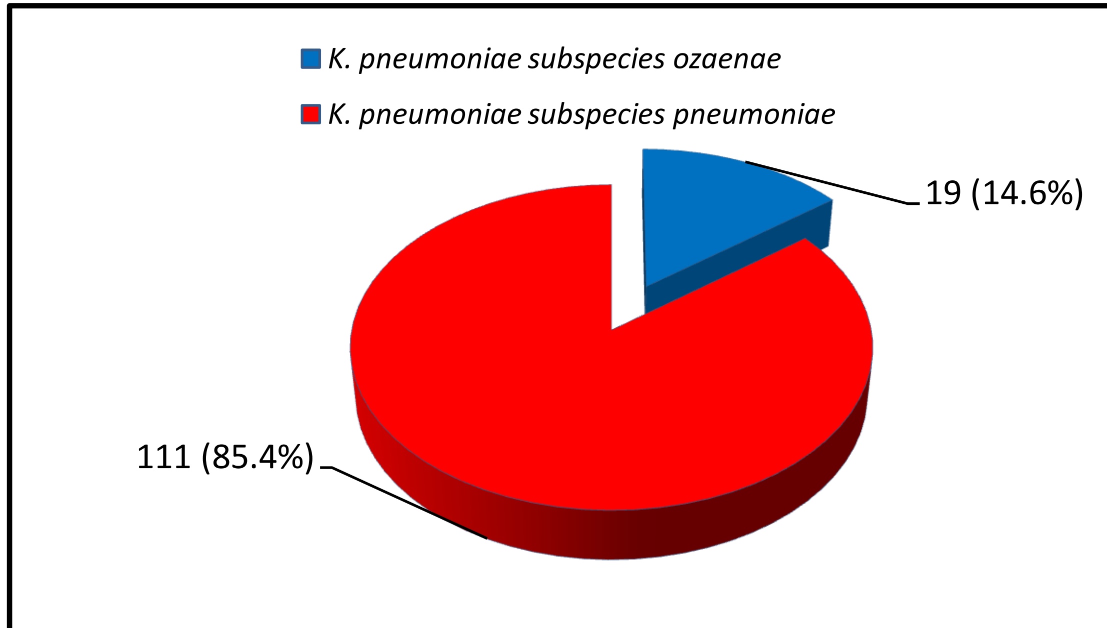

Figure (4-1): *Klebsiella pneumoniae* subspecies isolated from clinical and hospital environmental samples (n=130).

Three (6.0%) were isolated from wards, 6 (13.3%) were isolated from kitchen and 6 (13.3%) from bathrooms. Results also showed that 7 (31.8%) isolates were belonged to *K. pneumoniae* subsp. *ozaenae*.

Table (4-2): Distribution of *K. pneumoniae* subspecies among different clinical samples

Clinical sample	**No. of samples**	**No. (%) of *K. pneumoniae* isolates**	**No. (%) of *K. pneumoniae* subspecies**	
			K. pneumoniae subsp. ***pneumoniae***	***K. pneumoniae*** subsp. ***ozaenae***
Sputum	450	72 (16.0%)	65 (14.4%)	7(1.6%)
Urine	210	19 (9.5%)	17 (8.1%)	2(1.0%)
Burn wound	110	17 (15.5%)	14 (12.7%)	3(2.7%)
Total	770	108 (14.0%)	96(88.9%)	12(11.1%)

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One (4.0%) was isolated from birth hall, 4 (8.9%) from kitchen and 2 (4.4%) from bathrooms. Adversely, no *K. pneumoniae* isolates were recovered from samples of operation halls, intensive care units and burn unit.

Table (4-3): Distribution of *Klebsiella pneumoniae* subspecies among hospital environment samples

Hospital environmental sample	No. of samples	No. of *K. pneumoniae* isolates	No. (%) of *K pneumoniae* subspecies	
			K. pneumoniae subsp. ***pneumoniae***	***K. pneumoniae*** subsp. ***ozaenae***
Operation hall	30	0	0 (0%)	0 (0%)
ICU*	20	0	0 (0%)	0 (0%)
Birth hall	25	1	0 (0%)	1 (4.0%)
Burns unit	30	0	0 (0%)	0 (0%)
Wards	50	3	3(6.0%)	0 (0%)
Kitchen	45	10	6 (13.3%)	4 (8.9%)
Bathrooms	45	8	6 (13.3%)	2 (4.4%)
Total	245	22	15 (68.1%)	7 (31.8%)

***ICU: intensive care unit.**

4.2. Primary Detection of β-lactam Resistant Phenotype

All the 130 *K. pneumoniae* isolates were primarily screened for β-lactams resistance by growing on Muller-Hinton agar supplemented with ampicillin and amoxicillin (each alone) at final concentrations of 100 and 50 µg/ml, separately. Results of Table (4-4) show that a total of 103(79.2%) of β-lactam resistant isolates were grown normally in presence of ampicillin and amoxicillin. The table shows that 95/108 (88.0%) and 8/22 (36.4%) β-lactam resistant isolates were obtained from clinical and hospital environmental samples, respectively.

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Table (4-4): β-lactam resistant *Klebsiella pneumoniae* isolates obtained from clinical and hospital environmental samples

Source of sample	No. of *K. pneumoniae* isolates	No. (%) of isolates resistant to ampicillin and amoxicillin
Clinical sample	108	95 (88.0%)
Hospital environmental sample	22	8 (36.4%)
Total	130	103 (79.2%)

4.3. Antibiotic Susceptibility of *K. pneumoniae* Isolates

All the 103 β-lactam resistant *K. pneumoniae* isolates were tested for their antibiotic susceptibility against the selected 26 antibiotics.

Figure (4-2) shows that the resistant effect of isolates to carboxypenecillin including (carbenicillin and ticarcillin) are different with rates of resistance of 100% and 78.6%, respectively. Whereas 92.2% of the isolates were resistant to piperacillin. Such results of this study highlight serious alarming situation of highly diverse antibiotics resistance rates (60-100%) against cephalosporin generations (first, second, third and fourth). The study also revealed that there were a high resistant rates for amoxiclave (73.8%) and cefoxitin (70.9%). Most isolates were resistant to aztreonam antibiotic (60%), and the most effective β-lactam antibiotics imipenem and meropenem (3.9% resistance, each).

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High percentage rates of resistance to aminoglycoside, gentamicin, tobramycin and amikacin were detected (50, 48.5 and 30.8%), respectively.

The most active quinolones against all tested *K. pneumoniae* levofloxacin (51.5%) followed by ciprofloxacin (50%) and gatifloxacin (50%); while the least active were norofloxacin (46.2%) and naldixic acid (48.5%).

The resistance rate of isolates to the remaining antibiotics was as follows: tetracycline (63.1%), nitrofurantoin (59.2%), chloramphenicol (46.6%), and trimethoprim (42.3%). Results of susceptibility revealed that non of the isolates was fully resistant or susceptible to antibiotics tested. Moreover, any tested isolates resistant to a minimum of 3 classes of antibiotics was considered to be a multidrug resistant.

4.4. Detection of β-lactamase Producing Isolates

All the 103 *K. pneumoniae* isolates resistant to β-lactam antibiotics were tested for ability to produce β-lactamase enzyme Results are described in each of the two methods used.

4.4.1. Production of β-Lactamase by Rapid Iodometric

Results revealed that more than half β-lactam resistance *K. pneumoniae* isolates 63 (61.2%) were able to produce β-lactamase (Table 4-5). Of they 58(61.1%) were recovered from clinical samples and 5(62.5%) were from hospital environments. However, 40 (38.8%) of the isolates gave negative results.

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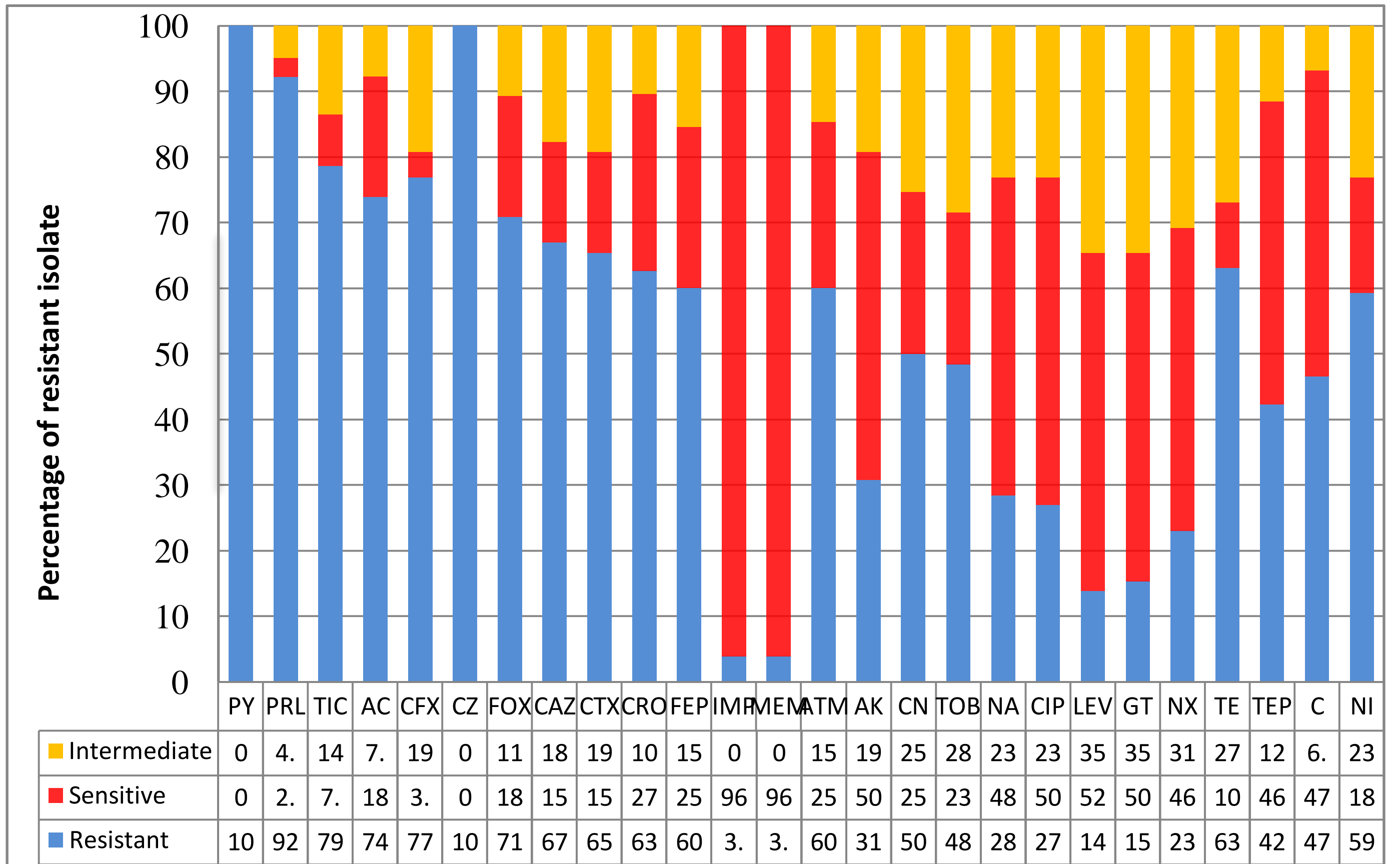

Figure (4-2): Antibiotics susceptibility profile of β–lactam resistant *Klebsiella pneumoniae* isolates by disk diffusion method (n=103). PY, Carbenicillin; PRL, Piperacillin; TIC, Ticarcillin; AC, Amoxi-clav; CFX, Cefexime; CZ,Cefazolin; FOX, Cefoxitin; CAZ, Ceftazidime; CTX, Cefotaxime; CRO, Ceftriaxone; FEP, Cefepime; IMP, Imipenem; MEM, Meropenem; ATM, Aztreonam; AK, Amikacin; CN, Gantamycin; TOB, Tobramycin; NA, Nalidixic acid ;CIP, Ciprofloxacin; LEV, Levofloxacin; GT, Gatifloxacin; NX, Norofloxacin; TE, Tetracyclin; TEP, Trimethoprime; C, Chloramphenicol;

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Table (4-5): Number and percentage of β-lactamases producing *Klebsiella pneumoniae* isolates by rapid iodometric method

Source of sample	No. β-lactam resistant *K. pneumoniae* isolates	No. (%) of positive isolates	No. (%) of negative isolates
Clinical sample	95	58(61.1%)	37(38.9%)
Hospital environmental sample	8	5(62.5%)	3(37.5%)
Total	103	63(61.2%)	40(38.8%)

4.4.2. Production of β-Lactamase by Nitrocefin Disk

For confirmatory purpose, presence of β-lactamase in the β-lactam resistance *K. pneumoniae* isolates was performed by the nitrocefin disk method. Among 103 isolates tested, 78 (75.7%) produced β-lactamase, by changing color of the nitrocefin disk from yellow to reddish-orange within few seconds (Table 4-6 and Figure 4-3)From these 70 (73.7%) isolates were from clinical samples, while all of hospital environmental samples were β-lactamase producers.

Table (4-6): Number and percentage of β-lactamases producing *Klebsiella pneumoniae* isolates by nitrocefin disk method

Type of sample	No. β-lactam resistant *K. pneumoniae* isolates	No. (%) of positive isolates	No. (%) of negative isolates
Clinical sample	95	70(73.7%)	25 (26.3%)
Hospital environmental sample	8	8 (100%)	0 (0%)
Total	103	78 (75.7%)	25(24.3%)
L.S.D. (0.05) = 3.413			

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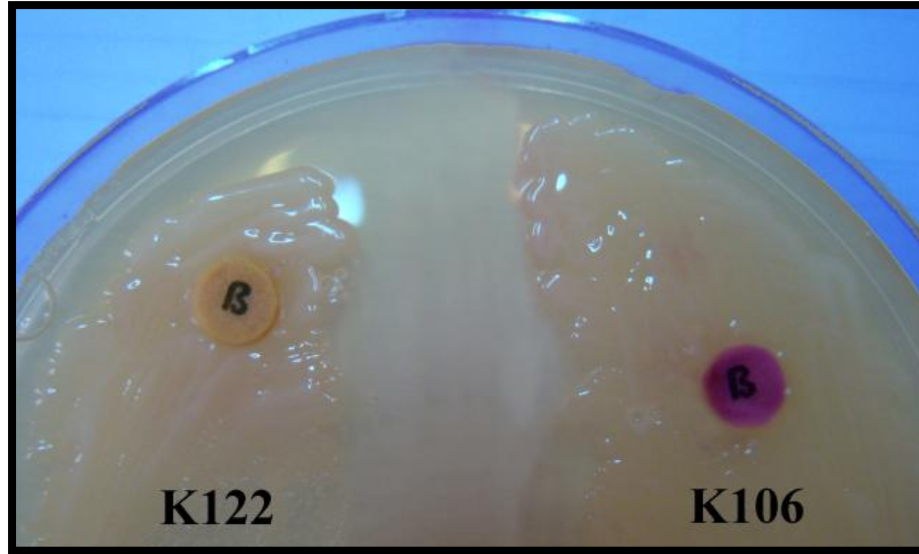

Figure (4-3): β-lactamase detection by nitrocefin disk method; *Klebsiella pneumoniae* isolate symboled K106 exhibited positive test (red disk), while K122 exhibited negative test (yellow disk).

The above results revealed that β-lactamase production in *K. pneumoniae* isolates by nitrocefin method was significantly high ($P < 0.05$), and none of the remaining 25 (24.3%) β-lactam resistant isolates exhibited β-lactamase activity.

4.5. Production of Extended-Spectrum β-lactamases (ESBL)

Of all the 130 *K. pneumoniae* isolates included in this study, a total of 78 (60.0%) isolates were screened and verified for ESBL production according to the Clinical and Laboratory Standard Institute (CLSI) criteria. All these isolates were β-lactam resistance and able to produce β-lactamase enzyme as mentioned above. Detection of ESBL producing isolates were based on their phenotypical properties. In this work, functionality of the disk diffusion, CHROMagar, disk combination and disk approximation methods were evaluated.

Performance of the test isolates in the ESBL initial screen disk test was assessed using ceftazidime disks. According to the CLSI, the isolate is considered to be a potential ESBL producers, if the inhibition zone of

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ceftazidime disks (30 µg) was ≤ 22 mm. The study found that 76 (97.4%) of the 78 β-lactamase-producing *K. pneumoniae* isolates were ESBL positive during the initial screening (Table 4-7), which considered as significant potential ESBL ($P < 0.05$). The majority of the suspected ESBL-producing isolates were found to be from clinical origin compared to only 6 (75%) of the 8 hospital environment isolates.

A positive result from initial screening was followed up with a phenotypic confirmatory tests. Results revealed that, ESBL-production was verified with the three different methods (Table 4-8). From CHROMagar confirmatory test of ESBL-producing isolates, only 37 (47.4%) of the 78 isolates of β-lactamase producers mostly suspected ESBL producers, gave positive result. All these isolates showed overnight growth with blue colonies on the ESBL supplemented CHROMagar orientation medium (Figure 4-4). Of which, 32 (45.7%) isolates were detected in the 70 clinical samples and 5 (62.5%) the in 8 hospital environmental samples with obvious significant differences at ($P < 0.05$) (Table 4-8).

In the disk combination method was cefotaxime disk was combined with clavulanic acid and compared with cefotaxime disk alone. The isolate was considered ESBL producer, when the inhibition zone of combined disks more than or equal to 5 mm increased than inhibition zone of disk alone (Figure 4-5).

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Table (4-7): Initial screening for potential ESBL production by *Klebsiella pneumoniae* isolates obtained from clinical and hospital environmental samples

Source of sample	No. β-lactamase-producing *K. pneumoniae* isolates	No. and (%) Suspected ESBL* of producing *K. pneumoniae* isolates
Clinical sample	70	70 (100%)*
Hospital environmental sample	8	6 (75%)
Total	78	76 (97.4%)
* χ^2 is significant in ($P<0.05$)		

*ESBL: extended spectrum β-lactamase

Table (4-8): Frequency of phenotypic ESBL producing *Klebsiella pneumoniae* isolates by three confirmatory methods

Source of sample	No. β-lactamase producing *K. pneumoniae* isolates	Number and (%) of isolates by:		
		ESBL CHROMagar technique	disk combination method (CTC/CTX)*	Disk approximation method
Clinical sample	70	32 (45.7%)	32 (45.7%)	0 (0%)
Hospital environmental sample	8	5 (62.5.%)	3 (37.5%)	0 (0%)
Total	78	37 (47.4%)	35 (44.8%)	0 (0%)
L.S.D. (0.05) of Samples = 3.419, Methods = 3.172, Interaction = 4.154				

*CTC: cefotaxime-clavulanic acid disk (30/10 µg) / CTX: cefotaxime disk (30µg). *ESBL: extended spectrum β-lactamase.

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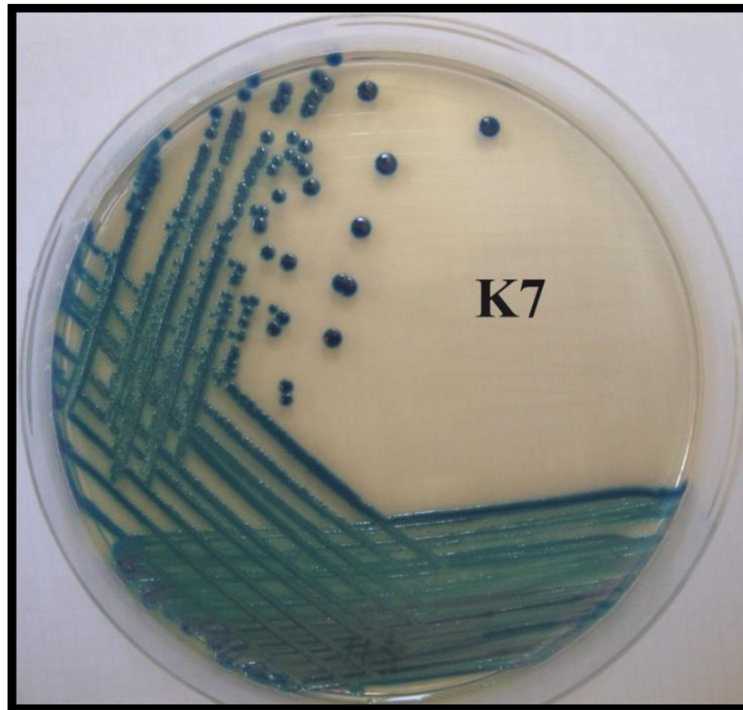

Figure (4-4): Appearance of ESBL production by *Klebsiella pneumoniae* K7 isolate after incubation at 37°C for 24hr. Deep blue colonies appear on the ESBL supplemented CHROMagar medium.

Results in this regard showed that out of the 78 *K. pneumoniae* isolates β-lactamase producers, 35 (44.8%) exhibited zones enhancement with clavulanic acid, confirming their ESBL production. Of these, 32 (45.7) isolates were of clinical samples and 3 (37.5%) of hospital environmental samples (Table 4-8).

ESBL production was also carried out by the disk approximation method in which augmentation of the inhibition zone between a 30μg antibiotic disks (ceftazidime, ceftriaxone, cefotaxime and aztreonam) toward amoxicillin-clavulanate disk (20/10μg), was interpreted as synergy, indicating the presence of an ESBL. Same table (4-8), revealed that no ESBL producing isolates was confirmed by this method in both clinical and hospital environmental samples.

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Eventually, in spite of CHROMagar technique was recorded a high percent of ESBL detection. This study noticed that clear indicative differences ($P<0.05$) among all previous confirmatory methods.

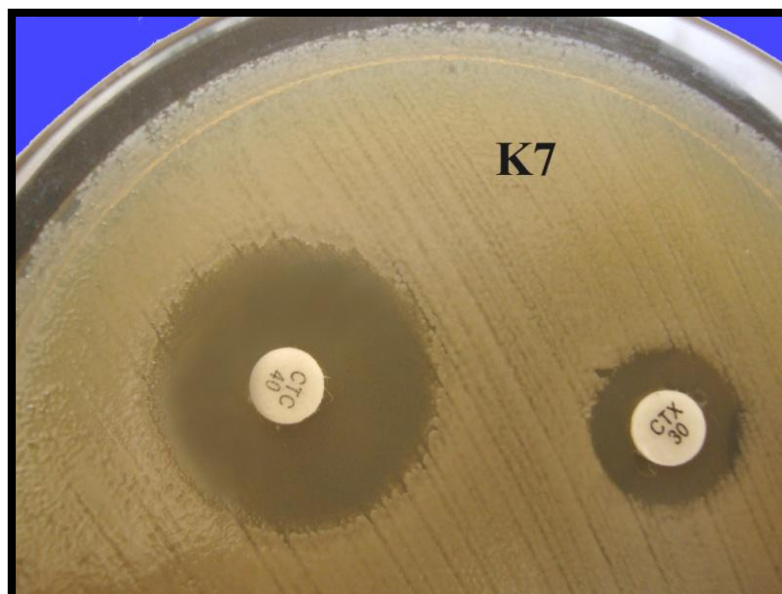

Figure (4-5): Disk combination test exhibiting ESBL *Klebsiella pneumoniae* K7 isolate with a significant inhibition zone difference(>5 mm) between cefotaxim-clavulante (CTC) on left side and cefotaxim alone (CTX) on right side. Plate was incubated at 37°C for 24hr.

4.6. AmpC-Mediated β-Lactams Resistance

Four methods were applied to detect production of AmpC β-lactamases in the β-lactam resistant *K. pneumoniae* isolates. Results confirmed that 73 (70.9%) out of the 103 β-lactam resistant *K. pneumoniae* isolates tested for cefoxitin susceptibility by the standard Kirby-Bauer disk diffusion method. Yielded cefoxitin zone diameter less than 18 mm. According to Clinical and Laboratory Standards Institute recommendation, such resistant isolates were significantly ($P < 0.05$) suspected as AmpC β-lactamase producers. Cefoxitin resistant isolates were as probable AmpC producers for the purposes of this study. The phenotypic data generated in this study indicated that 19 (18.4%) and 11

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(10.7%) of the remaining isolates were phenotypically confirmed to be cefoxitin susceptible and intermediate resistance, respectively (Figure 4-6).

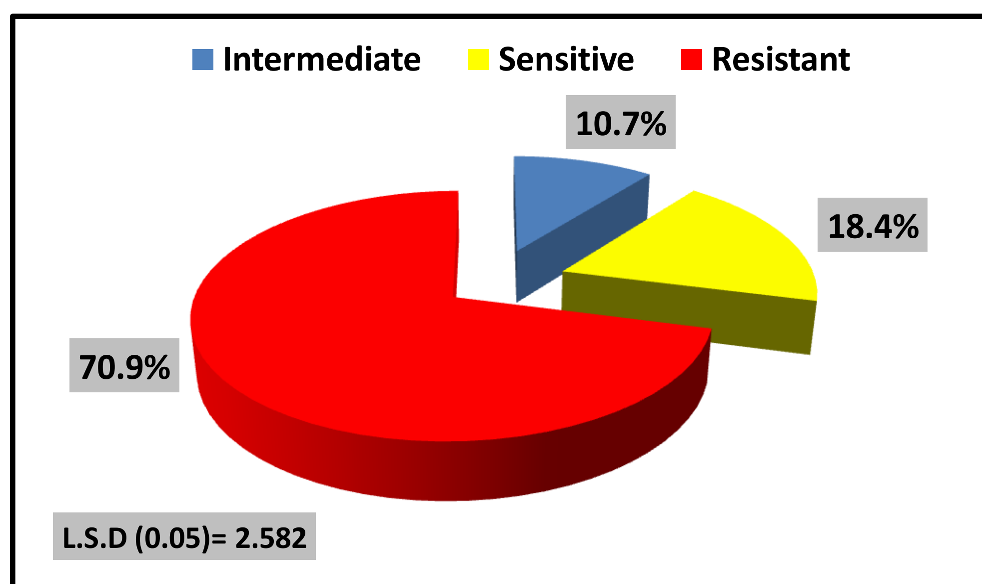

Figure (4-6): Cefoxitin susceptibility of β-lactam resistant *Klebsiella pneumoniae* isolates (n=103).

AmpC β-lactamase production was confirmed in all cefoxitin resistance (n=73), intermediate (n=11), and susceptible (n=19) *K. pneumoniae* isolates by the modified three dimension test. A clear distortion of the zone of inhibition of cefoxitin was observed in 31 (30.1%) of the 103 isolates tested (Table 4-9, Figure 4-7). As many as 31 (42.5%) of the 73 cefoxitin resistant isolates were confirmed to be AmpC β-lactamase positive by this method. Whereas, all the cefoxitin susceptible and intermediate resistance isolates showed no distortion zone of inhibition of cefoxitin and were considered as none AmpC β-lactamase producers.

AmpC β-lactamase production was further confirmed by the AmpC disk test. Indentation indicating strong AmpC producer was observed in 30 (29.1%) of the 103 isolates.

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Table (4-9): Phenotypic characterization of AmpC β-lactamase producing *Klebsiella pneumoniae* by confirmation methods (n=103)
*MTDT: modified three-dimensional test

Cefoxitin-Susceptibility of isolates	No. (%) of phenotypic AmpC β-lactamase producer isolates			No.(%) of non AmpC β-lactamase producer isolates
	MTDT*	AmpC disk method	Disk-based inhibitor method	
Resistant (n=73)	31(42.5%)	30(41.1%)	31(42.5%)	42(57.5%)
Susceptible (n=19)	0(0%)	0(0%)	0(0%)	19(100%)
Intermediate resistant (n=11)	0(0%)	0(0%)	0(0%)	11(100%)
Total (n=103)	31(30.1%)	30(29.1%)	31(30.1%)	72(69.9%)
L.S.D. (0.05) of isolate susceptibility and methods = 1.019, Interaction= 3.245				

All these isolates were resistant to cefoxitin (41.1%) and gave positive results with a modified three dimension test (Table 4-9, Figure 4-8). It was found that no flattening (weak AmpC) or distortion was observed in any of the 31 modified three dimension test positive isolates as well as in all cefoxitin susceptible or intermediate resistant isolates. The above results were compared against a disk-based inhibitor assay using various combinations of ceftazidime and cefoxitin as antibiotic substrates and cloxacillin as an AmpC inhibitor (Figure 4-9). Results revealed synergism effect between antibiotic substrates was exhibited in 31 (42.5%) of the 73 cefoxitin resistance isolates.

On the other hand, non of the cefoxitin susceptible and intermediate resistant isolates gave synergism zones of inhibition between antibiotic substrates and AmpC inhibitor (Table 4-9).

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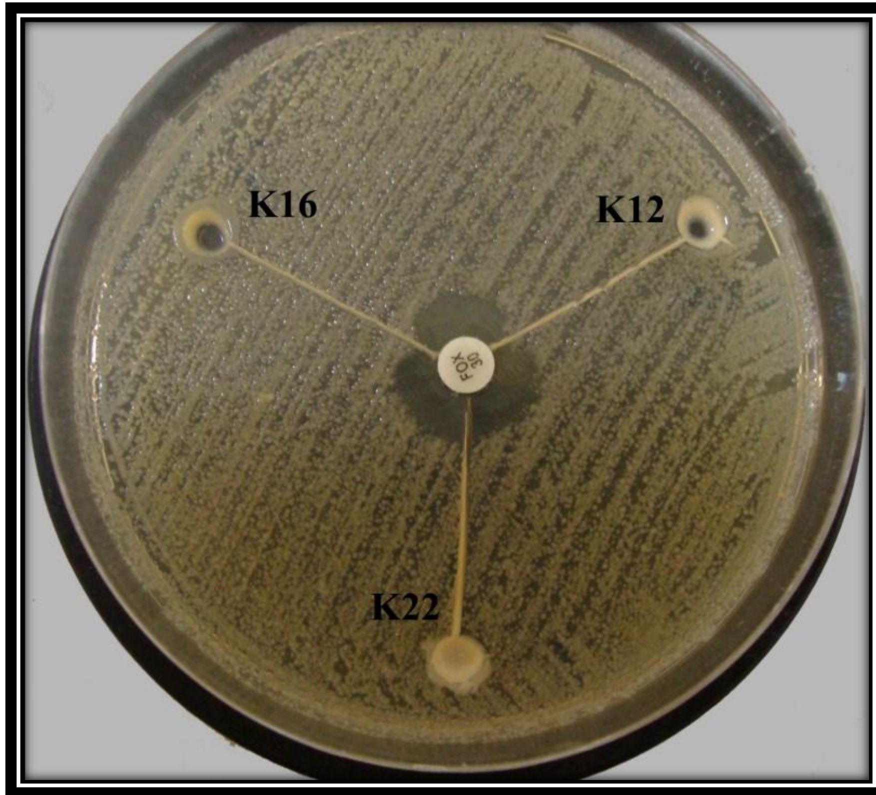

Figure (4-7): AmpC β-lactamase production in *Klebsiella pneumoniae* by modified three dimentional test; **Growth of *E. coli* ATCC 25922 strain around slits containing AmpC enzyme extractions of K16 and K12 test isolates exhibit clear distortion of the inhibition zone of cefoxitin, K22 isolate exhibits negative result. Plate incubated at 37°C for 24hr.**

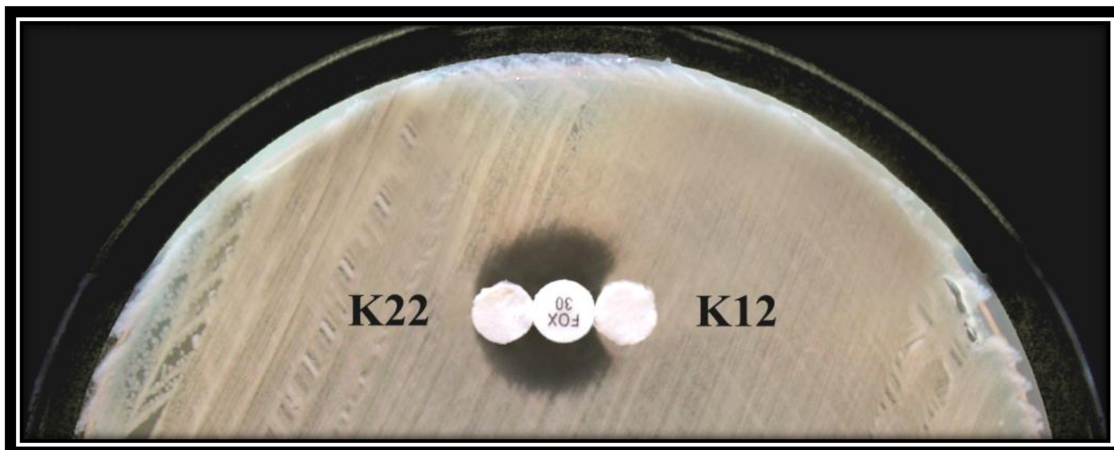

Figure (4-8): AmpC β-lactamase production in *Klebsiella pneumoniae* by AmpC disk test; **A lawn culture of *E. coli* ATCC 25922 strain with cefoxitin disk placed between two loaded disk with tested isolates, K12 test isolates, exhibit clear distortion of the inhibition zone of cefoxitin, K22 isolate exhibit negative result. Plate incubated at 37°C for 24hr.**

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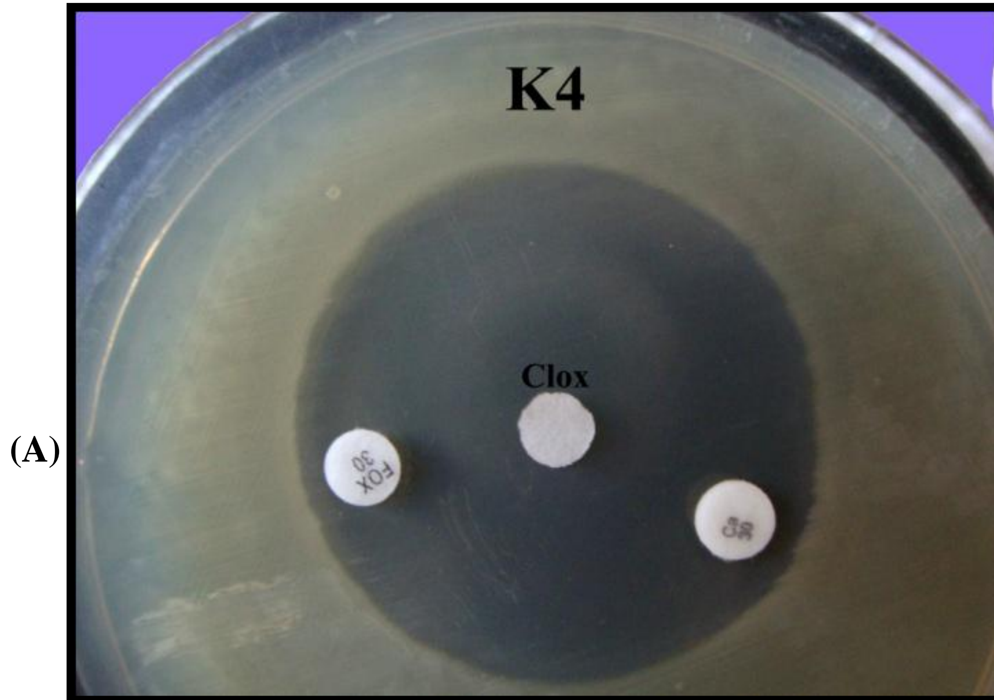
(A)



(B)

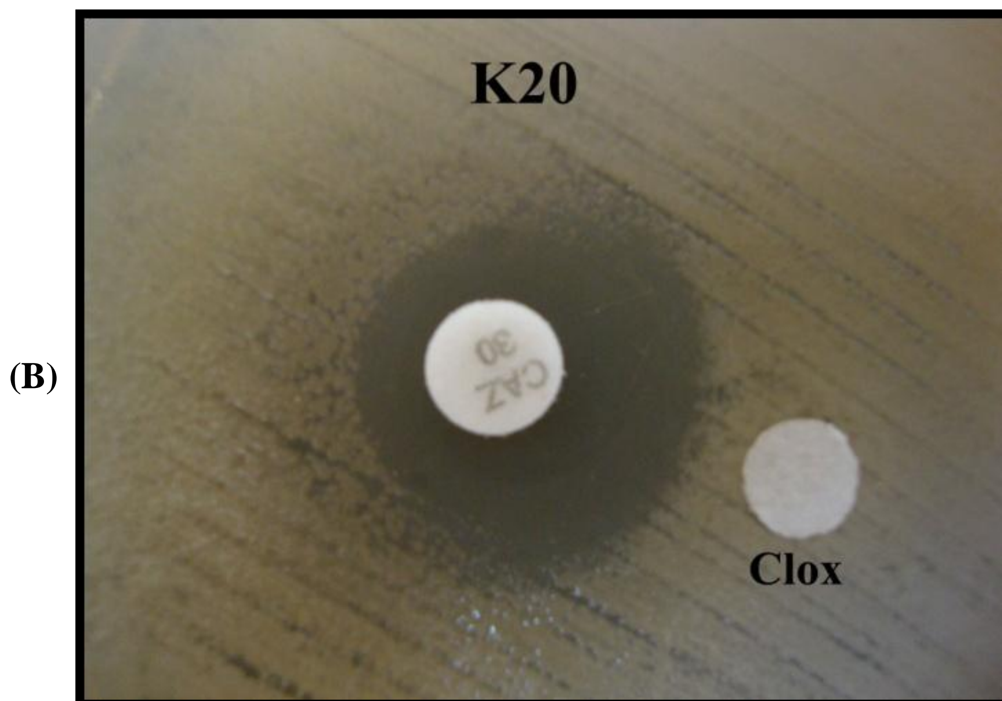


Figure (4-9): AmpC β-lactamase production in *Klebsiella pneumoniae* by disk based inhibitor test; **(A) shows K4 test isolate with synergism between ceftazidime (Ca), cefoxitin (FOX) and cloxacillin disk was placed between them, (B) shows K20 test isolate with synergism between ceftazidime (CAZ) and cloxacillin. Plates incubated at 37°C for 24hr.**

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No significant differences ($P < 0.05$) were distinguished among the above three tests. However, the detection of AmpC β-lactamase production in cefoxitin resistant *K. pneumoniae* isolates was more significant ($P < 0.05$) than in the cefoxitin intermediate and susceptible isolates. Data in table (4-9) also revealed that 72 (69.9%) isolates were none AmpC β-lactamase producer; including 42 (57.5%) resistant to cefoxitin, 19 (100%) isolates susceptible to cefoxitin and 11 (100%) intermediate susceptible to cefoxitin.

4.7. Inducible AmpC β-Lactamase Production

Results in Figure (4-10) show that only 2 (1.9%) of the β-lactam resistant *K. pneumoniae* isolates were ceftazidime-imipenem antagonism test positive, indicating the production of inducible AmpC β-lactamases. one of these isolates gave strong visible reduction and the other one gave moderately visible. However, the remaining 101 isolates (98.1%) were phenotypically negative (Table 4-10).

4.8. Molecular Detection of AmpC β-Lactamase

When presence and occurrence of bla_{AmpC} genewere evaluated in the 103 β-lactam resistant *K. pneumoniae* isolates, only 20 (19.4%) isolates were able to yield amplification products with AmpC-PCR specific primers. PCR detection of these isolates is presented in Figure (4-11). The isolation frequency of *K. pneumoniae* having this gene were detected only in the cefoxitin resistant isolates (20/73, 27.4%). Figure (4-12) shows that none of the 19 cefoxitin susceptible and 11 cefoxitin intermediate resistant isolates harbored bla_{AmpC} gene as determined by PCR.

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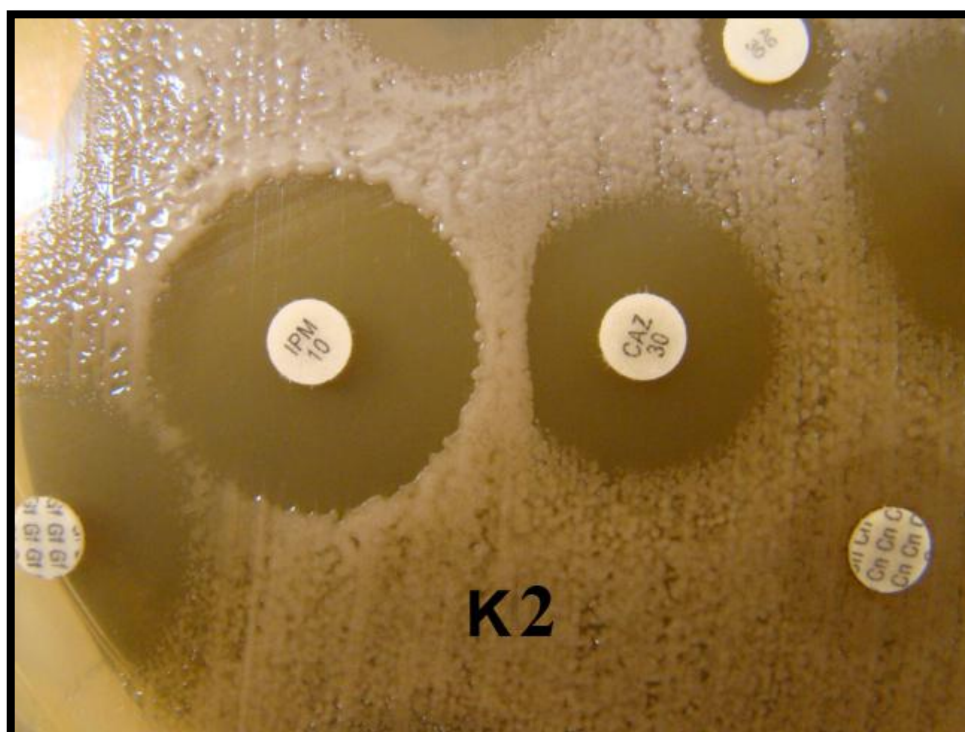

Figure (4-10): Inducible AmpC β-lactamase production in *Klebsiella pneumoniae* by ceftazidime-imipenem antagonism test (CIAT); K2 test isolate exhibit antagonism between ceftazidime (CAZ) disk and imipenem (IPM) disk. Plate incubated at 37°C for 24hr.

Table (4-10): Numbers and percentages of *Klebsiella pneumoniae* isolates able to produce inducible AmpC β-lactamase by ceftazidime-imepenem antagonism test (CIAT)

Cefoxitin susceptibility	No.(%) of *K. pneumoniae* isolates	No. (%) of ceftazidime-imipenem antagonism screened isolates		
		Strong positive	Moderate positive	Negative
Resistant	73(71%)	1(1.4%)	1(1.4%)	71(97.2%)
Intermediate	19(18.4%)	0(0%)	0(0%)	19(100%)
Susceptible	11(10.6%)	0(0%)	0(0%)	11(100%)
Total	103	2(1.9%)		101(98.1%)

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Frequency of the AmpC β-lactamase-producing *K. pneumoniae* isolates and their sites of isolation are listed in Table (4-11). It has been noticed that the frequencies of cefoxitin resistant isolates were 65 (68.4%) and 8 (100%) in clinical and hospital environmental samples, respectively. The AmpC β-lactamase-production was phenotypically detected in 30 (31.6%) isolates of clinical samples and in only 1 (12.5%) isolate of hospital environment samples by using modified three dimension test. Results also revealed that the occurrence of AmpC β-lactamase was quite high in the clinical samples compared with the environmental samples. However, the isolates harbored bla_{AmpC} gene were most prevalent in the hospital environmental (2/8, 25%). Interestingly, there was no significant difference ($P < 0.05$) in distribution of the isolates harbored bla_{AmpC} gene between source of samples.

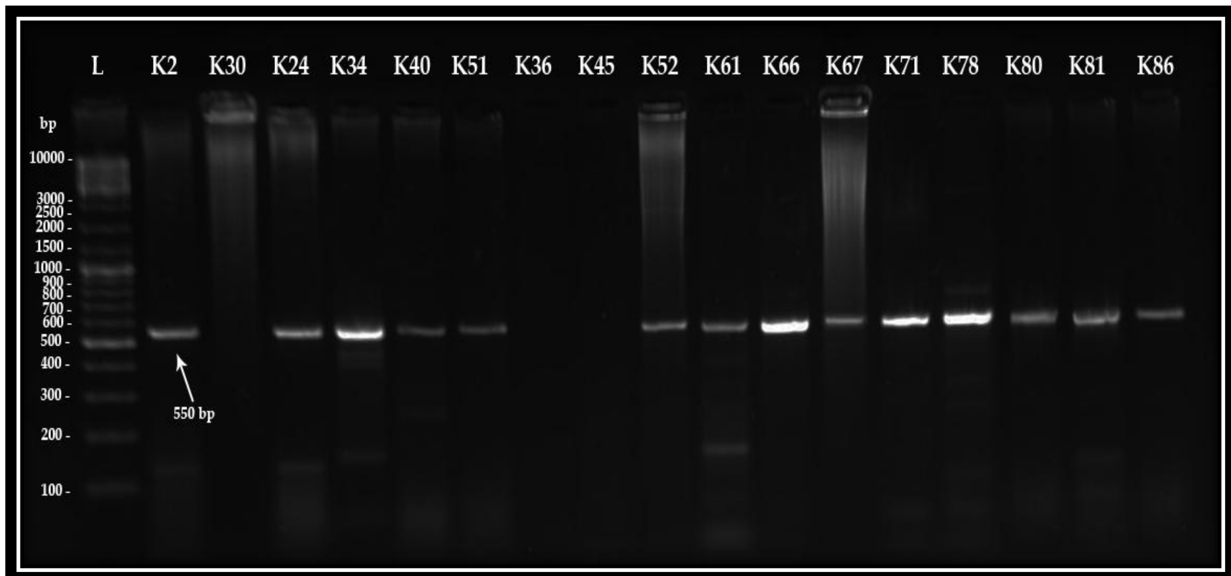

Figure (4-11): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of cefoxitin resistant *Klebsiella pneumoniae* isolates and amplified with bla_{AmpC} gene primers. The electrophoresis was performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (l0000-bp ladder), Lanes (K 2, 24, 34, 40, 51, 52, 61, 66, 67, 71, 78, 80, 81 and 86) show positive results with bla_{AmpC} gene, Lanes (K 30, 36 and 45) show negative results with bla_{AmpC} gene.

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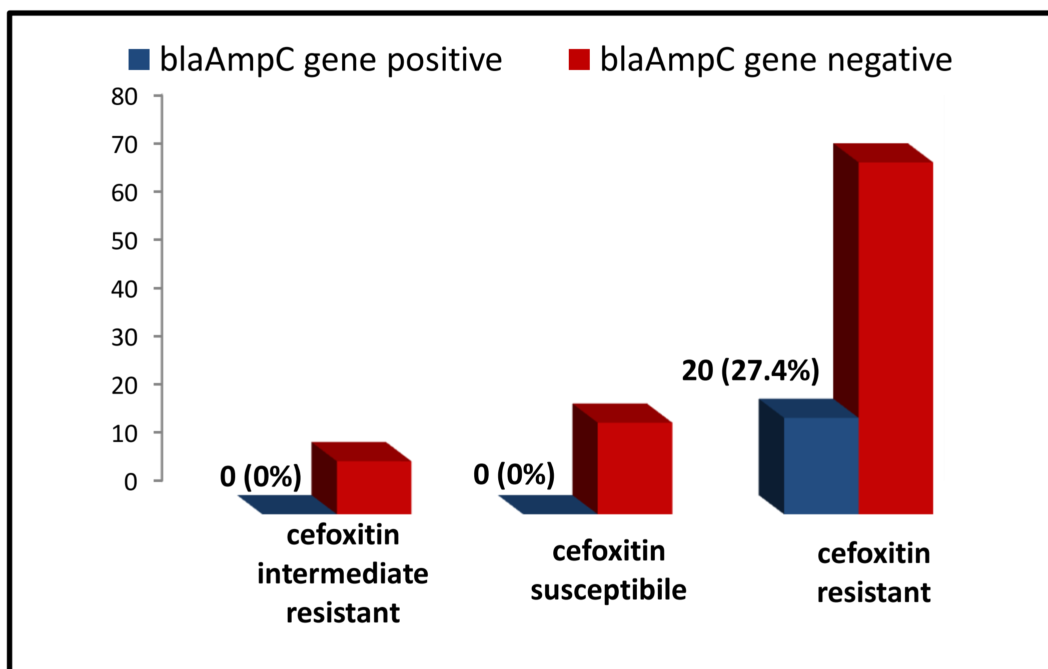

Figure (4-12): Occurrence of *bla*$_{AmpC}$ gene among β-lactama resistant *Klebsiella pneumoniae* isolates (n=103).

Table (4-11): Occurrence of AmpC β-lactamase *Klebsiella pneumoniae* isolates recovered from clinical and hospital environmental samples

Source of sample	No. (%) of cefoxitin resistant *K. pneumoniae* isolates	No.(%) of MTDT* positive isolates	No.(%) of *bla*$_{AmpC}$ gene positive isolates
Clinical (n=95)	65 (68.4%)	30(31.6%)	18(19.0%)
Hospital environmental (n=8)	8 (100%)	1(12.5%)	2(25.0%)
Total (n=103)	73 (70.9%)	31(31.1%)	20 (19.4%)

***MTDT: Modified three dimensional test**

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4.9. Emergence of *Klebsiella pneumoniae* Carrying bla_{AmpC}

Table (4-12) revealed that 18 bla_{AmpC} positive *K. pneumoniae* isolates were obtained from the clinical samples. Although, there was no indicative significance (P< 0.05) among age and gender of patients. Isolates having bla_{AmpC} gene were detected more commonly in the both extremes of ages; 51-72 years (12/325, 3.7%) and 2-10 years (1/40, 2.5%). Less number (5/270, 1.9%) was isolated from the age group of 31-50 years. None of the isolates carrying bla_{AmpC} were detected in the age group of 11-30 years (0/135, 0%). Results revealed that ages more than 51 years were significantly associated with bla_{AmpC} positive *K. pneumoniae* isolates. *K. pneumoniae* isolates having bla_{AmpC} gene were 3.5 times more likely to be recovered from male (14/510, 2.7%) than female patients (4/260, 1.5%) among all age groups.

Ten of the 18 *K. pneumoniae* isolates carrying bla_{AmpC} were classified as hospital onset (obtained from 290 inpatients), and 8 isolates as community onset (recovered from 480 outpatients). In spite of, high number of samples were collected from outpatients, results show that bla_{AmpC} gene positive isolates were more common in inpatients (3.4%) compare with the outpatients (1.7%). Moreover, all isolates carrying bla_{AmpC} gene were enrolled among immune suppressed patients (18/302, 6.0%) (Table 4-12). In addition, all the 770 clinical samples were collected from patients had previously taken different antibiotics within the distinct periods. However, all bla_{AmpC} gene positive isolates were recovered from these patients (18/770, 2.3%). The results show that significant relationship (P<0.05) was between existed of bla_{AmpC} isolates and immunity state, hospitalization and antibiotic receiving.

Table (4-13) show that the bla_{AmpC} positive *K. pneumoniae* isolates were also frequent among hospital environmental samples. Out of the 245 hospital environmental samples, only 2 (0.8%) were bla_{AmpC} positive

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K. pneumoniae isolates. One (2.2%) of them was isolated from kitchen and the other from bathrooms. Adversely, non of the bla_{AmpC} positive *K. pneumoniae* isolates were detected among other samples collected from operation halls, ICU, birth halls, burn units and wards.

Table (4-12): Clinical characteristics of patients infected with bla_{AmpC} gene positive *Klebsiella pneumoniae*
***L.S.D. (0.05)= 1.239**

Patient profile	Status	No. of sample (n=770)	No.(%) bla_{AmpC} gene positive *K. pneumoniae* isolates (n=18)*
Age group (Years)	(2-10) (11-30) (31-50) (51-72)	40 135 270 325	1 (2.5%) 0 (0%) 5 (1.9%) 12 (3.7%)
Gender	Male Female	510 260	14 (2.7%) 4 (1.5%)
Immunity state	Immune suppressed Not	302 468	18 (6.0%) 0 (0%)
Hospitalization	Inpatient Outpatient	290 480	10 (3.4%) 8 (1.7%)
Antibiotic administration	Yes No	770 0	18 (2.3%) 0 (0%)

4.10. Antibiotic Susceptibility of bla_{AmpC} Positive *K. pneumoniae*

Results of antibiotic susceptibility testing of the 20 bla_{AmpC} carrying *K. pneumoniae* isolates are shown in Figure (4-13). Susceptibility determination for the 26 antibiotics and the respective percentage of resistant phenotypes showed that all isolates (100%) were resistant to carbenicillin, piperacillin, ticarcillin, amoxi-clav, cefazolin, cefoxitin,

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Table (4-13):Distribution of *bla*$_{AmpC}$ gene positive *Klebsiella pneumoniae* isolates recovered from hospital environmental samples

Hospital environmental sample	No. of sample	*bla*$_{AmpC}$ gene positive *K. pneumoniae* isolates (n=2)
Operation hall	30	0 (0%)
ICU*	20	0 (0%)
Birth hall	25	0 (0%)
Burns unit	30	0 (0%)
Wards	50	0 (0%)
Kitchen	45	1 (2.2%)
Bathrooms	45	1 (2.2%)
Total	245	2 (0.8%)

***ICU: Intensive care unit**

cefotaxime, nalidixic acid and trimethoprim. In addition to high resistance level against cefexime (95%), ceftazidim, cefetriaxone, cefepime, gentamicin, tetracycline, nitrofurantoin (90% each), and aztreonam (85%).

The resistance to amikacin, tobramycin, and chloramphenicol among isolates was 70%. Moderate resistance was seen with fluroquinolone antibiotics including; ciprofloxacin (55%), gatifloxacin (40%), levofloxacin (35%), and norofloxacin (35%). Nevertheless, antibiotics which seem more effective had lower resistance among these bacterial isolates were imipenem (20%) and meropenem (20%). No intermediate resistant results were seen with all *bla*$_{AmpC}$ carrying *K. pneumoniae* isolates. However, results also showed that no antibiotic were fully active against these isolates.

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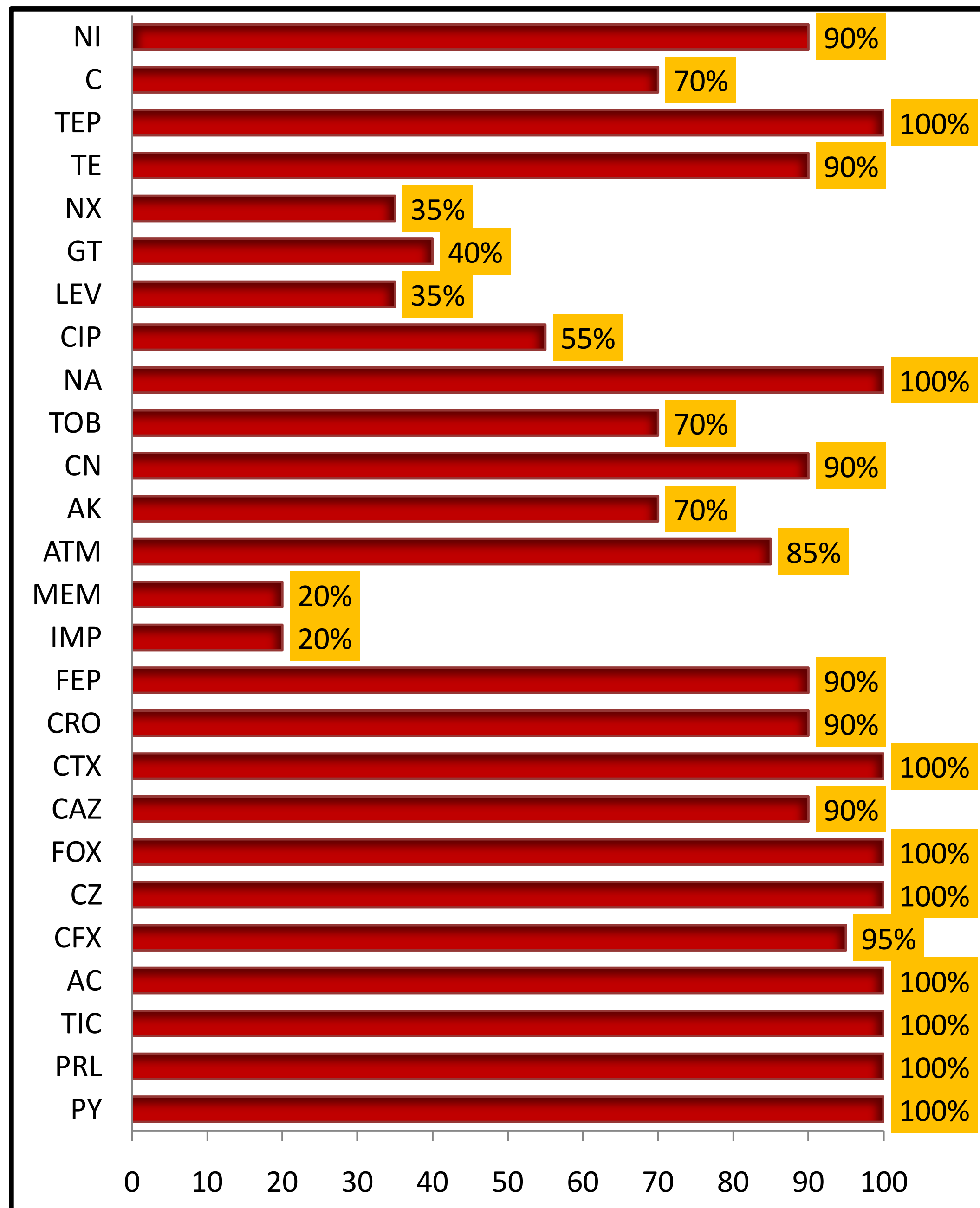

Figure (4-13): Antibiotics resistance pattern of *bla*$_{AmpC}$ harboring *Klebsiella pneumoniae* isolates by disk diffusion method (n=20).
PY, Carbenicillin; PRL, Piperacillin; TIC, Ticarcillin; AC, Amoxi-clav; CFX, Cefexime; CZ,Cefazolin; FOX, Cefoxitin; CAZ, Ceftazidime; CTX, Cefotaxime; CRO, Ceftriaxone; FEP, Cefepime; IMP, Imipenem; MEM, Meropenem; ATM, Aztreonam; AK, Amikacin; CN, Gantamicin; TOB, Tobramycin; NA, Nalidixic acid ;CIP, Ciprofloxacin; LEV, Levofloxacin; GT, Gatifloxacin; NX, Norofloxacin; TE, Tetracyclin; TEP, Trimethoprime; C, Chloramphenicol; NI, Nitrofuradantin.

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4.11. Multi-drug resistance among *bla*$_{AmpC}$ Positive *K. pneumoniae*

Present study also focused on the present of multidrug resistant (MDR) in the 20 *bla*$_{AmpC}$ positive *K. pneumoniae* isolates. A strain is considered a MDR, if an isolate is resistant to representatives of three or more classes of antibiotics (penicillins, cephalosporins, aminoglycosides, monobactams, fluoroqinolones, tetracyclines and carbapenems).

While the definition of extensive drug resistance (XDR) is an isolate that is resistant to all but one or two classes. A pandrug resistant (PDR) isolate is resistant to all available classes of antimicrobial agents (Falagas and Karageorgopoulos, 2008).

All isolates were found to be resistant to at least 3 antibiotic classes tested. Of these, 11 (55.0%) isolates were considered as MDRs isolates. one (5%) of them were resisted to three antibiotic classes, while 2 (10.0%) and 8 (40.0%) were resisted to four and five antibiotic classes, respectively (Table 4-14).

Moreover, 9 (45.0%) of the *bla*$_{AmpC}$ carrying *K. pneumoniae* isolates were resistant to at least 6 antibiotic classes and hence considered XDR isolates. Of which, 7 (35.0%) were resistant to 6 antibiotic classes, and 2 (10.0%) to 7 antibiotic classes (Table 4-14). Pandrug-resistant pattern was not detected among the *bla*$_{AmpC}$ harbouring *K. pneumoniae* isolates tested.

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Table (4-14): Antibiotics resistant pattern of harboring *Klebsiella pneumoniae* isolates.

Type of resistance	No. and (%) of *bla*$_{AmpC}$ positive *K. pneumoniae* isolates (n=20)	No. of resisted antibiotic classes
*MDR (n=11)	1 (5%) 2 (10%) 8 (40%)	3 4 5
*XDR (n=9)	7 (35%) 2 (10%)	3 7

***MDR: multi-drug resistant; XDR: extensive drug resistant**

4.12. Minimum Inhibitory Concentrations of *bla*$_{AmpC}$ Positive *Klebsiella pneumoniae*

Minimum inhibition concentrations were determined according to the Clinical and Laboratory Standards Institute by a standard agar dilution method on Mueller-Hinton medium containing antibiotics. Table (4-15) indicates that all the 20 *bla*$_{AmpC}$ carrying *K. pneumoniae* isolates were highly resistant for ampicillin, piperacillin and cephalothin with concentrations beyond the break point values (64-128) µg/ml, (16-256) µg/ml, and (16-64) µg/ml, respectively, compared with *E. coli* ATCC 25922 as a negative control.

In the same manner, Table (4-15) shows that *bla*$_{AmpC}$ positive *K. pneumoniae* isolates were highly resistant for cefotaxime with an MIC range of 8-128 µg/ml. While that of ceftazidime was 16-128 µg/ml. On the other hand, 4 (20.0%) of the isolates were resistant to meropenem with an MIC range of 2-32 µg/ml, compared with *E. coli* ATCC 25922 as a negative control.

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Table (4-15): Minimum Inhibitory Concentration values of β-lactam antibiotics for *bla*AmpC Positive *Klebsiella pneumoniae* isolates.

Antibiotic	Break point (μg/ml)	MIC (μg/ml) for *bla*AmpC positive *Klebsiella pneumoniae* isolates (n=20)		
		Range	*MIC_{50}	*MIC_{90}
Ampicillin	≥32	64-128	128	128
Piperacillin	≥32	16-256	256	256
Cephalothin	≥128	16-64	32	64
Cefotaxime	≥64	8-128	64	128
Ceftazidime	≥32	16-128	64	128
Meropenem	≥16	2-32	2	16

***Cumulative percentage of the MIC concentration at which 50% (MIC_{50}) and 90% (MIC_{90}) of the bacterial isolates were inhibited from growth.**

Table (4-15) also shows the cumulative values of minimum inhibition concentrations at which 50% (MIC_{50}) and 90% (MIC_{90}) of bacterial isolates were inhibited. The MIC_{50} and MIC_{90} were detected in all *bla*$_{AmpC}$ carrying *K. pneumoniae* isolates, and were highly elevated for ampicillin (128/128 μg/ml), piperacillin (256/256 μg/ml) and cephalothin (32/64 μg/ml).

Activities of the β -lactams other than meropenem were found to be the order of cefotaxime equal to ceftazidime with MIC_{50} and MIC_{90}, were 64 μg/ml and 128 μg/ml for all isolates, respectively. The increment in these values may be due to their low efficient against *bla*$_{AmpC}$ positive *K. pneumoniae* isolates. Although, the MIC_{50} of meropenem was in zone of susceptible (2 μg/ml), and the MIC_{90} (16 μg/ml) was reached beyond the break point value. Upon such finding, this antibiotic was the most efficient against *bla*$_{AmpC}$ positive *K. pneumoniae* isolates (Table 4-15).

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4.13. Screening and Detection of Plasmid-Mediated AmpC β-Lactamase Genes

All *K. pneumoniae* isolates with an intrinsic bla_{AmpC} β-lactamases (n=20) were further investigated for the presence of plasmid-mediated AmpC β-lactamases (PABLs) using six families of genes. Presence of genes included bla_{FOX}, bla_{CIT}, bla_{DHA}, bla_{EBC}, bla_{ACC} and bla_{MOX} were detected by the conventional PCR technique. Table (4-16) shows the distribution of plasmid mediated *ampC*-genes among the isolates. Results (by using monoplex PCR assay) revealed that all examined isolates of *K. pneumoniae* (100%) had plasmid-mediated AmpC β-lactamases. With a varying presence of plasmid-mediated *ampC*-genes. Gene that encode FOX was the most frequently identified among the isolates (11, 55.0%) (Figure 4-14). The bla_{CIT} gene was detected in 8 (40.0%) isolates (Figure 4-15). The bla_{DHA} and bla_{EBC} was presented in 4 (20.0%) isolates, separately (Figures 4-16 and 4-17). In addition, bla_{ACC} gene was observed in only 1 (5.0%) isolate (Figure 4-18). On the other hand, all isolates tested did not possess the MOX-type plasmid-mediated AmpC β-lactamase.

Table (4-16) also show that only 2 (10%) bla_{DHA} harbored *K. pneumoniae* isolates were verified previously as inducible AmpC β-lactamase positive by ceftazidim-imepenem antagonism test.

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Table (4-16): Distribution of plasmid-mediated AmpC β-lactamase gene families in *bla*$_{AmpC}$ positive *Klebsiella pneumoniae* isolates.

Isolate symbol	Plasmid mediated AmpC β-lactamase gene family						Production of inducible AmpC β-lactamase	genes profile
	FOX	CIT	DHA	EBC	ACC	MOX		
K1	+	-	-	-	-	-	-	1
K2	-	-	+	-	-	-	+	1
K3	+	-	-	-	-	-	-	1
K4	+	+	-	-	-	-	-	2
K5	+	+	-	-	-	-	-	2
K6	-	-	-	+	-	-	-	1
K7	-	+	-	-	-	-	-	1
K8	+	+	-	-	-	-	-	2
K9	+	-	-	-	-	-	-	1
K10	-	+	-	-	-	-	-	1
K11	+	-	-	-	-	-	-	1
K12	+	+	-	+	-	-	-	3
K13	-	-	-	+	-	-	-	1
K14	-	-	+	-	-	-	-	1
K15	-	-	+	-	-	-	-	1
K16	+	+	-	+	-	-	-	3
K17	+	-	-	-	-	-	-	1
K18	-	-	+	-	-	-	+	1
K19	+	+	-	-	-	-	-	2
K20	-	-	-	-	+	-	-	1
Total positive	11 (55%)	8 (40%)	4 (20%)	4 (20%)	1 (5%)	0 (0%)	2 (10%)	

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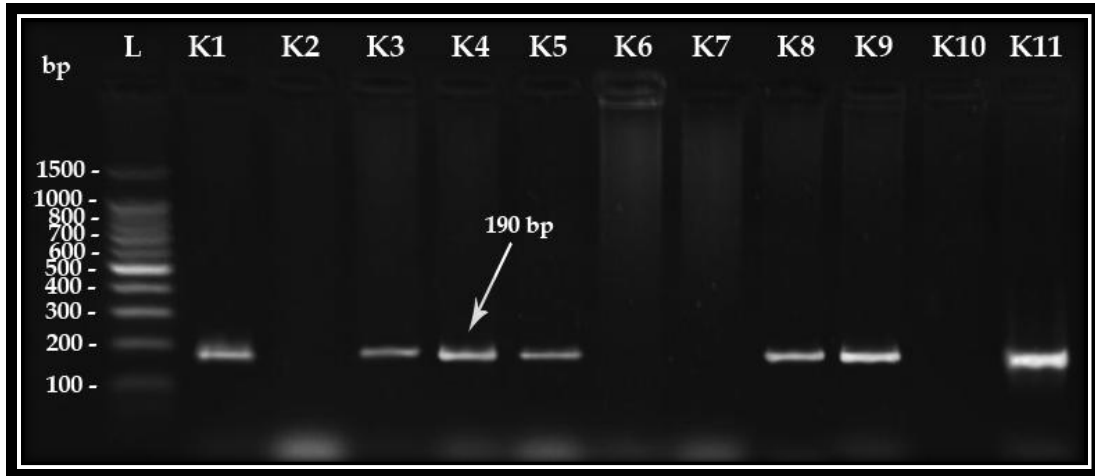

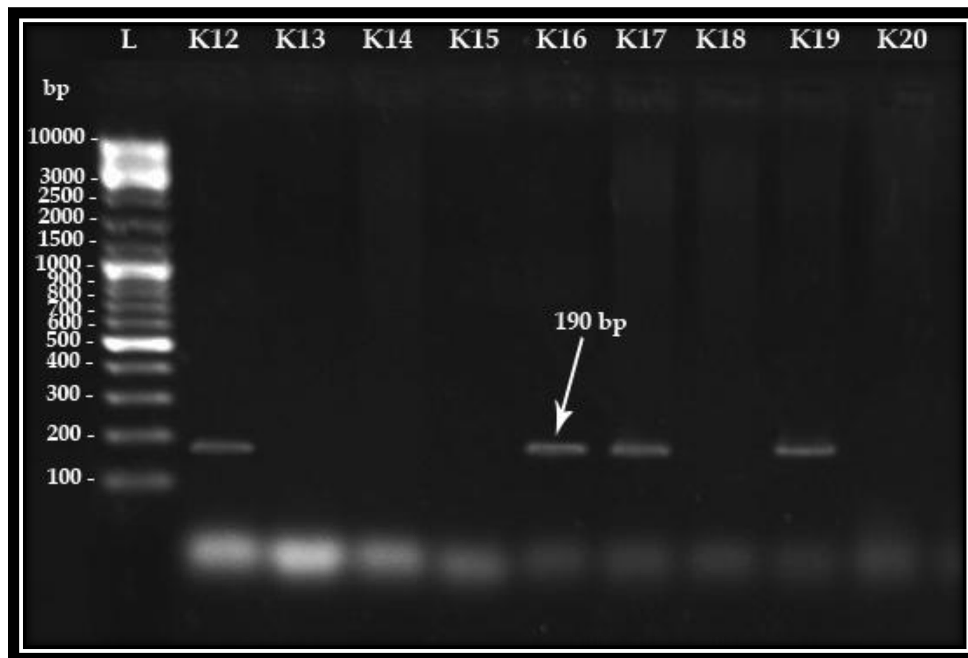

Figure (4-14): Ethidium bromide-stained agarose gel of monoplex PCR amplified products from extracted DNA of *bla*$_{AmpC}$ positive *K. pneumoniae* isolates and amplified with *bla*$_{FOX}$ genes primers (forward and reverse). The electrophoresis was performed at 70 volt for 2 hr. Lane (L), DNA molecular size marker (1500 and l0000-bp ladder), Lanes (K 1, 3, 4, 5, 8, 9, 11, 12, 16,17 and 19) of *K. pneumoniae* isolates show positive results with *bla*$_{FOX}$ (190 bp), Lanes (K 2, 6,7, 10, 13, 14, 15, 18 and 20) show negative results with *bla*$_{FOX}$ gene.

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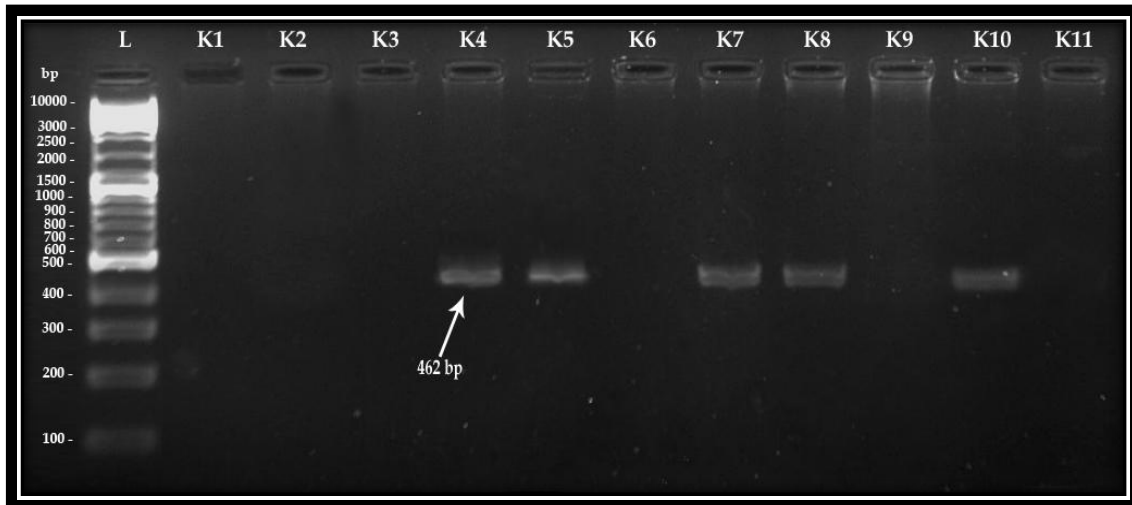

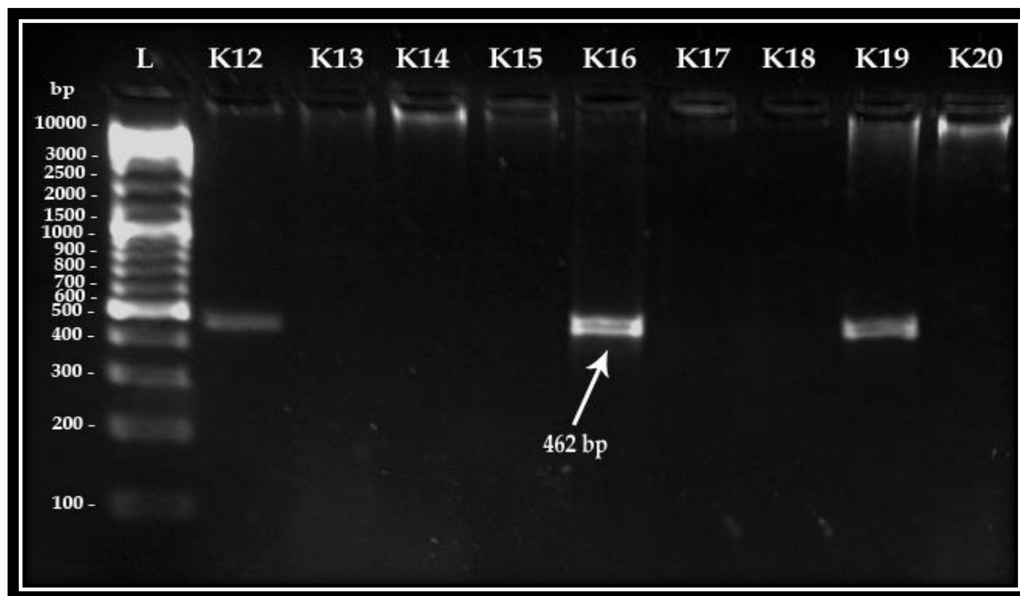

Figure (4-15): Ethidium bromide-stained agarose gel of monoplex PCR amplified products from extracted DNA of *bla*$_{AmpC}$ positive *K. pneumoniae* isolates and amplified with *bla*$_{CIT}$ genes primers (forward and reverse). The electrophoresis was performed at 70 volt for 2 hr. Lane (L), DNA molecular size marker (l0000-bp ladder), Lanes (K 4, 5, 7, 8, 10, 12, 16 and 19) of *K. pneumoniae* isolates show positive results with *bla*$_{CIT}$ (462 bp), Lanes (K 1, 2, 3, 6, 9, 11, 13, 14, 15, 17, 18 and 20) show negative results with *bla*$_{CIT}$ gene.

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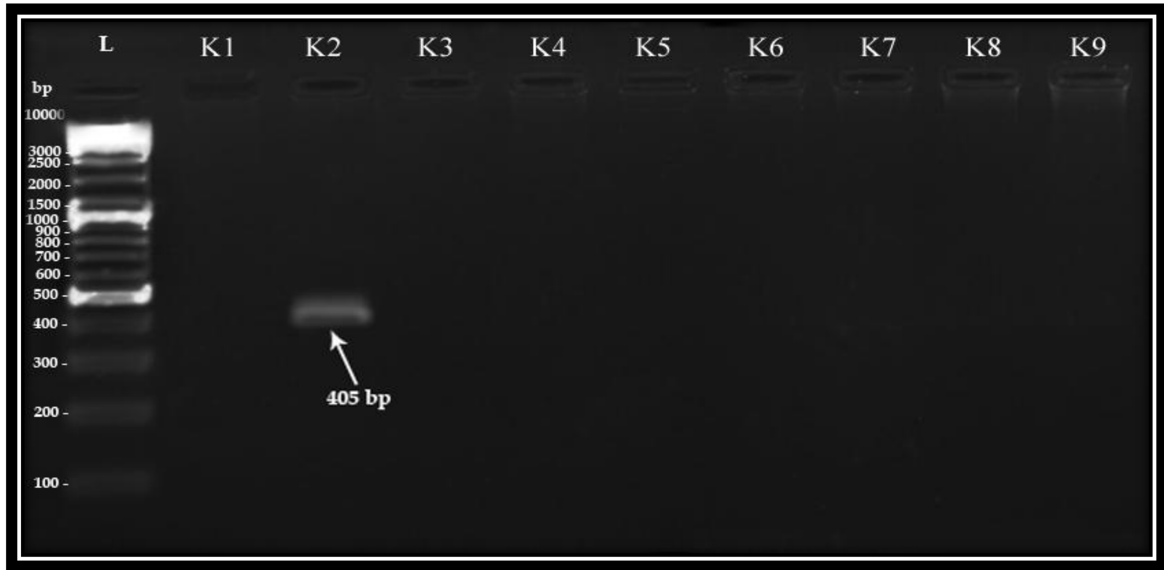

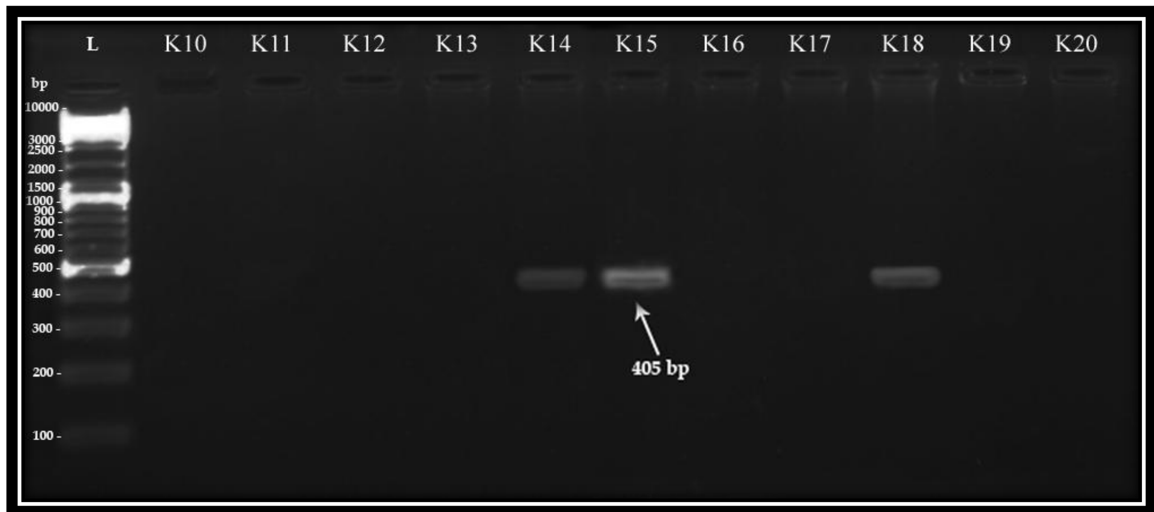

Figure (4-16): Ethidium bromide-stained agarose gel of monoplex PCR amplified products from extracted DNA of *bla*$_{\text{AmpC}}$ positive *K. pneumoniae* isolates and amplified with *bla*$_{\text{DHA}}$ genes primers (forward and reverse). The electrophoresis was performed at 70 volt for 2 hr. Lane (L), DNA molecular size marker (l0000-bp ladder), Lanes (K 2, 14, 15 and 18) of *K. pneumoniae* isolates show positive results with *bla*$_{\text{DHA}}$ (405 bp), Lanes (K 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 19 and 20) show negative results with *bla*$_{\text{DHA}}$ gene.

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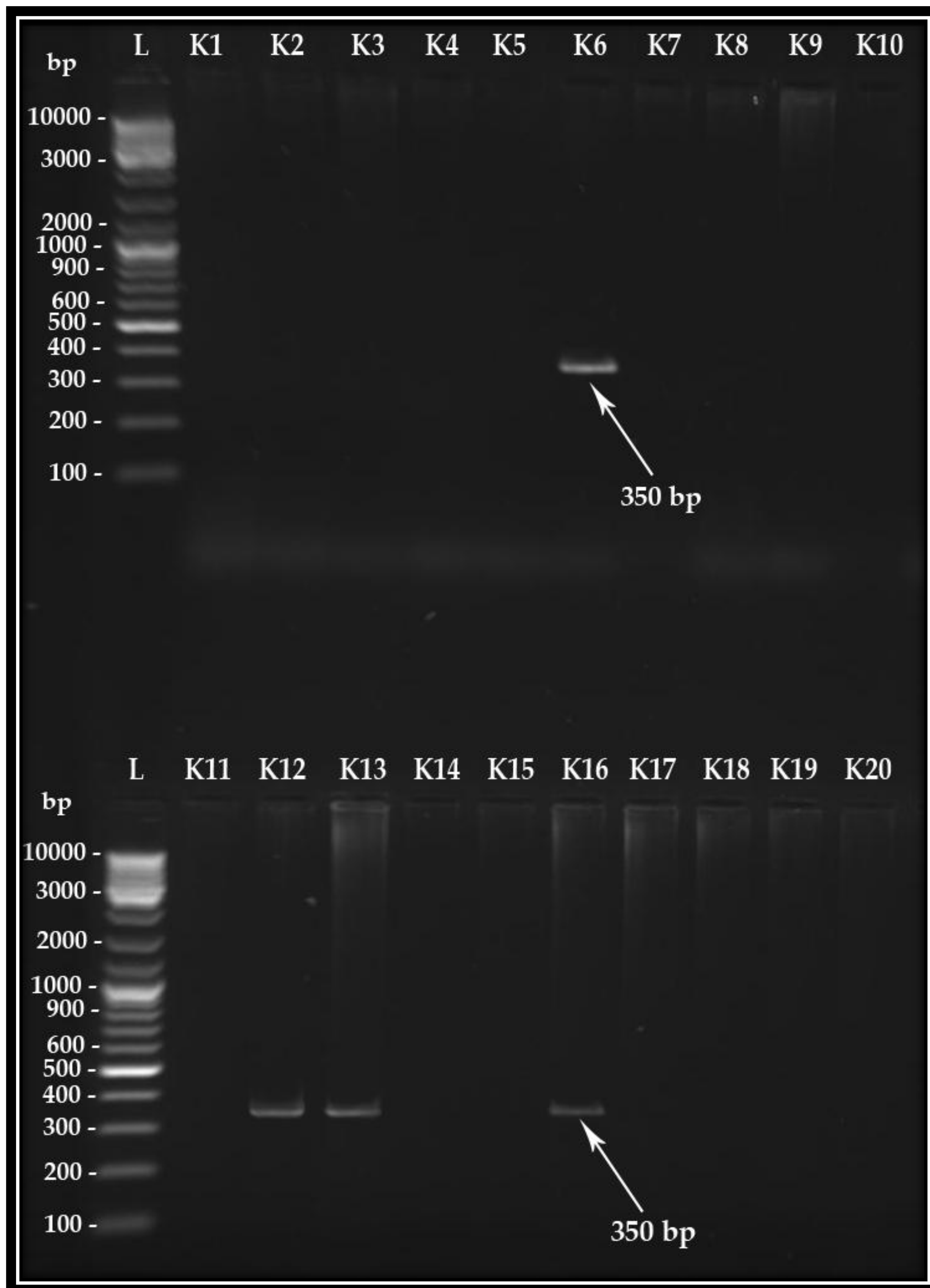

Figure (4-17): Ethidium bromide-stained agarose gel of monoplex PCR amplified products from extracted DNA of *bla*$_{AmpC}$ positive *K. pneumoniae* isolates and amplified with *bla*$_{EBC}$ genes primers (forward and reverse). The electrophoresis was performed at 70 volt for 2 hr. Lane (L), DNA molecular size marker (l0000-bp ladder), Lane (K 6, 12, 13, 16) of *K. pneumoniae* isolates shows positive results with *bla*$_{EBC}$ (350 bp), Lanes (K 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 14, 15, 17, 18, 19 and 20) show negative results with *bla*$_{EBC}$ gene.

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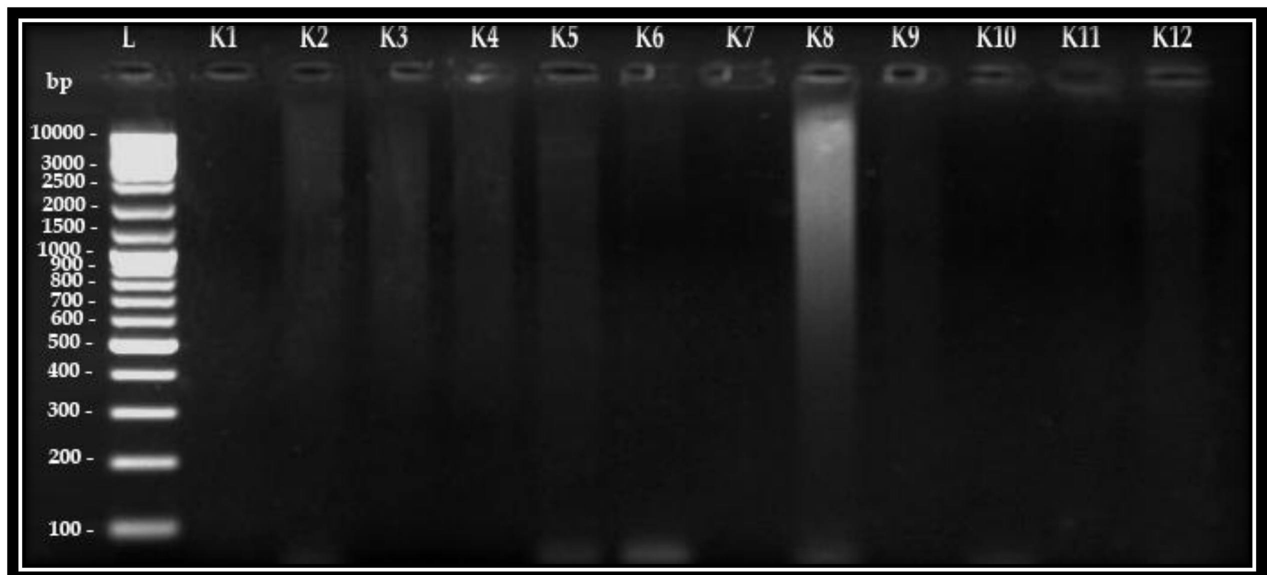

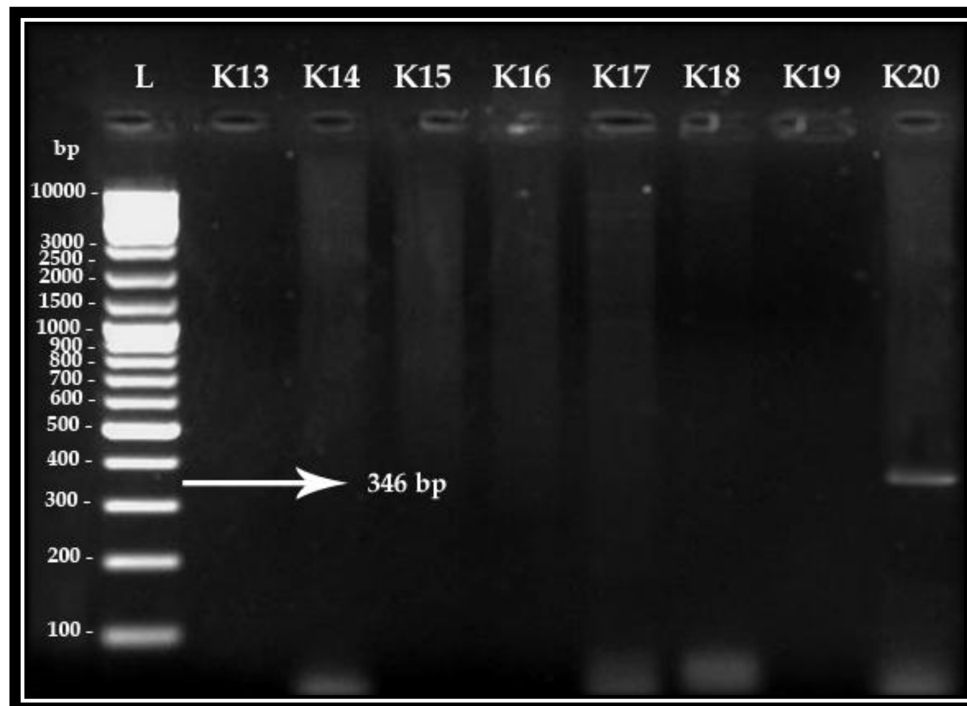

Figure (4-18): Ethidium bromide-stained agarose gel of monoplex PCR amplified products from extracted DNA of *bla*$_{AmpC}$ positive *K. pneumoniae* isolates and amplified with *bla*$_{ACC}$ genes primers (forward and reverse). The electrophoresis was performed at 70 volt for 2 hr. Lane (L), DNA molecular size marker (l0000-bp ladder), Lane (K 20) of *K. pneumoniae* isolates shows positive results with *bla*$_{ACC}$ (346 bp), Lanes (K 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19) show negative results with *bla*$_{ACC}$ gene.

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4.14. Multiplex PCR-Based Screening for Plasmid-Mediated AmpC β-Lactamase Genes

A multiplex PCR assay was designed to identify simultaneously the six families of plasmid-mediated AmpC β-lactamase genes in the 20 bla_{AmpC} β-lactamase positive *K. pneumoniae* isolates. The amplification products of isolates were previously identified as positive for plasmid-mediated *ampC*-genes by using monoplex PCR assay (Table 4-16), and the positive results were confirmed by multiplex PCR assay. Overall, plasmid-mediated *ampC*-genes were identified in all isolates (Table 4-17). Three *ampC*-genes (bla_{FOX}, bla_{CIT}, bla_{EBC}) were detected in two (10%) isolates (K12, K16), and two *ampC*-genes (bla_{FOX}, $bla_{CIT)}$ in four (20%) isolates (K4, K5, K8, K19) (Figure 4-19). While, five (25%) isolates (K1, K3, K9, K11, K17) contained only the bla_{FOX} gene, four (20%) isolates (K2, K14, K15, K18) contained only the bla_{DHA} gene, two (10%) isolates (K7, K10) contained bla_{CIT} gene, two isolates (10%) (K6, K13) contained bla_{EBC} gene and one (5%) isolate (K20) contained bla_{ACC} gene (Table 4-17).

Table (4-17): Plasmid-mediated AmpC β-lactamase multiple genes among bla_{Ampc} positive *Klebsiella pneumoniae* isolates

Isolate code No.	Plasmid mediated multiple genes pattern
K12, K16	bla_{FOX}, bla_{CIT}, bla_{EBC}
K4, K5, K8, K19	bla_{FOX}, bla_{CIT}
K1, K3, K9, K11, K17	bla_{FOX}
K2, K14, K15, K18	bla_{DHA}
K7, K10	bla_{CIT}
K6, K13	bla_{EBC}
K20	bla_{ACC}

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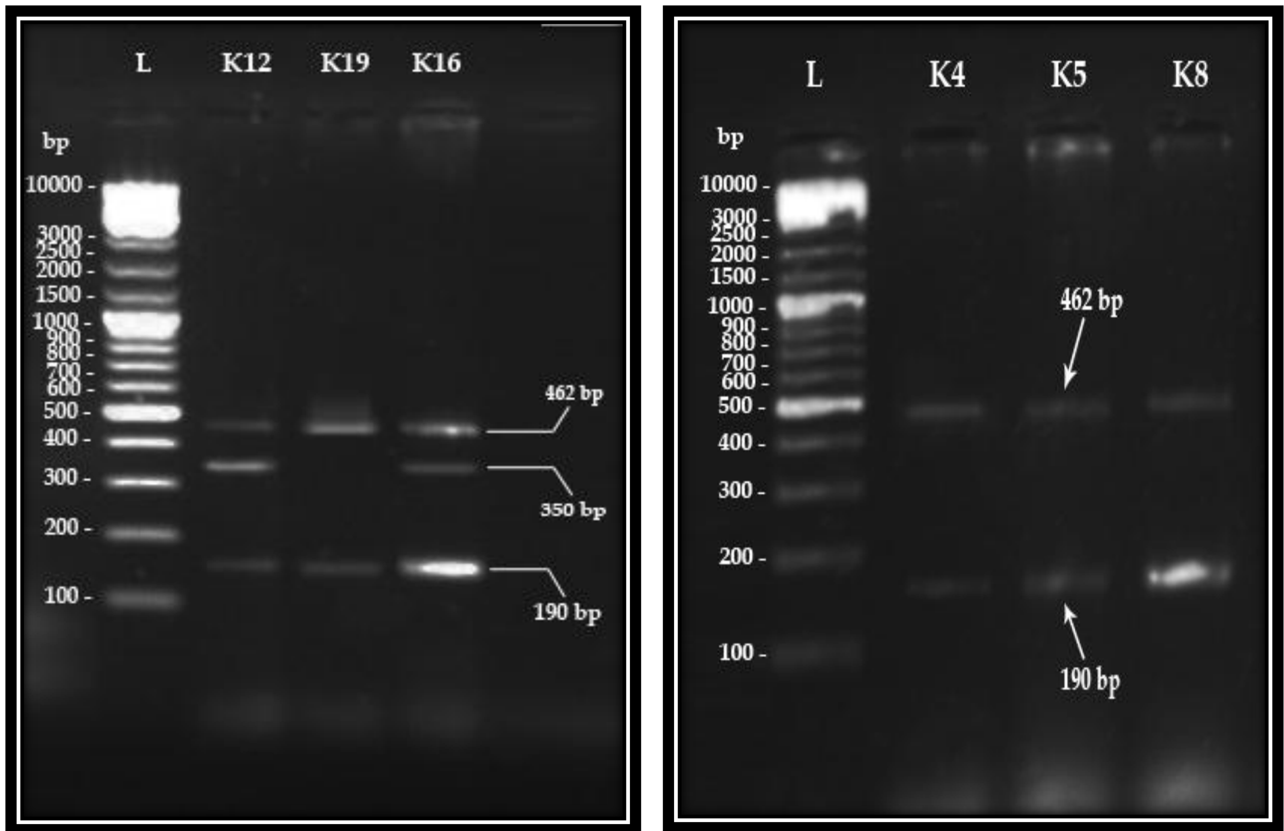

Figure (4-19): Ethidium bromide-stained agarose gel of multiplex PCR amplified products from extracted DNA of *bla*$_{AmpC}$ positive *K. pneumoniae* isolates and amplified with six PABL genes primers (forward and reverse). The electrophoresis was performed at 70 volt for 2 hr. Lane (L), DNA molecular size marker (l0000-bp ladder), Lanes (K 4, 5, 8 and 19) of *K. pneumoniae* isolates show positive results with *bla*$_{FOX}$ (190 bp) and *bla*$_{CIT}$ (462 bp) genes, Lanes (K 12, and 16) show positive results with *bla*$_{FOX}$ (190 bp), *bla*$_{CIT}$ (462 bp) and *bla*$_{EBC}$ (350 bp) genes.

4.15. Screening Criteria for ESBL Presence among *K. pneumoniae* Isolates Carrying *bla*$_{AmpC}$ Genes

All 20 *bla*$_{AmpC}$ gene positive isolates of *K. pneumoniae* were furthers investigated to determine the occurrence and types of extended-spectrum-lactamases (ESBLs). ESBL-production was tested phenotypicaly by CHROMagar. Detection of *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX\text{-}M}$, *bla*$_{OXA}$, *bla*$_{VEB}$, *bla*$_{PER}$, and *bla*$_{GES}$ genes was performed with PCR. Compared with phenotypic results. Table (4-18) that out of the 20 isolates, 10 (50.0%) isolates were confirmed as molecular analysis positive ESBL, but were

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identified as phenotypic negative. While, 6 (30.0%) isolates were verified as ESBL in both phenotype and molecular analysis. The remaining 4 (20.0%) isolates gave negative results in both phenotypic and molecular detections (Table 4-18).

Table (4-18): Phenotype and molecular co-existence of ESBL among *bla*$_{AmpC}$ positive *K. pneumoniae* isolates

No.of *bla*$_{AmpC}$ gene positive Isolates (n=20)	ESBL detection	
	Molecular analysis	Phenotype
10 isolates	+	-
6 isolates	+	+
4 isolates	-	-

Extended-spectrum β-lactamase genes had been vary prevalent among the 20 *bla*$_{AmpC}$ gene harbouring isolates of *K. pneumoniae*. Table (4-19) shows the distribution of ESBL enzymes determined by the consistent results of primers specific PCR. However, 16 (80%) of the isolates carried at least one type of *bla* genes. The most commonly identified ESBL gene was *bla*$_{CTX-M}$ type in 14 (70.0%) of the isolates (Figure 4-20). Amplification by PCR using primers specific to *bla*$_{SHV}$ gene showed positive results for 10 (50.0%) isolates (Figure 4-21). Presence of the *bla*$_{TEM}$ gene was detected in 5 (25.0%) of the tested isolates (Figure 4-22). Two (10.0%) of the examined isolates harbor a gene for the OXA-type enzyme (*bla*$_{OXA}$) (Figure 4-23). Whereas, a *bla*$_{VEB}$ type gene alone was detected in one (5.0%) isolate (Figure 4-24). Notable, none of the 20 *bla*$_{AmpC}$ gene carrying *K. pneumoniae* isolates had *bla*$_{PER}$, and *bla*$_{GES}$ ESBL genes.

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Table (4-19): Distribution of ESBL genes among bla_{AmpC} positive *K. pneumoniae* isolates

Isolate symbol	***bla* genes of ESBL**						
	CTX-M	**SHV**	**TEM**	**OXA**	**VEB**	**PER**	**GES**
K1	+	+	-	-	-	-	-
K2	+	+	-	-	-	-	-
K3	-	-	-	-	-	-	-
K4	-	-	-	+	-	-	-
K5	+	-	-	-	-	-	-
K6	+	+	+	-	-	-	-
K7	+	-	-	-	-	-	-
K8	+	+	-	-	-	-	-
K9	-	-	+	-	+	-	-
K10	-	-	-	-	-	-	-
K11	+	-	+	-	-	-	-
K12	+	+	+	-	-	-	-
K13	+	+	-	-	-	-	-
K14	-	-	-	-	-	-	-
K15	+	-	-	+	-	-	-
K16	+	+	+	-	-	-	-
K17	+	+	-	-	-	-	-
K18	+	+	-	-	-	-	-
K19	-	-	-	-	-	-	-
K20	+	+	-	-	-	-	-
Total positive	14 (70.0%)	10 (50.0%)	5 (25.0%)	2 (10.0%)	1 (5.0%)	0 (0.0%)	0 (0.0%)

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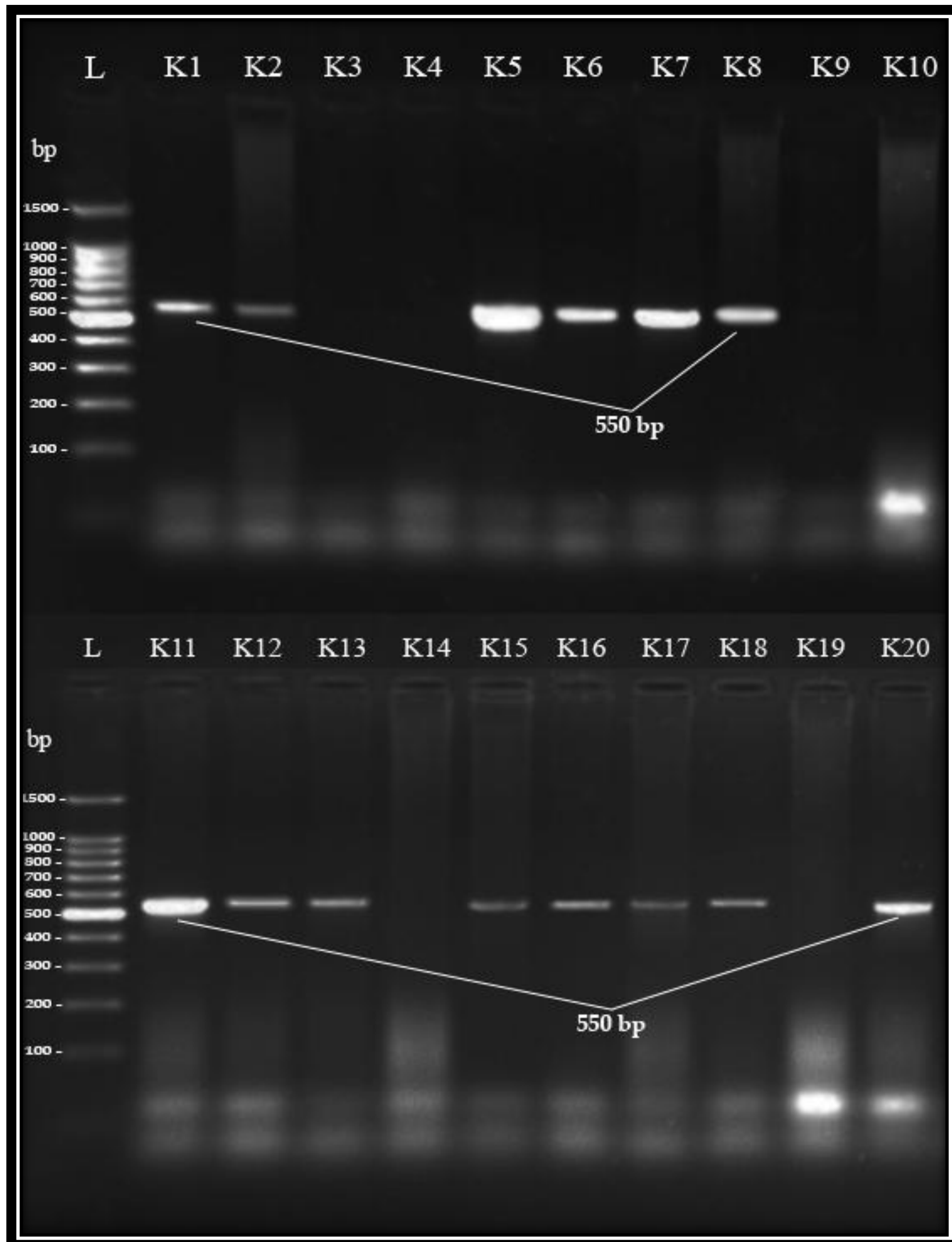

Figure (4-20): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *bla*$_{AmpC}$ positive *K. pneumoniae* isolates and amplified with primers *bla*$_{CTX-M}$ forward and *bla*$_{CTX-M}$ reverse. The electrophoresis was performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (l500-bp ladder), Lanes (K 1, 2, 5, 6, 7, 8, 11, 12, 13, 15, 16, 17, 18 and 20) show positive results with *bla*$_{CTX-M}$ gene (550 bp), Lanes (K 3, 4, 9,10,14 and 19) show negative results with *bla*$_{CTX-M}$ gene.

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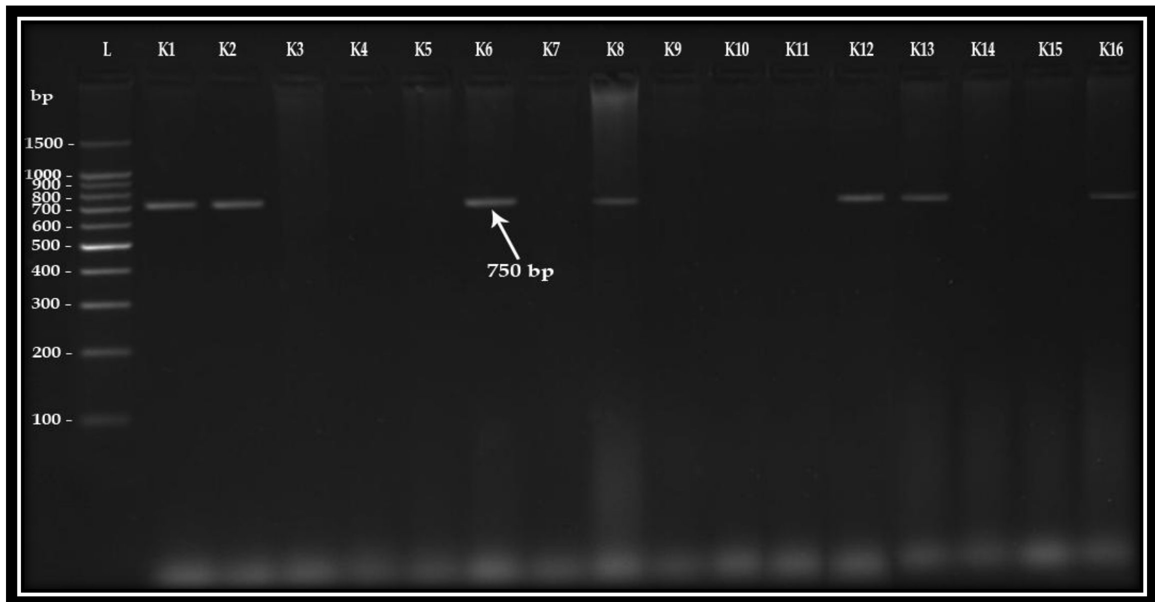

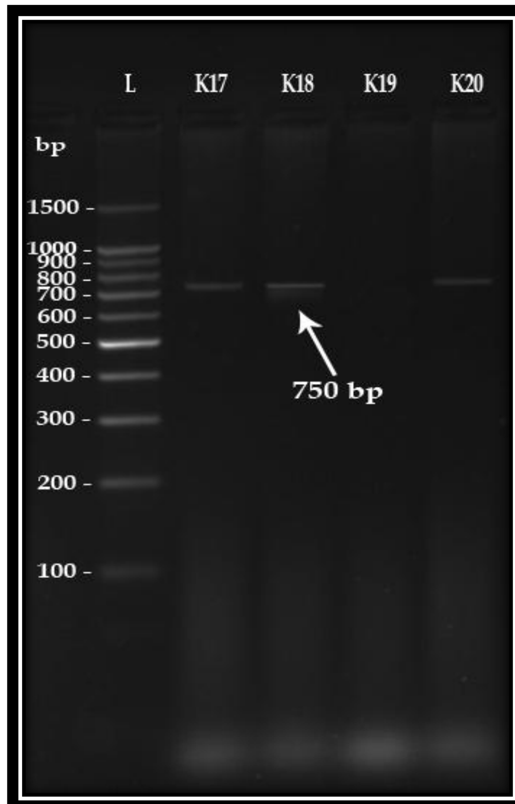

Figure (4-21): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *bla*$_{\text{AmpC}}$ positive *K. pneumoniae* isolates and amplified with primers *bla*$_{\text{SHV}}$ forward and *bla*$_{\text{SHV}}$reverse. The electrophoresis was performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (l500-bp ladder), Lanes (K 1, 2, 6, 8, 12, 13, 16, 17, 18 and 20) show positive results with *bla*$_{\text{SHV}}$ gene (750 bp), Lanes (K 3, 4, 5, 7, 9, 10, 11, 14, 15 and 19) show negative results with *bla*$_{\text{SHV}}$ gene.

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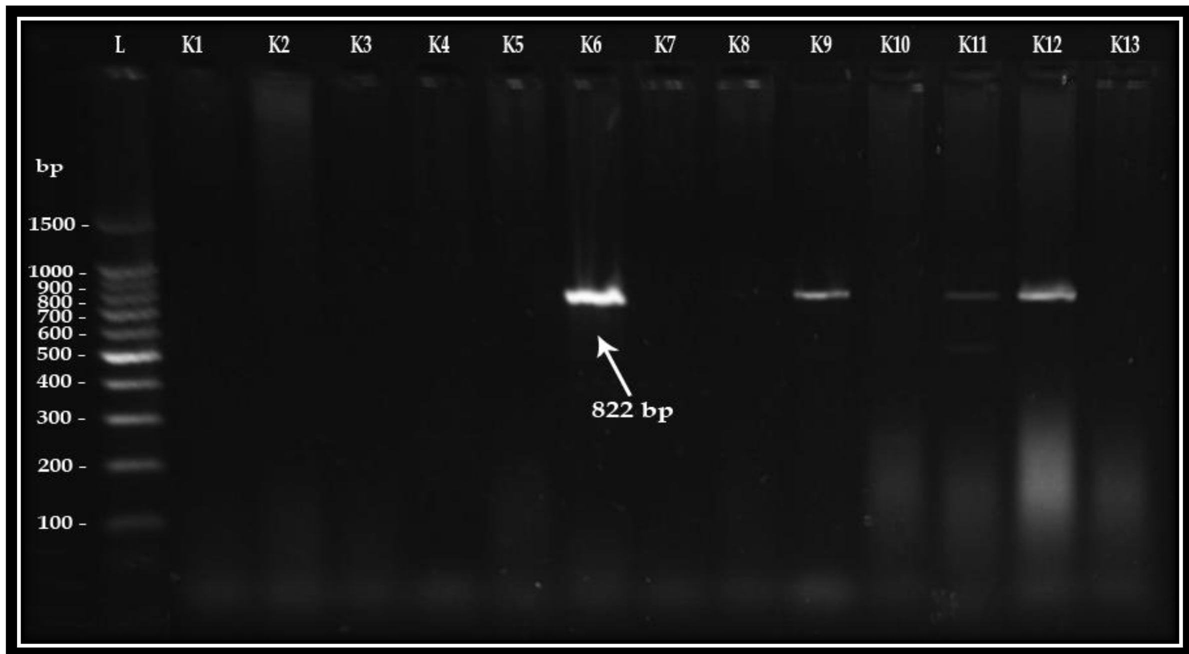

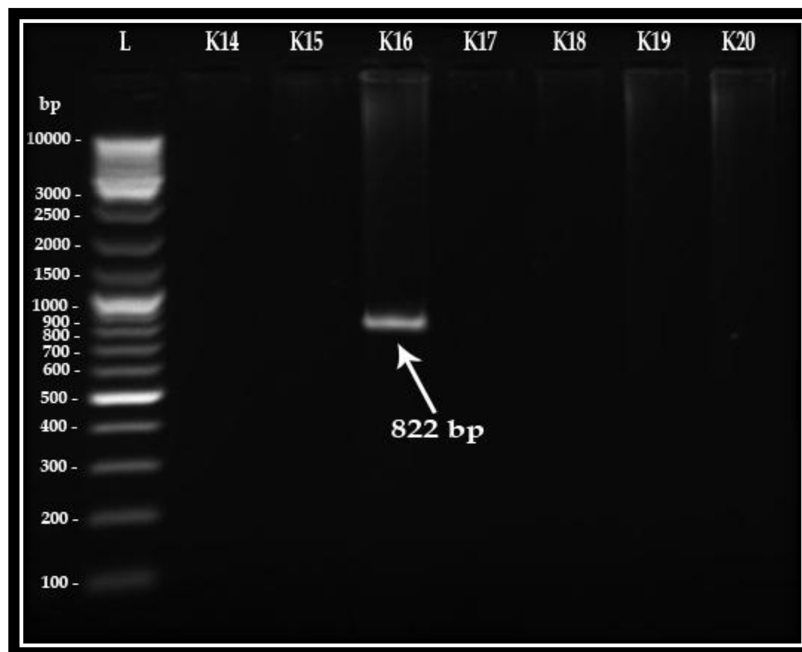

Figure (4-22): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *bla*$_{AmpC}$ positive *K. pneumoniae* isolates and amplified with primers *bla*$_{TEM}$ forward and *bla*$_{TEM}$ reverse. The electrophoresis was performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (1500 and 10000 bp ladder), Lanes (K 6, 9, 11, 12 and 16) show positive results with *bla*$_{TEM}$ gene (822 bp), Lanes (K 1,2, 3, 4, 5, 7, 8, 10, 13, 14, 15, 17, 18,19 and 20) show negative results with *bla*$_{TEM}$ gene.

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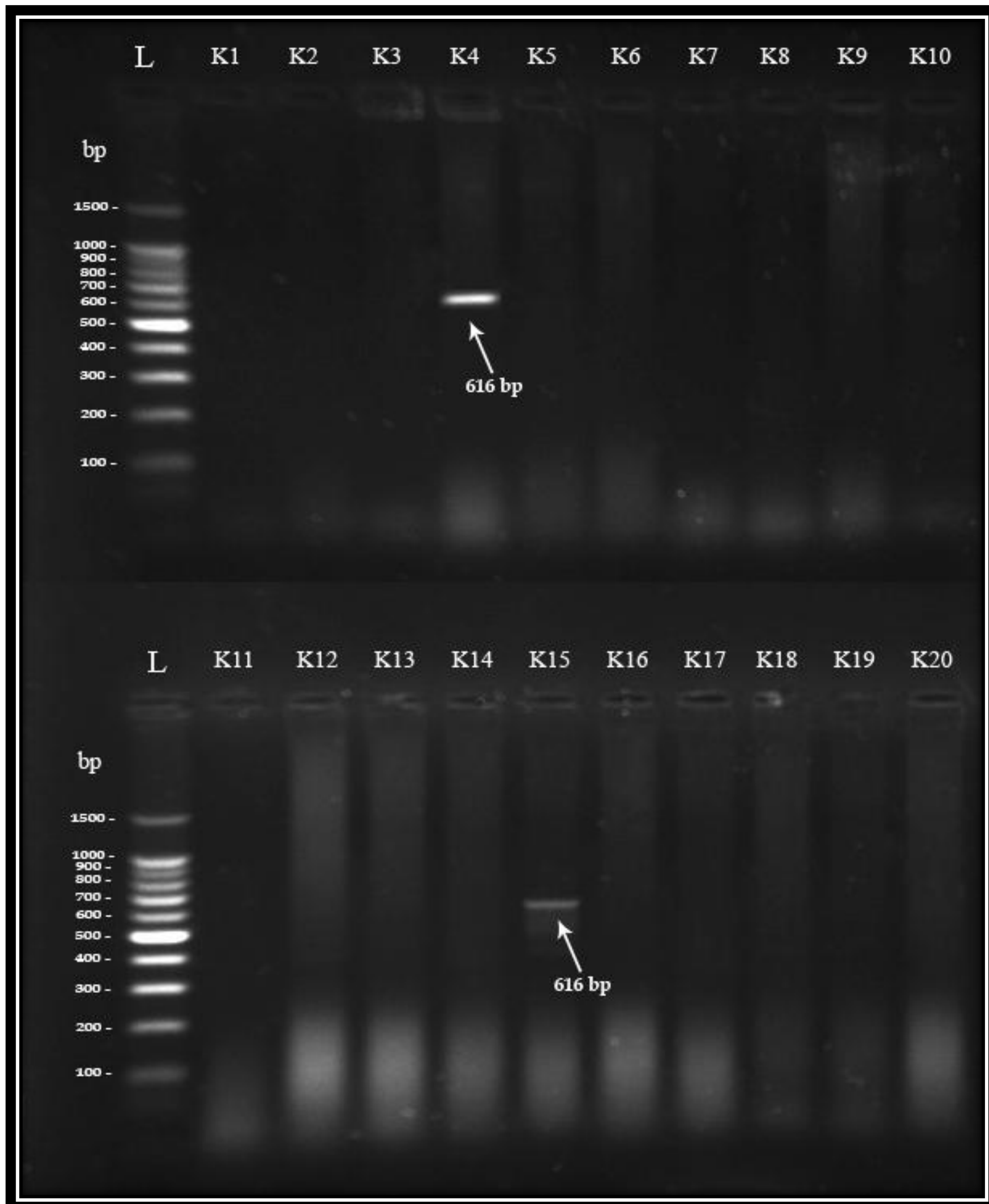

Figure (4-23): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *bla*$_{\text{AmpC}}$ positive *K. pneumoniae* isolates and amplified with primers *bla*$_{\text{OXA}}$ forward and *bla*$_{\text{OXA}}$ reverse. The electrophoresis was performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (l500 bp ladder), Lanes (K 4 and 15) show positive results with *bla*$_{\text{OXA}}$ gene (616 bp), Lanes (K 1, 2, 3, 5, 6, 7,8, 9, 10, 11, 12, 13, 14, 16, 17, 18,19 and 20) show negative results with *bla*$_{\text{OXA}}$ gene.

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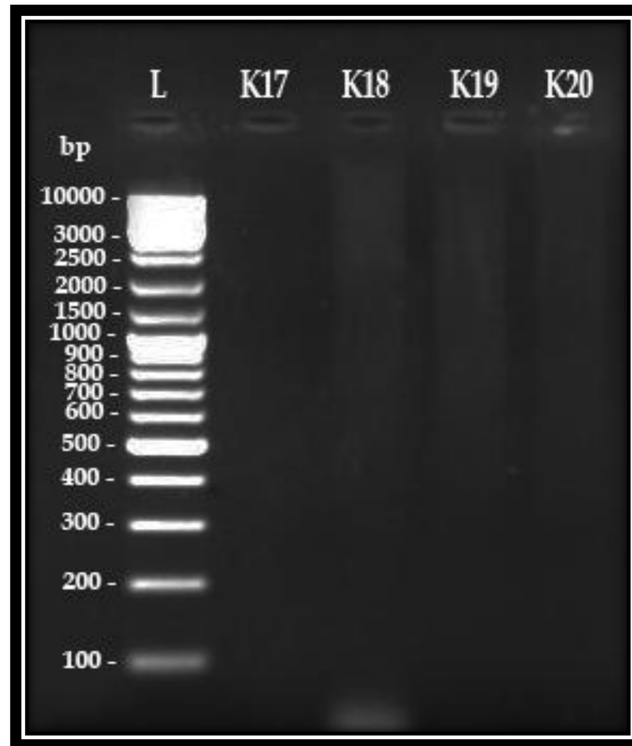

Figure (4-24): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *bla*$_{AmpC}$ positive *K. pneumoniae* isolates and amplified with primers *bla*$_{VEB}$ forward and *bla*$_{VEB}$ reverse. The electrophoresis was performed at 70 volt for 1.5 hr. Lane (L), DNA molecular size marker (10000 bp ladder), Lane (K9) shows positive result with *bla*$_{VEB}$ gene (961bp), Lanes (K1, 2, 3, 4, 5, 6, 7,8, 10, 11, 12, 13, 14, 15, 16, 17, 18,19 and 20) show negative results with *bla*$_{VEB}$ gene.

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4.16. Carbapenemase Production in *bla*$_{AmpC}$ Positive *K. pneumoniae* Isolates

Among the 103 β-lactam resistant *K. pneumoniae* isolates, 4 had previously been identified as resistant to both imipenem and meropenem (3.9%) by standard disk diffusion method (Figure 4-2). These isolates(K6, K12, K16, K20) were harbored *bla*$_{AmpC}$ gene (Table 4-16) and recovered from clinical samples. No carbapenems resistance were recorded among isolates obtained from hospital environmental samples. carbapenems resistant isolates were selected here. Production of carbapenemase was confirmed by three different methods, imipenem-EDTA disk, modified Hodge methods and KPC CHROMagar.

Phenotypic detection of metallo-β-lactamases was performed by using the imipenem-EDTA disk method and confirmed by the meropenem MIC test. Only two (50%) isolates (*K. pneumoniae* isolate K6 and K20) demonstrated enhancement of inhibition zone, suggesting the production of metallo-βlactamases, while no remarkable distinct change was noticed in the others isolates, in the PCR experiments using specific primers for *bla*$_{IMP}$ and *bla*$_{VIM}$. Negative results were recorded among all carbapenems resistant *K. pneumoniae* isolates (Table 4-20).

When the modified Hodge test was used as a phenotypic confirmatory method for both KPC and metallo-β-lactamases-production, presence of a distorted inhibition zone was interpreted as a positive result for carbapenem hydrolysis screening. However, this test was positive for all (n=4, 100%) carbapenems resistant *K. pneumoniae* isolates, indicating carbapenemase production (Table 4-20 and Figure 4-25).

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Table (4-20): Numbers and percentages of carbapenemase producing isolates of bla_{AmpC} harbored *Klebsiella pneumoniae*

Source of sample	No. of Carbapen-em disk resistance isolates	No. (%) of phenotypic confirmed carbapenemase producer isolates			Molecular detection of carbapenemase		
		Imipenem-EDTA disk test	Modified Hodge test	KPC* CHROM-agar techinque	bla_{IMP}	bla_{VIM}	bla_{KPC}
Clinical	4	2 (50%)	4 (100%)	4 (100%)	-	-	-
Hospital environment	0	0 (0%)	0 (0%)	0 (0%)			
Total	4	2 (50%)	4 (100%)	4 (100%)	0 (0%)	0 (0%)	0 (0%)
L.S.D. (0.05) Samples = 9.143, Methods = 8.942							

* KPC: *K. pneumoniae* carbapenemase

The KPC supplemented CHROMagar test (Figure 4-26) was also evaluated for detection of KPC-mediated resistance in all *K. pneumoniae* isolates positive to modified Hodge test. The isolates were able to give overnight heavy growth onto the modified Hodge medium. Isolates that yielded a positive result with the KPC supplemented CHROMagar test underwent KPC polymerase chain reaction (PCR) testing to confirm the presence of a KPC β-lactamase. No isolate of *K. pneumoniae* was found to harbour the bla_{KPC} gene. Using modified Hodge test and KPC CHROMagar technique were more significant ($P < 0.05$) than imipenem-EDTA disk test for phenotypic carbapenemase detection.

4.17. Combination of β-lactamases and Plasmied-Mediated *ampC* Genes

The relatedness between plasmid *ampC*-gene families and other *bla*-genes in the 20 bla_{AmpC} harbouring *K. pneumoniae* isolates was investigated.

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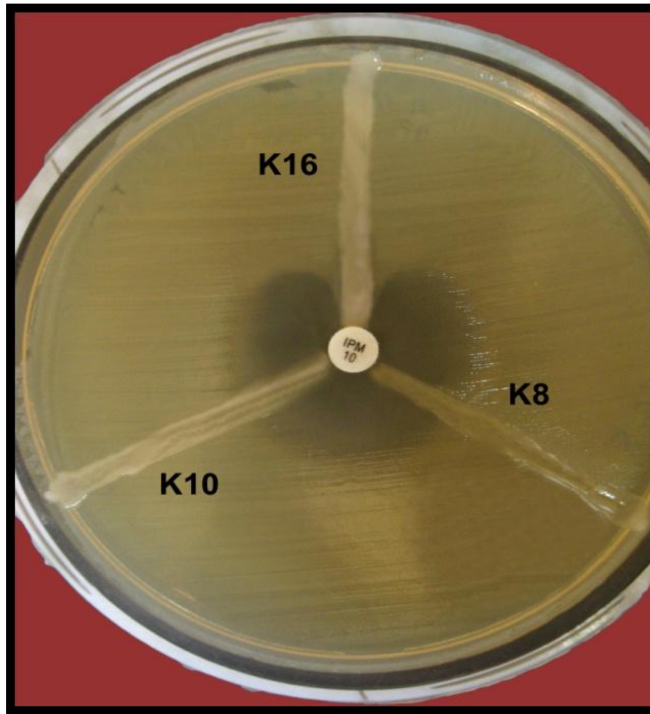

Figure (4-25): Carbapenmase producing *Klebsiella pneumoniae* isolate by modified Hodge test. **Growth of *E. coli* ATCC 25922 strain around straight line of test isolates, K16 isolate, exhibits clear distortion of the inhibition zone of imipenem disk. K8 and K10 isolates exhibit negative result, (IPM: imipenem). Plate incubated at 37°C for 24hr.**

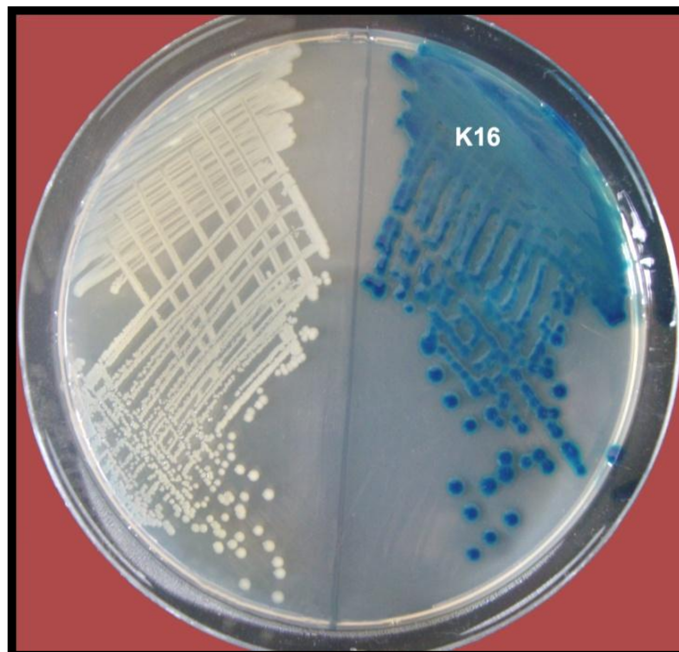

Figure (4-26): Carbapenmase producing *Klebsiella pneumoniae* isolate by KPC CHROMagar medium, **K16 test isolate exhibits blue growth indicate to KPC positive on the right side, and KPC positive of non fermentative Gram negative bacteria exhibit white to creamy growth on the left. Plate incubated at 37°C for 24hr.**

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As shown table (4-21), distribution of the ESBL genes among isolates were so varied, when 16 (80.0%) of the isolates carried at least one type of ESBL *bla*-genes. PCR assay revealed that 3 (15.0%), 2 (10.0%), 11 (55.0%), 2 (10.0%), and 2 (10.0%) isolates carried 1, 2, 3, 4, and 6 ESBL and plasmid mediated AmpC *bla*-genes, respectively.

Detection of carbapenemases production was done previously by phenotypic (imipenem-EDTA disk, modified Hodge, and KPC CHROMagar) methods. Moreover, PCR amplification with primers derived from the most usual carbapenemases (KPC, IMP and VIM) was used as a primary attempt to identify the genes responsible of the detected carbapenemase activity. In the present study, no amplification of any carbapenemase genes was detected (Table 4-21).

4.18. Partial purification of AmpC β-lactamase and Enzymatic Activity

AmpC β-lactamases of one isolate (K19) belonged to bla_{FOX} and bla_{CIT} positive *K. pneumoniae* was extracted and partially purified.

Result in table (4-22) revealed that the isolate was resistant to cefoxitin, gave positive results to all three phenotypic AmpC β-lactamases production, harbored bla_{FOX} and bla_{CIT} genes, but was negative for ESBL and carbapenemase production. During the purification steps, penicillin G were used for enzymatic activity assay. According to what is mentioned above, the enzymatic activity, specific activity and total activity of the sonicated crude extract centrifugation were 1.1 U/ml, 0.18 U/mg and 44 U, respectively as shown in Table (4-23).

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Table (4-21): Dissemination of *bla* genes among *ampC*-gene possessed *Klebsiella pneumoniae*

Isolate symbol	Plasmid mediated *ampC*-genes						ESBL genes							Carbapenemase genes			Total genes
	bla$_{FOX}$	*bla*$_{CIT}$	*bla*$_{DHA}$	*bla*$_{EBC}$	*bla*$_{ACC}$	*bla*$_{MOX}$	*bla*$_{CTX-M}$	*bla*$_{SHV}$	*bla*$_{TEM}$	*bla*$_{OXA}$	*bla*$_{VEB}$	*bla*$_{PER}$	*bla*$_{GES}$	*bla*$_{KPC}$	*bla*$_{IMP}$	*bla*$_{VIM}$	
K1	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	3
K2	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	3
K3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
K4	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	3
K5	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	3
K6	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	4
K7	-	+	-	-		-	+	-	-	-	-	-	-	-	-	-	2
K8	+	+	-	-	-	-	+	+		-	-	-	-	-	-	-	4
K9	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	3
K10	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
K11	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	3
K12	+	+	-	+	-	-	+	+	+	-	-	-	-	-	-	-	6
K13	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	3
K14	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	1
K15	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-	3
K16	+	+	-	+	-	-	+	+	+	-	-	-	-	-	-	-	6
K17	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	3
K18	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	3
K19	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
K20	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	3

isolates

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Total	11	8	4	4	1	0	14	10	5	2	1	0	0	0	0	0	

Table (4-22): Phenotypic and molecular description of *ampC*-gene positive *Klebsiella pneumoniae* isolate K19.

Isolate symbol	AmpC β-lactamase				ESBL		Carbapenemase	
	Type of *ampC*-genes	Phenotype			ESBL *bla*-genes	Phenotype	Carbapenm-ase genes	Phenotype
		MTDT*	DBIT**	AmpC disk				
K19	Multiple gene of bla_{FOX} and bla_{CIT}	+	+	+	-	-	-	-

***MTDT: modified three dimensional test **DBIT: disk based-inhibitor test**

Protein in the sonicated crude extract centrifugation was precipitated by adding up to 30% ammonium sulfate and then to 75% of saturation. The enzymatic activity, specific activity, total activity, purification fold and yield of AmpC β-lactamases in this step of

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purification were as 3.4 U/ml, 0.32 U/mg, 8.5 U, 1.7 fold and 19.3%, respectively (Table 4-23).

AmpC β-lactamases extracted from *K. pneumoniae* isolate K19 obtained by the above step was dialyzed and then purified by the gel filtration chromatography on sephadex G75 column. The equilibration and elution were carried out with sodium phosphate buffer of pH 7.0. However, a typical enzymatic activity and protein elution profile with the wave length of 280 nm is shown in Figure (4-27). Scanning of collected fractions by measurement of absorbance revealed that an increase in protein absorbance at a fraction number 13, then decreased gradually. On the other hand, two peaks of homologous enzyme activities were separated and enclosed at fractions number 12-15 and 19-21 for first and second peaks, respectively.

Table (4-23): Purification steps of AmpC β-lactamase extracted from *Klebsiella pneumoniae* K19 isolate

Purification step	Volume (ml)	Activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purificatio n fold	Yield (%)
Crude extract centrifugation	40	1.1	6	0.18	44	1	100
Precipitation by (30-75%) of $(NH4)_2SO_4$	2.5	3.4	10.5	0.32	8.5	1.77	19.31
Gel filtration chromatograph y (Sephadex G75)							
First peak	12	2.2	0.28	7.8	26.4	43.3	60
Second peak	9	0.54	0.20	2.7	4.86	15	11

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Enzymatic activity, specific activity, total activity, purification fold and yield of the *K. pneumoniae* isolate K19 protein-enzyme(s) for gel filtration chromatography sachem are shown in Table (4-23). The yield of protein (total) from 6 mg/ml of crude extract centrifugation was 0.28 mg/ml and 0.2 mg/ml for first and second peaks, respectively. The purification fold of enzyme(s) was 43.3 and 15 fold for first and second peaks, respectively, as compared with the starting material (crude extract centrifugation). Results, showed that enzymatic and specific activities of the *K. pneumoniae* isolate K19 enzyme(s) varied from 2.2 U/ml and 7.8 U/mg at the first step of purification to 0.54 U/ml and 2.7 U/mg for first and second peaks, respectively, at the final step of purification. Results also showed that the yield of the *K. pneumoniae* isolate K19 enzymatic activity ranged from 100% of bacterial crude extract centrifugation to 60% (for first peak) and 11% (for second peak) in the final purification step (Table 4-23).

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4.19. Description of AmpC β-Lactamase

4.19.1. Effect of Temperature and pH on AmpC β-Lactamase Activity

Optimal activity of partial purified AmpC β-lactamase was found at 37°C, using penicillin G as a substrate. Below optimum temperature, the rate of reaction increased as the temperature rises. Above the optimum, a sharp falling off of reaction occurred when the activity decreased slightly at 40°C and dropped at higher temperature (Figure 4-28). Figure (4-29) represents the relationship between partial purified AmpC β-lactamase activity and pH. The optimum activity was detected at plateau of pH 7.5. While at pH values higher than 7.5 reaction rates decreased sharply.

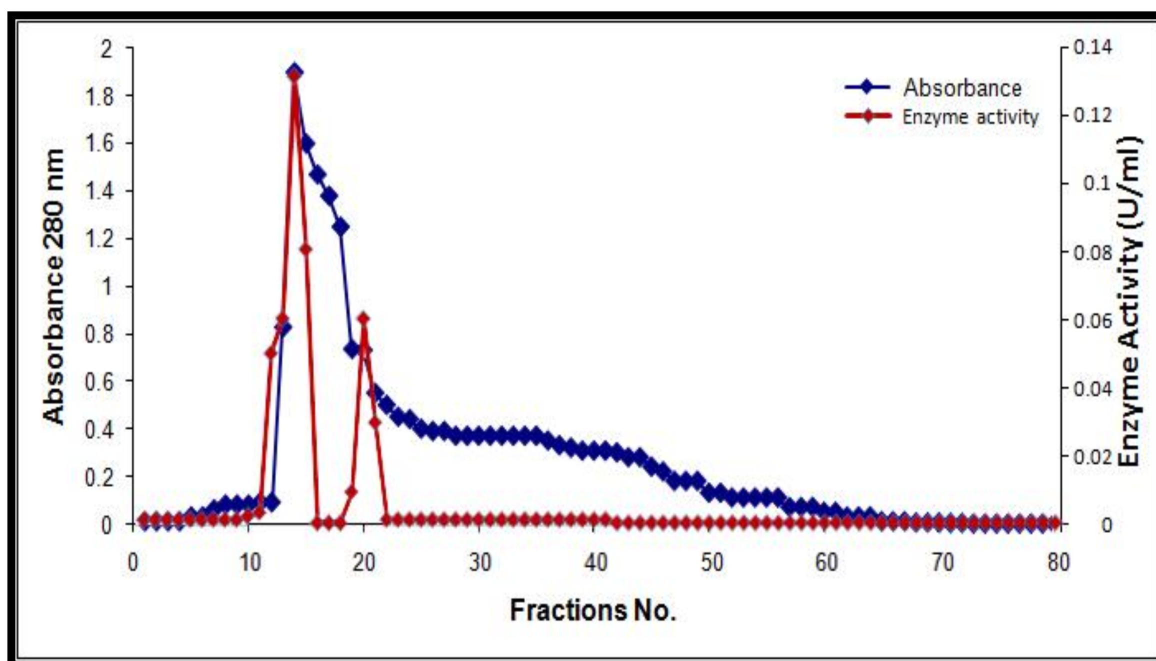

Figure (4-27): Gel filtration chromatography for AmpC β-lactamase extracted from PABL positive *Klebsiella pneumoniae* K19 isolate, using sephadex G75 column with dimensions (1.5 x 45 cm), column equilibrated by 0.05M sodium phosphate buffer (pH 7.0) at a flow rate of 12 ml/hr the eluted fractionated size was 3 ml for each fraction.

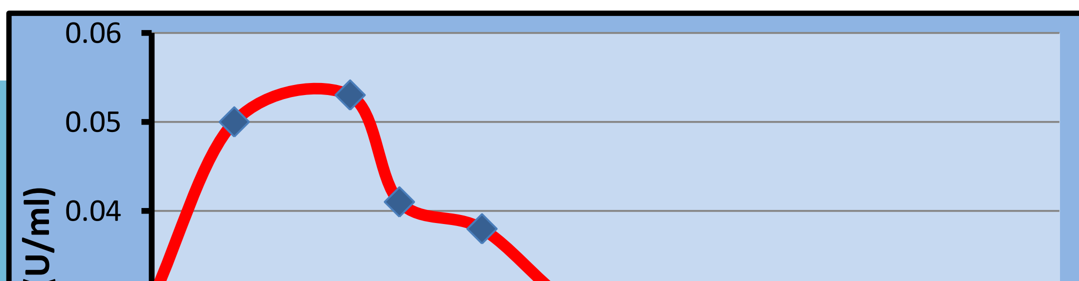

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Figure (4-28): Effect of temperature on the AmpC β-lactamase activity extracted and partially purified from *ampC*-gene positive *Klebsiella pneumoniae* K19 isolate.

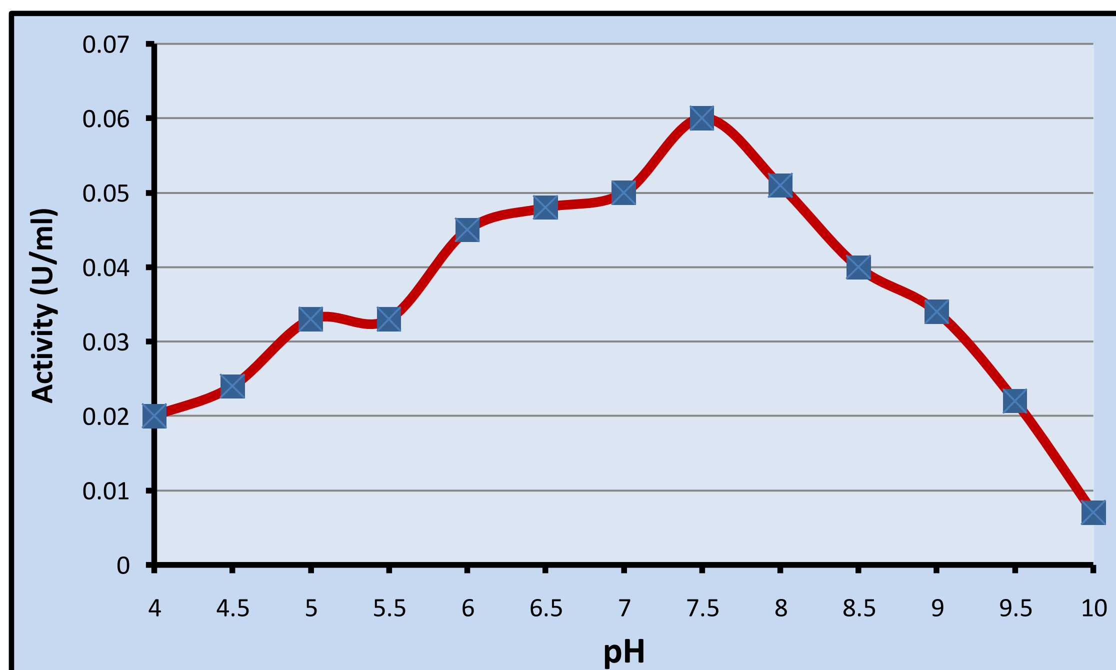

Figure (4-29): Effect of pH on the AmpC β-lactamase activity extracted and partially purified from *ampC*-gene positive *Klebsiella pneumoniae* K19 isolate at 37^{0}C.

4.19.2. Kinetic of Partial Purified AmpC β-Lactamases

Kinetic parameters of the various β-lactam antibiotics hydrolyzed by partial purified AmpC β-lactamase are shown in Table (4-24). The

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kinetic values for each of the antibiotics was calculated mathematically between the reciprocal of enzyme rate and 1/V and reciprocal of various concentrations of the antibiotics 1/[S]. The Lineweaver-Burk reciprocal plot method was adopted to determine the kinetic parameters in which comprise K_m , V_{max} and hydrolysis efficiency (relative V_{max} / K_m) (Figure 4-30). Penicillin G was used as a standard antibiotic used in the calculation of antibiotics relative values. Generaly, the partial purified AmpC β-lactamases hydrolyzed penicillin G was more rapid (V_{max} was 0.02 µmol/min), and high affinity (low K_m, 2.8 mM). Relatively, the catalytic efficiency of partial purified AmpC β-lactamases against penicillin G was set as 100%.

The kinetic properties of partial purified AmpC β-lactamase toward cfotaxime were elevated. Table (4-24) revealed that AmpC enzyme exhibited relatively high values of K_m (3.36 mM) and V_{max} (0.045 µmol/min) compared to penicillin G, However, the hydrolysis rates was very high (191.32%). The K_m of meropenem (9.3mM) was much higher than that penicillin G. Eventhough, the V_{max} for meropenem (0.03 µmol/min) was lower than those for other tested substrates. Same table shows that the hydrolysis of meropenem by partial purified AmpC β-lactamase was too slow (46.08%).

Table (4-24): Kinetic properties of partial purified AmpC β-lactamases for selected β-lactam antibiotics

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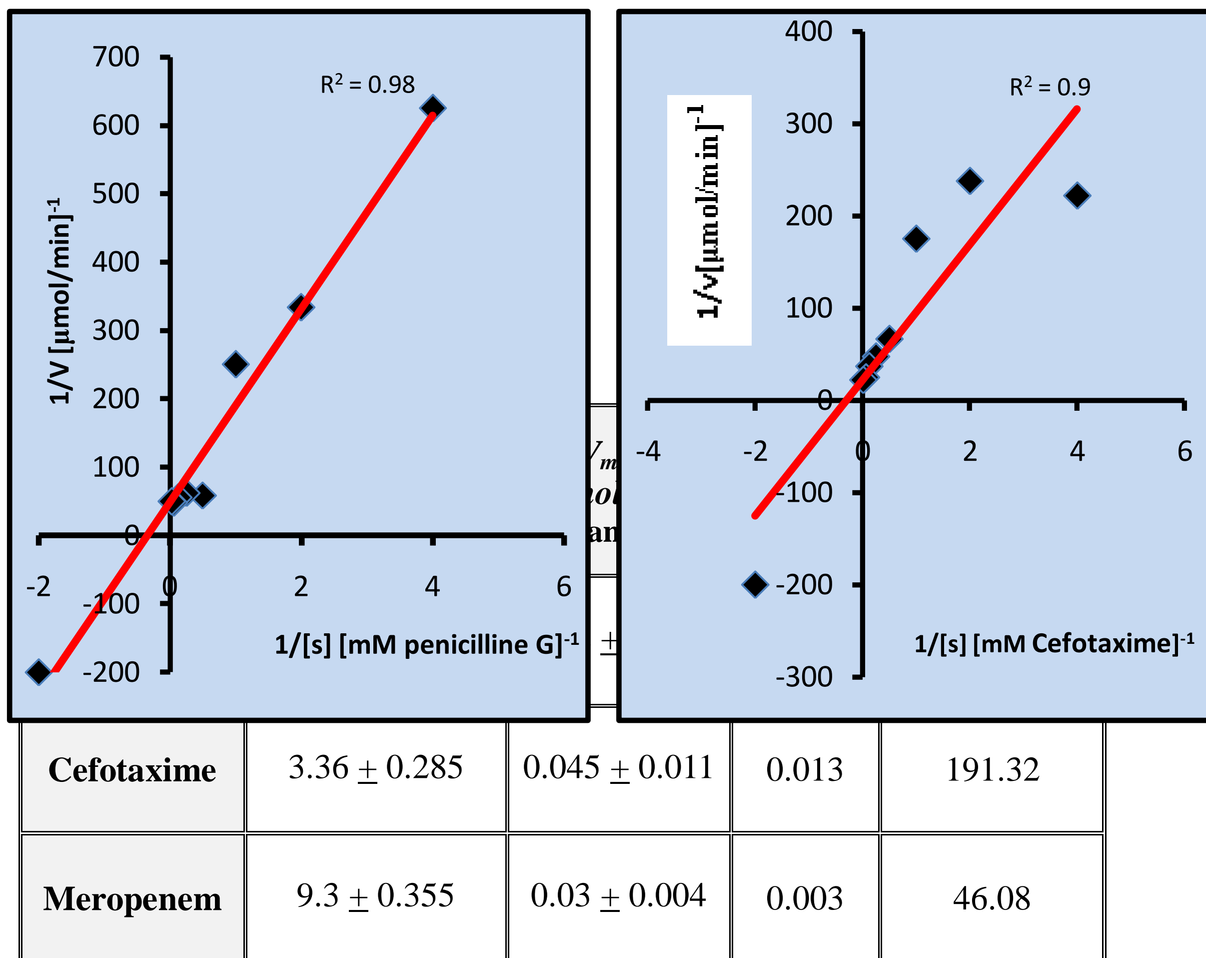
*K_m : Michaelis constant, V_{max} : maximum velocity at saturating concentration of substrate. **values relative with penicillin G which was set at 100% ,37°C and pH 7.5, SD: standard deviation.



Cefotaxime	3.36 ± 0.285	0.045 ± 0.011	0.013	191.32
Meropenem	9.3 ± 0.355	0.03 ± 0.004	0.003	46.08

(A) **(B)**

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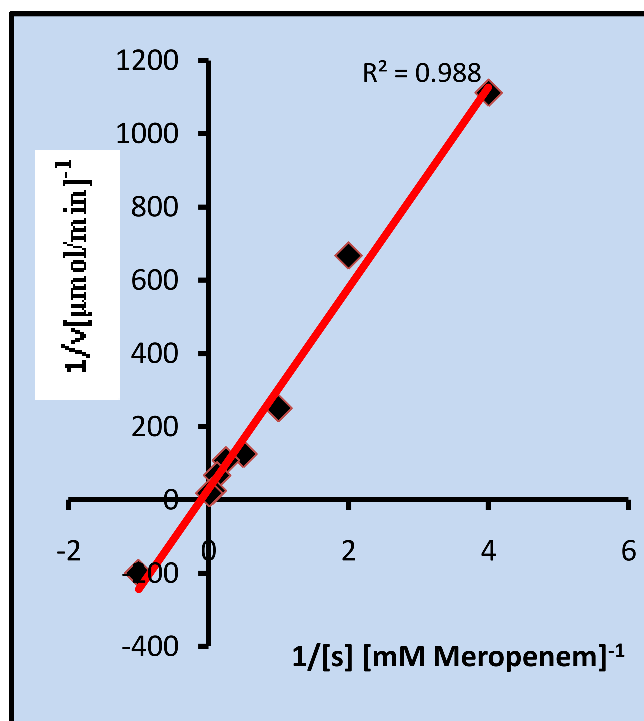

(C)

Figure (4-30): Kinetic parameters plot of partial purified AmpC β-lactamases at 37^{0}C and pH 7.5, extracted from PABL positive *Klebsiella pneumoniae* isolate K19 with different substrates, calculated by the Lineweaver-Burk method: (A); penicillin, (B); cefotaxime, (C); meropenem.

5.1. Survey of *K. pneumoniae* Isolates

Results of cultural, microscopical and biochemical characterization revealed that presence of 217 (21.4%) isolates belongto *Klebsiella* spp. (Table 4-1). Of these 182 (23.6%) and 35 (14.3%) from clinical and hospital environmental sources, respectively. This result corresponds with many studies that focused on predominant of *Klebsiella* spp. among clinical and hospital environmental samples. In a local study by Al-Charrach (2005), 29 *Klebsiella* spp. isolates were obtained from 209 clinical samples in Hilla. While, Hadi (2008) found that *E. coli* was the most common (42.6%) organism isolated from patients with significant bacteriuria followed by *Klebsiella* spp. In the United States, Sahly and

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Podschun (1997) comprise *Klebsiella* spp. as 3 to 7% of all nosocomial bacterial infections, placing them among the eight most important pathogens in hospitals. The basic pathogenic reservoirs for transmission of *Klebsiella* are the gastrointestinal tract of patients and the hands of hospital personnel's. While, reports mentioned the carrier rates in hospitalized patients are 77% in the feces, 19% in the pharynx and 42% on the hands of patients (Podschun and Ullmann, 1998).

Klebsiella pneumoniae was detected in 130 (12.8%). However, 108 (14.0%) isolates were identified in clinical samples (n=770) alone (Table 4-1).

Feglo *et al.* (2010) found that *Klebsiella* spp. *K. pneumoniae* was the highest (74.1%) identified species in 205 clinical samples, followed by *K. oxytoca* (24.4%). As a result, *K. pneumoniae* is a well known a prominent nosocomial pathogen mainly responsible for urinary tract, respiratory tract, or blood infections. However, the great majority of infections are hospital-acquired pneumonia (Brisse *et al.,* 2009). Moreover, its high prevalence in clinical samples may be due to thes producing exhibit different virulence factors, such as capsular polysaccharides, type 1 and type three adhesions. The relevance of pili to bacterial virulence is thought to arise mainly from binding of the bacteria to mucus or to epithelial cells of the urogenital, respiratory, and intestinal tracts (Damian *et al.,* 2009).

From the perspective of the world community, acquired lower respiratory tract infections are an important cause of morbidity and mortality for all age groups. Each year, approximately 7 million people die as a direct consequence of acute and chronic respiratory infection (WHO, 1995). Lower respiratory tract infections are very common, with an incidence in the world population of 4-5%. Since the etiology agents of lower respiratory tract infections cannot be determined clinically,

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microbiological investigation is critical for both treatment and epidemiological purpose (Ozyilmaz *et al.,* 2005). In the present study (Table 4-2) from a total of 450 lower respiratory tract infected patients 72 (16.0%) *K. pneumoniae* isolates were obtained. The National Nosocomial Infected Surveillance (NNIS) of the USA reported that 60.0% of nosocomial lower respiratory tract infections is caused by aerobic Gram-negative bacteria (Veena Kumari *et al.,* 2007). In Najaf, Al-Muhannak (2010) the occurrence of Gram-negative in the lower respiratory tract of hospitalized and outpatients, isolation rate of *K. pneumoniae* has been reported 34.9%. Additionally, the prevalence of respiratory isolates of *Klebsiella* spp.in som asian conteries varied from 10.8% in Thailand (Srifuengfung *et al.,* 2005) to 15.0% in China (Ding *et al.,* 2009) and up to 37.1% in India (Taneja *et al.,* 2009)

Results of Table (4-2) showed that from 210 patients with significant bacteriuria, 9.5% *K. pneumoniae* isolates were obtained. Hadi, (2008) found that *K. pneumoniae* comprised 23.1% of all nosocomial bacterial infection of the urinary tract infection In Najaf city.

Regarind burning wound infection, *K. pneumoniae* was reported in 17 (15.5%) of the 110 burn wound samples. It is an opportunistic pathogen found along with other bacteria as part of the transient normal flora of the human skin, when the host is immunocompromized, as in the case of a thermal burn or surgical wound(Obiaguru *et al.,* 2010). These opportunistic bacteria can quickly colonize and infect the burn and wound sites. Increasing incidence of *Klebsiella* spp. in wound infections was observed by researchers. Al-Muhannak (2010) and Kaur *et al.* (2006) demonstrated that prevalence rates of *Klebsiella* spp. isolate from burn wound infections were 30.6% and 7.5% , respectively. *Klebsiella* spp. is regarded simply as transient members of the flora. The carrier rates change drastically in the hospital environment, where colonization rates

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increase in direct proportion to the length of stay. Even hospital personnel have elevated rates of *Klebsiella* carriage (Podschun *et al.,* 2001).

Klebsiella pneumoniae has factors that seem to be composed of either capsule-like extracellular material or fimbrial KPF-28 and non-fimbrial adhesions CF29K involved in aggregative adhesions (Huang *et al.,* 2009). Subsequently, the biofilm formation may increase the aggregative adherence on the medical devices and hospital furniture surfaces. All these factors may contribute to the *K. pneumoniae* prevalence among hospital staff, sub staff, and patients or through the hospital devices and instruments. However, the results of the present study showed that out of all the hospital environmental culture positive, 9.0% exhibited *K. pneumoniae* isolates. In a study on the occurrence of Gram-negative pathogens in Marjan hospital, Babylon city in Iraq, carried out by Al-Hilli (2010), *K. pneumoniae* the most occurrence organism (13.3%) followed by *E. coli* (20%), while Ali Shah *et al.* (2003) reported that of the 200 nosocomial *Enterobacteriaceae* isolates collected from different hospital wards in Pakistan, *E. coli* was the most frequent (35.0%) followed by *K. pneumoniae* (25%).

The resent study also revealed that other Gram-negative bacterial isolates were frequented as (32.2%) in the clinical and hospital environmental samples (Table 4-1). There is no doubt that hospitals are the typical environments for the presence of pathogens like *E. coli, Klebsiella, Proteus, Morganella, Enterobacter, Citrobacter, Serratia, Acinetobacter,* and *Pseudomonas* spp. (Kucukates and Kocazeybek, 2002). However, the study revealed that 37.1% and 16.7% of Gram-negative isolates were recovered from clinical and hospital environmental samples, respectively. However, the dissemination of Gram-negative bacteria in clinical and environmental samples may be due to its ability to cause different nosocomial infections and resistance to awide range of

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antibiotics. However, some authors claim that the source of Gram-negative infection perhaps from endogenous routes (from the own gastrointestinal flora) rather than the exogenous routes via sink taps and hands of hospital personnel (Thomson *et al.,* 2004). Hence, the food could be a potential source for acquisition of this opportunistic pathogen.

Identification of *Klebsiella* to the species and subspecies levels by the conventional identification tests remains difficult in the clinical microbiology laboratory (Monnet and Freney, 1994). Therefore, in this study the multi test API 20E system was used to confirm subspecies identification and to avoid the variability in findings of biochemical tests. Results confirmed that *K. pneumoniae* subspecies *pneumoniae* was predominant subspecies (85.4%), followed by *K. pneumoniae* subspecies *ozaenae* (14.6%) (Figure 4-1). Monnet and Freney (1994) identified 198 non motile strains of clinical origin by API 20E, API 20EC, API 50 CH, API 50 AO, and API 50 AA systems, and reported the following subspecies: 101 strains of *K. pneumoniae* subsp. *pneumoniae*, 2 strains of *K. pneumoniae* subsp. *ozaenae* and 2 strains of *K. pneumoniae* subsp. *rhinoscleromatis*. In a study in Babylon city, Al-Charrach (2005) found that *K. pneumoniae* subsp. *pneumoniae* was the most frequently 87% occurring subspecies in clinical and environmental samples followed by *K. pneumoniae* subsp. *ozaenae* (9.5 %) and *K. pneumoniae* subsp. *rhinoscleromatis* (3.5 %). In this regard Brisse *et al (*2009) related the low percent (14.6%) of *K. pneumoniae* subsp. *ozaenae* and absence of *K. pneumoniae* subsp. *rhinoscleromatis* in their study, to the differently in separated from *K. pneumoniae* by DNA relatedness. For this reason, *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* were treated as subspecies of *K. pneumoniae* in the early editions of the Bergey's Manual. However, *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* are causative agents for a certain

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disease of ozena and rhinoscleroma, respectively. The reason after investigating such diseases and theicausative may be due to the very rare occurrence of there two diseases, th the specific pathologic-anatomic changes often (Goldstein *et al.,* 1978; Sahly and Podschun, 1997).

French and Ransjo (2006) accounted that reduction of gastric pH due to some drugs is associated with colonization of the upper respiratory tract and oropharynx by aerobic Gram-negative bacteria derived from the patients own bowel. These microorganisms may then pass into the lower respiratory tract and cause infection. These microbes may also be introduced into the respiratory tract via contaminated equipments or the hand of staff (Dancer, 2004).

Results ensured that 14.4% of *K. pneumoniae* subsp. *pneumoniae,* and 1.6% of *K. pneumoniae* subsp. *ozaenae* of sputum samples (Table 4-2). Al-Muhannak (2010) found that the detection rates of *K. pneumoniae* subsp. *pneumoniae* and *K. pneumoniae* subsp. *ozaenae* in patients with lower respirtory tract infections in Najaf were 11.94% and 6.72%, respectively. In humans, they may colonize the pharynx, or gastrointestinal tract. Whereas, Oropharyngeal carriage with endotracheal intubation, impaired host defenses, and antimicrobial use (Osazuwa *et al*., 2010). Subsequently, infection with this organism occurs in the lungs, where they cause destructive changes. Necrosis, inflammation, and hemorrhage occur within lung tissue, sometimes producing thick, bloody, mucoid sputum described as currant jelly sputum (Chan *et al*., 2009).

Results of Table (4-2) also showed that 8.1% of *K. pneumoniae* subsp. *pneumoniae* and 1.0% of *K. pneumoniae* subsp. *ozaenae* were recovered from urine of patients with urinary tract infection patients. Felgo *et al.* (2010) obtained only 1(0.5%) isolates of *K. pneumoniae* subsp. *ozaenae* from 205 *Klebsiella* spp. from clinical samples. This may be regarded *K. pneumoniae* as normal flora in many parts of the colon

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and intestinal tract and in the biliary tract. However, one of the most common reasons of urinary tract infections is the transmission of bacteria from the gastrointestinal tract to the beginning of urethra, especially in the cases of women. In addition, the secreted vaginal fluids nearby urethra, which create a suitable condition, may help in the presence of bacteria predominantly (Wing *et al*., 1999). Moreover, the urine is an appropriate media for the growth and multiplication of bacteria that cause those infections (Bartges, 2007), especially, in the case of infections associated with the use of the urinary catheter techniques (Godfrey and Evans, 2000).

Eventually, the predominant of *K. pneumoniae* subsp. *pneumoniae* in burn wound samples (12.7%) could be due to the ability of *K. pneumoniae* subsp. *pneumoniae* to colonize of sterile wound. However, the damaged infected tissue may cause suppression of the local immunity that increases the chance of infection.

Regarding distribution of *K. pneumoniae* subspecies among hospital environmental samples results showed that *K. pneumoniae* subsp. *pneumoniae* was highly coexisted in hospitals environment. A total of 15 (68.1%) of *K. pneumoniae* subsp. *pneumoniae* isolates and, 7 (31.8%) *K. pneumoniae* subsp. *ozaenae* isolates were obtained from hospital environmentes. Slightly high rates of *K. pneumoniae* subsp. *pneumoniae* and subsp. *ozaenae* isolates in kitchen, bathrooms and ward in comparison with other local environments. On the other hand, the present study revealed that none of the isolates was detected in operation hall, ICU, and burns unit. This may be due to the frequent use of disinfectant in cleaning and available of high care conditions. In the study of Al-Hilli (2010) *K. pneumoniae* isolates were more commonly isolated from the waste containers, bathrooms and kitchen. Osazuwa *et al* (2010) related the presence of *K. pneumoniae* in hospital environment, to its ability to

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adhere on invasive devices, causes contamination of respiratory support equipment, and urinary catheters but also due to the frequent personal use, accumulation of wastes and little of disinfections procedures (Wang *et al.*, 2005).

5.2. Screening for β-Lactam Resistant Isolates

frequency of β-lactam resistance was evaluated after *K. pneumoniae* isolates were primarily screened for resistance using ampicillin and amoxicillin (Bush *et al.*, 1995; Al-Charrakh, 2005). However, the isolate that is resistant to carbenicillin and cephalosporins, is already resistant to ampicillin and amoxicillin. As result of table (4-4), a total of 103 (79.2%) of *K. pneumoniae* isolates were able to grow in presence of ampicillin and amoxicillin. This result agreed that of Hadi (2008) who found that 78.6% of *K. pneumoniae* isolates recovered from patients suffering significant bacteriuria exhibited resistant to both antibiotics, when Al-Hilli (2010) found it 89.3% of *K. pneumoniae* isolated from Merjan hospitals in Hilla were ampicillin and amoxicillin resistant. Perhaps, Gram-negative bacteria are intrinsically resistant to penicillin-G by virtue of their double membrane structure, which prevents the antibiotic from accessing the cell wall target. Moreover, the acquired resistance to β-lactams operates through different mechanisms; production of β-lactamases, changes in the outer membrane permeability or alterations in the PBPs (Wilke *et al.*, 2005). Reduced permeability through porin losing may reduce the steady state of periplasmic drug concentrations and thereby reduces PBP inactivation. Therefore, decreased permeability may act synergistically with the expression of β-lactamases or active efflux to confer higher levels of β-lactam resistance (Livermore and Woodford 2006).

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Table (4-4) show that out 95 (88.0%) of the 108 of *K. pneumoniae* clinical isolates, were resistant to ampicillin and amoxicillin. Such high percentage may be due to frequently use β-lactam antibiotics by patients.

However, the study revealed that only 8 (36.4%) of β-lactam resistant isolates were belonged to hospital environmental samples. The presence of β-lactam resistance among hospital environmental isolates may be due to the predominant exposure of these isolates to antibiotics effect. That makes hospital environment as selective pressure, which causes acquired antibiotics resistance. Additionally, the most nosocomial infections caused by resistant pathogens may arise from patients and carriers that transmitted by person to person or by contaminated of medical equipments (Wang *et al.*, 2005; Al-Hilli, 2010). However, β-lactam resistance mostly associated with transmissible plasmids can be transferred between different bacterial species among hospital isolates (Carattoli, 2008).

5.3. Antibiotic Susceptibility Profile

Reports hypothesized that the versatility of plasmids together with the usage of antimicrobials in human medicine and animal husbandry may have largely contributed to the spread of antimicrobial resistance worldwide (Carattoli, 2008). Results of figure (4-2) show high resistant rates to carboxypenicillin; to (100%) carbenicillin and (78.6%) to ticarcillin. Highe resistance was also found against piperacillin (92.2%). Al-Hilli (2010) reported in Hilla that 100% and 81% of *K. pneumoniae* isolates were resistant to carbenicillin and piperacillin, respectively. Recently, this fact indicates to increment of carboxypenicillin and ureidopenicillins resistant among β-lactam resistant *K. pneumoniae*, may be because the frequently use and abuse of these antibiotics in Iraq.

All tested β-lactam resistant *K. pneumoniae* isolates showed a high degree to moderately resistance to cephalosporins. Cefazolin is a classic

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antibiotic represents the first generation of cephalosporins and the bacterial resistance to cefazolin may arose from the multiuse in hospitals for different infections as parenteral antibiotic. Most the third generation cephalosporins and aztreonam are administrated in a hospital setting. (Nasser *et al.,* 2007). In this regards Cao *et al.,* (2002) found that chromosomally encoded resistance to first generation of cephalosporins in strains of *Klebsiella* spp. has emerged in many hospitals.

During the entire study period, antibiotic susceptibility testing revealed that more than 60% of β-lactam resistant *K. pneumoniae* isolates were resistant to the third generation cephalosporins including ceftazidime, cefotaxime, and ceftriaxone; also, the isolates also showed a wide range of resistance to amoxiclave (73.8%) and aztreonam (60.0%). In Turkey, Gunseren *et al.* (1999) reported that the resistance rates of *Klebsiella* spp. isolates to cefotaxime and ceftazidime were 96.7% and 85.5%, respectively. These antibiotics usually used for treating urinary tract, respiratory tract and burn wound infections caused by *Enterobacteriaceae* (Nasser *et al.,* 2007). The resistance to third generation cephalosporins was caused mainly by mutations in the common group of class A β-lactamases, consisting of TEM, SHV and CTX-M β-lactamases that has extended hydrolytic spectrum activity on cephalosprins, but also a number of other rarer enzymes that often exhibit ESBL activity (Bush *et al.*, 1995). High resistance of ceftazidime and cefotaxime in this study may be related to the production of enzymes named cefotaximases (CTX-M), and these CTX-Ms showed a much higher degree of activity to cefotaxime than to ceftazidime (Walther-Rasmussen and Hoiby, 2004). Moreover, the multiple β-lactamases within a single isolate may confer complex phenotypic expression, like SHV and AmpC enzymes production exhibit resistance to third, fourth generation cephalosporins and aztereonam (Hammond, 2004).

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As showen in figure (4-2), presence of amoxiclave resistant among β-lactam *K. pneumoniae* isolates (73.8%) may indicate to the most β-lactamase inhibitors have a low antimicrobial activity. Nevertheless, they contain β-lactam ring and their sole purpose is to prevent the inactivation of the β-lactam antibiotics. These inhibitors have a strong affinity for the β-lactamases (higher than the β-lactam antibiotics), to which they will attach and form an irreversible union, protecting the β-lactam antibiotics from the hydrolysing action of the β-lactamases (Marti, 2008).

In Iraq, the cefoxitin use is restricted in treatment of bacterial infections, but the study showed that 70.9% of β-lactam *K. pneumoniae* isolates were cefoxitin resistant. Others such as Al-Muhannak (2010) and Al-Hilali (2010) reported similar findings in Najaf. The major causes of cefoxitin resistant in *K. pneumoniae* are encoding of plasmid mediated AmpC β-lactamase and/or reduced outer membrane permeability which might cause the reduce susceptibility of *K. pneumoniae* to cefoxitin (Tan *et al*., 2009).

The results also revealed that imipenem and meropenem were the most effecient β-lactam antibiotics against β-lactam *K. pneumoniae* isolates due to high susceptibility rate (95.1% for each one). Closed results were achieved by Hadi (2008) who found that none of the *K. pneumoniae* isolates recovered from patients with significant bacteriuria in Najaf were resistant to imipenem. This may be because the carbapenems are the treatment of choice and many times used as therapy of last resort for serious infections caused by ESBL or AmpC producing pathogens. Moreover, imipenem is highly stable to hydrolyze by TEM and SHV β-lactamases (Jacoby and Munoz-Price, 2005).

Figure (4-2) shows that 4 (3.9%) of the 103 β-lactam resistant *K. pneumoniae* isolates resisted both imipenem and meropenem. In spite that using these antibiotics is limited in Iraq, carbapenems showed gradual

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development of resistance. However, SENTRY Program data encompassing 5 years (2000-2004) of testing bacteria recovered from various sites of infections (bloodstream, pneumonia, skin and skin structure, and urinary tract) has presented a longitudinal view of this global evolution, reported that greater than 99% of evaluated *Enterobacteriaceae* were susceptible to the imipenem and meropenem. However, carbapenem should be administrated as empirical therapy for Gram-negative infections that are not life threatening because their overuse can pose a significant problem(Deshpande *et al.,* 2006).

Aminoglycosides were relatively effective against β-lactam *K. pneumoniae* isolates tested, hence amikacin was more effecient (30.8% resistance) than gentamicin (50% resistance) and tobramycin (48.5% resistance). High effeciency 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 amikacin were relatively effective against *Klebsiella* spp. isolates from various clinical samples, 40.6% of isolates were susceptible to this antibiotic. In both *Enterobacteriaceae* and other Gram-negative rods, gentamicin and other aminoglycosides resistance, is often due to the expression of a variety of modifying enzymes include aminoglycoside modifying enzymes (AME), acetylases, phosphorylases, and adenylases which can impair the effectiveness of antibiotics. Other resistance mechanisms include changes in bacterial membrane permeability and altered ribosomal proteins (Barros *et al*., 1999).

One of the goals of the present study was to evaluate the antibiotic resistance of clinical and hospital environmental isolates of *K. pneumoniae* to several fluoroquinolones antibiotics. There was a reduction activity of fluoroquinolones including levofloxacin (13.8%), gatifloxacin (15.4%), norofloxacin (23.1%) and ciprofloxacin (26.9%)

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and nalidixic acid (28.5%) against β-lactam resistant *K. pneumoniae* isolates. The resistance against fluoroquinolones in this study may reflect antibiotic pressure in Najaf hospitals. However Ali *et al.* (2010) reported that 72.22% of *K. pneumoniae* (collected from several hospital in Pakistan) were resistant to ciprofloxacin. Quinolones resistance is typically encoded chromosomally. However, the quinolones target is the DNA gyrase in order to inhibit bacterial growth. The mutations arising in the four genes involved, *gyrA, gyrB, parC* and *parE* are known to result in resistance to fluoroquinolones. More recently, plasmid-borne quinolone resistance genes (*qnr*) have been identified (Robiesek *et al.,* 2006). The resistance of β-lactam resistant *K. pneumoniae* isolates against fluoroquinolones in this study may reflect antibiotic pressure in hospitals rather than self-medication of people without obtaining prescriptions from physicians.

Results in figure (4-2) revealed that moderate and low resistant rates against tetracycline, trimethoprim and chloromphenicol (60%, 42.3% and 46.6%, respectively) might be related to β-lactam mult-iresistance, Mooij, (2009) pointed out that the multidrug resistant is mostly due to antibiotics resistance genes which bear on transferable conjugative plasmid, transposons, integrons class 1 or on transconjugants carrying gene cassettes that expression phenotypic multi antibiotics resistance.

The present study concluded that the increase of antibiotics resistance in *K. pneumoniae* isolates is often related to the overdose and mistreatment of the antibiotics prescribed. Heavy and widespread use of antibiotics in hospital does not only force the emergence of antibiotic resistance, but also promotes selection of drug-resistant organisms in the hospital environment (Beneiae *et al.,* 2001). Iraq is one of the developing countries where antibiotics are sold over the counter, an attitude that encourages self-medication. On the other hand, it is remarked that during

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period of time, a group of antibiotics become more used than others without susceptibility tests which may lead to variability in their resistance.

5.4. β-Lactamase Producing Isolates

To determine the susceptibility of specific microorganisms to β-lactam drugs should be examine their ability to produce β-lactamase. More than 60% of isolates were positive by rapid iodometric test (Table 4-5). Similarly, Al-Charrakh (2005) found that 58.5% of 65 β-lactam resistant *Klebsiella* spp. isolates were able to produce β-lactamases. The rapid iodometric method depends on the fact that hydrolysis of penicilloic or cephalospoic acid (products of β-lactamase) reduce iodine to iodide, consequently, decolorization starch-iodine (dark blue) complex occurs in an isolate is a β-lactamase producer but not when the enzyme is absent (Livermore, 1995).

In this investigation, the high dissemination of β-lactamases among *K. pneumoniae* isolates, which could be attributed to the production of either chromosomally constitutive or plasmid mediated β-lactamases (Babini and Livermore, 2000). However, the vast majority of *K. pneumoniae* isolates were resistant to ampicillin and amoxicillin by the production of plasmid-mediated and chromosome-encoded β-lactamases (Sanders *et al.,* 1996).

The results of table (4-5) indicates that 38.8% were negative with iodometric method, suggesting that these isolates may be either having no β-lactamases or production of low quantities of enzymes, making its detection more difficult, like class C β-lactamase in *K. pneumoniae* which produce in low basal level (Jacoby, 2009). On the other hand, the negative results may be due to the β-lactamase in *K. pneumoniae* isolates required more time to destruct their cell wall and released. However, iodometric method is not recommended to detection of β-lactamases in

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Gram-negative bacteria, since these enzymes are produced and secreted extracellularly by Gram-positive, whereas in Gram-negative accumulated in periplasimic space (MacFaddin, 2000). Although, this method is characterized by simplicity, available materials and cheaper than nitrocefin method, nevertheless may give fals positive results because the non-specific reaction of iodine with other bacterial proteins (Al-Charrakh, 2005). Additionally, factors such as temperature and pH may play an important role in the enhancement or reduction of enzyme activity (Foley and Perret, 1962).

Nitrocefin disk is an original test used in the present study, for confirming of β-lactamase production by iodometric method. Nitrocefin is chromogenic β-lactamase substrate (chromogenic cephalosporin) that undergoes distinctive color change from yellow to red at pH 7.0, during the amide bond in the β-lactam ring that hydrolyzed by β-lactamase (MacFaddin, 2000). Table (4-6) shows that 75.7% of β-lactam resistant *K. pneumoniae* isolates were significantly ($P<0.05$) positive with this test as compared with the rapid iodometric method. This may refer to the nitrocefin which is more sensitive to hydrolyzed with all known β-lactamases produced by Gram-negative bacteria (Bebrone *et al.,* 2001). In addition, this method is useful to the detection of β-lactamase patterns from bacterial cell extracts and susceptible for detecting low level of β-lactamases that produced constitutively or by induction in enteric bacteria.

5.5. Extended Spectrum β-Lactamases Production

ESBL-producing organisms are now distributed worldwide and their prevalence is increasing. The prevalence of these organisms varies according to species, antibiotic use, and geographical area. Despite the rise in the prevalence of ESBL in many countries, there are very few reports from Iraq (Al-Charrakh, 2005; Hadi, 2008; Al-Hilli, 2010; Al-

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Muhannak, 2010; A-Hilali, 2010; Belal, 2010; Shamkhi, 2011). ESBL detection is not carried out in many microbiology units in developing countries, including Iraq, and this could be attributed to lack of awareness and the lack of resources and facilities to conduct ESBL identification.

In this research, ceftazidime resistance was used to primarily detection of potential ESBL. However, ceftazidime was chosen to detect ESBL producers for the reason that it is the best third-generation cephalosporin substrates for most TEM, SHV and CTX-M derived ESBLs (Livermore and Brown, 2001). Figure (4-2) revealed that the antimicrobial susceptibility profile against ceftazidime gave higher resistance percentage (66.9%) than other third generation cephalosporins like cefotaxime (65.4%), ceftriaxone (62.7%), and aztreonam (60%). However, ceftazidime is able to expose wide range of resistance isolates. Hence, this antibiotic should be used in early ESBL detection in Najaf hospital laboratories.

Earlier studies on the dissemination of ESBL producing *K. pneumoniae* isolates in Najaf hospitals (using ceftazidime disk) showed that the distribution of ESBL producing isolates during 2008-2010 was 64.3% and 72.1%, respectively (Hadi, 2008; Al-Muhannak, 2010). In India, Chiangjong (2006) found that 95% of *K. pneumoniae* isolates were suspected ESBL producers by using ceftazidime disk as initial screen test. From the results of this study, it can hence be concluded that the use of ceftazidime is the best cephalosporin for primarily screening of ESBLs.

Table (4-7) revealed that all β-lactamase producing *K. pneumoniae* isolates recovered from clinical samples and 75% of the isolates obtained from hospital environmental samples were suspected as ESBL producers. Generally, an isolate is suspected to be an ESBL producer, when it shows *in vitro* susceptibility to the cefoxitin but resistance to the third-generation cephalosporins and to aztreonam (Samaha-Kfoury and Araj,

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2003). Actually, the detection of ESBL by initial screening test perhaps doesn't reflect the fact of enzyme production in individual bacterium, resulted from the ESBL identification may lead to other erroneous phenotypic conclusions because of confounding several factors, such as the production of multiple ESBLs in one bacterium (Svard, 2007). However, Gram-negative bacteria have been isolated with multiple plasmids that carry resistance genes for different classes of antimicrobial agents or different β-lactamases (Bush, 2001).

The identification of ESBL producers is a major challenge for the clinical microbiology laboratory, due to the affinity of ESBL-producing isolates to the different substrates is variable and makes their detection difficult. Additionally, some ESBL isolates may appear susceptible to a third generation cephalosporins *in vitro* (Aggarwal and Chaudhary, 2004; Hadi, 2008).

The utility of CHROMagar technique for the detection of ESBL-producing isolates was first documented in Najaf, and no studies published the use of CHROMagar technique for detecting ESBL-producing *K. pneumoniae* isolates. Thus, table (4-8) revealed that 47.4% of the β-lactamase producing *K. pneumoniae* isolates were confirmed as ESBL producers. This may be due to ESBL CHROMagar allows growing of ESBL-producing organisms only, while inhibiting the growth of other bacteria including those carrying AmpC β-lactamase type. This is an important feature of CHROMagar medium, since intrinsic AmpC β-lactamase has no clinical relevance, but often leads to ESBL false positive reading in the classical testing methods (CHROMagar microbiology /www.CHROMagar.com). Present study concluded that CHROMagar technique is overall cost per single test, but had the advantage of saving time and materials.

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Failure to detect ESBL enzymes has contributed to their uncontrolled spread and sometimes to therapeutic failures (Thomson, 2001). Although, the β-lactam resistance mediated by ESBL is difficult to detect, therefore the Clinical Laboratories Standard Institute recommended more than one confirmatory test for ESBL detection. Disk combination test was performed according to CLSI (2010).

When the disk combination test was performed (Table 4-8), a number of 35/78 (44.8%) *K. pneumoniae* isolates were detected as ESBL producers. The increasing number of *Klebsiella* isolates producing ESBLs in Najaf, leads to limitation of therapeutic options and calls for enhancing surveillance and control measures of hospital acquired infections. Prevalence of ESBL producing *K. pneumoniae* strains causing community acquired urinary tract infections runs to 42% in India (Mohammed *et al.*, 2007) and to 40.3% in Saudi Arabia (Kadar and Angamathu, 2005). However, Obiajuru *et al.* (2010) noticed that 51/150 (34%) of ESBL phenotype positive *K. pneumoniae* were isolated from sputum, urine and wounds. The high occurrence 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.*, 1991; Saurina *et al.*, 2000). However, the dissemination of ESBL producing *K. pneumoniae* isolates in this study may be due to the majority of these enzymes have 1, 2, or 3 amino acid mutation from those of the parent enzymes TEM-1, TEM-2, and SHV-1 (Batchoun *et al.*, 2009). Finaly, the present study suggests that ESBL producing isolates are already endemic in Najaf hospitals.

Same table revealed that ESBL-producing *K. pneumoniae* isolates have significantly ($P<0.05$) more presence among hospital environmental samples than clinical samples in both CHROMagar and disk combination

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methods. This may confirm the fact that most of these enzymes are carried by plasmids and different elements, like insertion sequences and integrons, and they are involved in the mobilization of ESBL genes (Messai *et al.,* 2006). These factors have been facilitated the spread among members of the family *Enterobacteriaceae* and other Gram-negative bacteria. The present study confirmed that detection of ESBL production is of importance in hospital isolates. Firstly, these isolates 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.

Disk approximation 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). In this investigation (Table 4-8) , no ESBL-producing *K. pneumoniae* isolates were detected by disk approximation test. This may be attributed to the fact that presence of ESBLs in a bacterial cell does not always produce a resistance phenotype when using the disk diffusion interpretive criteria published by NCCLS (2003a). Several studies have also shown that the disk approximation test failed to detect some ESBL-producing strains (Coudron *et al*., 1997; Al-Muhannak, 2010). During the study period, 70.9 % of the isolates were cefoxitin resistant, and these isolates are possible AmpC β-lactamase producers, which can mask ESBL production in the disk approximation test. Negative results are supposed to occur if the AmpC activity is larger than activity of ESBL which may lead to failure results (Yan *et al.,* 2002).

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5.6. AmpC β-lactamase Production

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). However, these enzymes are only inhibited by fourth generation cephalosporins and carbapenems (Jacoby, 2009). At present, resistance to carbapenem has been described in *E. cloacae* resulted from, overproducing AmpC β-lactamases combining with the decreased permeability of membrane protein (Li *et al.,* 2009). In this study, cefoxitin was used as a marker for the production of AmpC β-lactamases. Results of figure (4-6) shows high significant ratio ($P<0.05$) of cefoxitin resistant *K. pneumoniae* isolates (70.9%). The frequency of cefoxitin resistance in the present study was higher than previously recorded in Iraq by Al-Hilli (2010) who found that 28.0% of *K. pneumoniae* isolates were resistant to this antibiotic. However, the prevalence of cefoxitin resistance among *K. pneumoniae* isolates varies from country to country, 10.5% in India (Arora and Bal, 2005), 32.1% in Canada (Mulvey *et al.*, 2005) and 9.4% in Korea (Yoo *et al.,* 2010).

In spite of, the restricted use of cefoxitin as prepared treatment in Najaf hospitals, cefoxitin resistant *K. pneumoniae* isolates are disseminating. This may be due to presence of contributed factors like; over expression of chromosomal *ampC* gene, acquisition of a plasmidic

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ampC gene, porin or permeability mutations, or a combination of these factors (Mulvey *et al.,* 2005). Moreover, the high rate of cefoxitin resistance is considered a risky factor that indicates the presence of AmpC β-lactamases producers isolates.

Although, some of AmpC types producing Gram-negative bacteria are susceptible to cefoxitin (Derbyshire *et al.,* 2009), the present study revealed that 18.4% and 10.7% of isolates were found to be susceptible and intermediate resistance to cefoxitin, respectively (Figure 4-6) . In a study reported during the SENTRY Antimicrobial Surveillance Program, cefoxitin was the most successful cephalosporin against European *K. pneumoniae* (4.4% resistance) (Nijssen *et al.,* 2004). The presence of these isolates could be due to the cephamycin included cefoxitin has increased activity against Gram-negative and sometimes cefoxitin is stable to certain β-lactamases and able to pass through the outer cell envelope (Yao and Moellering, 2003).

AmpC β-lactamases are one of the most important β-lactamases in Gram-negative bacteria. Nevertheless, the knowledge about the AmpC β-lactamases is still limited at present. The capability to detect AmpC is important in all hospitals, to improve the clinical management of infections and provide sound epidemiological data. Reduced susceptibility to cefoxitin in the *Enterobacteriaceae* may be an indicator of AmpC activity, but it should be confirmed by other tests. The detection of AmpC β-lactamase is a challenge for clinical laboratories, and there is no Clinical Laboratories Standards Institute (CLSI) guideline for its detection (Nasim *et al.*, 2004). Hence, the study design make a comparison between cefoxitin disk susceptibility and three confirmatory tests for phenotypic detection of AmpC β-lactamase.

The modified three-dimensional test can be used for routine screening of the AmpC enzyme in a clinical laboratory. In one study,

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Coudron *et al*. (2000) reported that the 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. In table (4-9), out of 73 cefoxitin resistant isolates, AmpC β-lactamase production was confirmed in 42.5% by using modified three dimension test. The occurrence of AmpC β-lactamase in *K. pneumoniae* isolates tested may reflect two modes of production: hyper production of chromosome-mediated and plasmid-mediated AmpC β-lactamase. However, a the previous study in India, Singhal *et al.* (2005) reported that 33.3% of cefoxitin resistant *K. pneumoniae* isolates were confirmed as AmpC producer by modified three-dimension test. In another study, Manchanda and Singh (2003) estimated that 25.1% of AmpC producing *K. pneumoniae* isolates (detected by modified three dimension test) were cefoxitin resistant and 7.5% were cefoxitin susceptible.

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). Another 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 increase cefoxitin resistance in *Klebsiella* spp. (Hernandez-Alles *et al.,* 1999). To date, several modifications of the three dimensional test to detect AmpCs were tried, but no satisfactory technique has been established. In routine laboratory, three-dimensional tests for detection of AmpC are not feasible as it is cumbersome and time consuming (Taneja *et al.,* 2008).

The AmpC disk test was an easier, reliable, and rapid method for detection of isolates that harbour AmpC β-lactamase, and provide confirmatory or alternative test for detection AmpC enzyme in *K. pneumoniae* isolate. This test discriminates between cefoxitin-resistance

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caused by cephamycinase activity in test positive isolates and other causes (e.g. loss of permeability or altered drug target) in test-negative isolates (Schonning *et al.,* 2007). Table (4-9) conducted that 30 (41.1%) of the 73 cefoxitin resistant isolates gave strong positive result with AmpC disk test. All these isolates gave positive results with modified three-dimension test. In similar study, Basak *et al.* (2009) found that 47 isolates of AmpC producers Gram-negative were confirmed by both modified three dimension and AmpC disk tests.

Furthermore, such previous study was to estimate the AmpC disk test for detection of plasmid mediated AmpC β-lactamases. Black *et al.* (2005) reported that the AmpC disk test exhibited 100% sensitivity and 98% specificity for detection of plasmid mediated AmpC β-lactamases. On the other hand, the phenotypic data pointed that all AmpC positive isolates were previously recorded as cefoxitin resistant. However, this test was unable to show the possibility of AmpC production in cefoxitin susceptible and intermediate resistant isolates. This may be because the AmpC disk test is based on permeabilization of cell membrane and release β-lactamases into the external environment (Basak *et al.,* 2009). This test perhaps is not sensitive to low level expression enzymes.

The low occurrence of AmpC β-lactamases producing *K. pneumoniae* isolates (if compared with cefoxitin exploration results) in the present study, may be due to the isolates having *ampC* genes, but might not be expressed in all the isolates. This means that they might have 'silent genes' or there might be low-level expression of *ampC* genes that was not detected (Jacoby, 2009).

The disk-based inhibitor test is a new assay based on the synergism effect in utility of cefoxitin and ceftazidime as antibiotics substrates and cloxacillin as AmpC inhibitor for detection of AmpC β-lactamases. In this study, all modified three dimension and AmpC positive tests *K.*

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pneumoniae isolates were verified as AmpC producers by disk-based inhibitor test. In agreement with the present study, Tan *et al.* (2009) evaluated several methods for screening of AmpC β-lactamase producing Gram-negative isolates and found that the disk based inhibitor and modified three-dimensional test yielded a maximum of AmpC positive isolates. Additionally, Manchanda and Singh (2003) reported that the using of cloxacillin discs confirmed the presence of AmpC enzyme in all cefoxitin resistant *K. pneumoniae* isolates. However, Hemalatha *et al.* (2007) also found that 47.3% of screened *Klebsiella* spp. and *E. coli* isolates harboured AmpC enzyme by using inhibitor based method. Disk based inhibitor could be a simple cost effective and first line tests for AmpC-type β-lactamase determination, and further providing a specific means of detection of the cefoxitin susceptible ACC-1 β-lactamase (Ruppe *et al.,* 2006).

Results, of table (4-9) also revealed that 42(57.5%) of cefoxitin resistant isolates, 11(100%) intermediate cefoxitin resistant isolates and 19 (100%) cefoxitin susceptible isolates were confirmed as non AmpC producers by all confirmatory tests. This study take for granted, these isolates basically may not have AmpC encoding genes. However, experimentaly *K. pneumoniae* strains harboring $bla_{DHA\text{-}1,2}$ can phenotypicaly expressed intermediate resistance to cefoxitin (Yan *et al.*, 2002). While, Fortineau *et al.* (2001) reported that all bla_{ACC1} *K. pneumoniae* strains showed cefoxitin susceptibility in standard disk diffusion test. All these reasons may lead to decrease the efficiency of AmpC β-lactamases detection by the present methods. The results showed no significant differences (P<0.05) among the AmpC β-lactamase detection methods, Moreover, the capability of AmpC enzyme detection is important to improve the clinical management of infections and provide perfect epidemiological data, but at present, there are no

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standardized phenotypic screening methods that are readily available to microbiology laboratories in Iraq. Therefore, the enzyme extraction methods have traditionally been cited as the optimum phenotypic detection method for AmpC activity, despite the labor intensive and they are not suitable for routine clinical use.

5.7. Inducible Mediated AmpC β-lactamase Production

The present study was undertaken to find out the presence of inducible AmpC type of β-lactamases producing *K. pneumoniae* isolates in Najaf using standard methods presently available for their detection. Furthermore, point mutation in these *ampC* genes may confer resistance to virtually all cephalosporins and monobactams, with the possible exception of cefepime, cefpirome and the carbapenems (Chow *et al.,* 1991). Chromosomal AmpC β-lactamases are usually inducible, while plasmid-mediated AmpC enzymes are not, except for DHA enzymes (Fortineau *et al.,* 2001). Inducible AmpC expression is regulated by *ampR* in the presence of two other gene products, *ampD* and *ampG* (Hanson and Sanders, 1999). However, AmpR does not regulate expression from the majority of the plasmid-associated *ampC* genes, and therefore, the mechanisms by which high-level AmpC expression from noninducible plasmid-associated *ampC* genes remain elucidated.

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 (Pai *et al.*, 2004). Thus, this type of drug can cause more harm than help (Arora and Bal, 2005). Present study documented here the utility of a phenotypic (ceftazidime-imipenem antagonism (Figure 4-10)) test to detect and confirm the presence of

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inducible AmpC β-lactamases among 103 β-lactam resistant *K. pneumoniae* isolates, based on the strong inducing effect of imipenem on these enzymes and the consequent antagonism with ceftazidime.

However, Table (4-10) reveals that only two (1.9%) isolates were detected to produce inducible AmpC β-lactamase. There is no published information on the prevalence of inducible AmpC β-lactamase in Najaf. However, this is the first report on the presence of inducible AmpC in *K. pneumoniae* isolates. The results of the present study suggest that the chromosomally or may be plasmid inducible AmpC β-lactamases were rare among the *K. pneumoniae* isolates collected from clinical and hospital environmental samples. Present results are in correspondence with study conducted by Coudron *et al*. (2003) who cited that two (40%) of *K. pneumoniae* isolates gave flattening inhibition zone near the ceftazidime disk toward cefoxitin disk as an induction of AmpC β-lactamase production. In similar study, Cantarelli *et al.* (2007) confirmed the presence of inducible AmpC β-lactamase in one *K. pneumoniae* of the 34 *Entetrobacteriaceae* isolates, which granted positive imipenem-ceftazidime antagonism test. However, in such molecular study, Ruppe *et al.* (2006) pointed that the one isolate of *K. pneumoniae* harboring bla_{DHA-1} gave positive antagonism test. Currently, many plasmid-mediated AmpC enzymes such DHA β-lactamases have been found in *K. pneumoniae* that naturally lack a chromosomal AmpC β-lactamase. It is believed that such β-lactamases arise through the transfer of chromosomal AmpC genes onto plasmids. Other study suggested that this inducibility may got by the presence of a regulator *ampR* gene, on the same plasmid (Phillippon *et al.,* 2002). Moreover, Reisbig and Hanson (2002) detected that the organization of plasmid bla_{ACT-1}/*amp*R in 225 isolates of *K. pneumoniae* were identical to the chromosomal *amp*R/*amp*C region in *Enterobacter cloacae.*

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Table (4-10) also indicates that 98.1% of isolates were phenotypically negative and the results showed that no inducible AmpC β-lactamase production were detected among susceptible and intermediate cefoxitin resistant isolates. Present study suggested that all inducible mediated negative isolates might be due to plasmid-mediated AmpC β-lactamases type that not expressed phenotypicaly.

Eventually, it has been recommended that inducible AmpC-producing *K. pneumoniae* should be recorded as resistant to all extended-spectrum β-lactams since the using of third generation cephalosporins in treatment may induce organism to produce AmpC enzymes, that may lead to arise resistant to all β-lactams except carbapenems.

5.8. Molecular Detection of AmpC β-lactamases

AmpC β-lactamases producing bacteria are a serious threat in treating bacterial infections. Existence of various mechanisms, which create resistance to antibiotics, accounts for treatment failure in infections with these bacteria. Presently, no CLSI or other approved criteria for AmpC detection (Jacoby, 2009). However, the testing for AmpC β-lactamases is not widely attempted by clinical laboratories because the available phenotypic tests are either inconvenient, subjective, lack sensitivity and/or specificity, or require reagents that are not readily available (Black *et al.,* 2005). As well, there is no molecular survey for AmpC enzymes in Iraqi hospitals. For this reasons, this study was designed to evaluate the phenotypic tests of AmpC β-lactamase production by PCR techniques to detect the bla_{AmpC} encoding gene. Polymerase chain reaction was used to determine bla_{AmpC} gene in all the 103 β-lactama resistant isolates (Figure 4-11). However, bla_{AmpC} gene by PCR was seen in 20 (27.4%) isolates (Figure 4-12). All these isolates were cefoxitin resistance and identified as AmpC β-lactamase producers by phenotypic tests. This is perhaps the first report of bla_{AmpC} gene

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horbouring *K. pneumoniae* isolates from Iraq. The present study like other study in Najaf conducted by Al-Hillali (2010) who found that 9.1% of the enteropathogenic *E. coli* were amplified with bla_{AmpC} primers. In a previous study in Birmingham, Brenwald *et al.* (2005) showed that 60 isolate of *E. coli* and *K. pneumoniae* were hydrolyzed cefoxitin, 14 (23.3%) were positive by PCR for bla_{AmpC} gene. Similarly, A Malaysian study carried out by Palasbramaniam *et al.* (2007) who found that all isolates (100%) of cefoxitin resistant *K. pneumoniae* obtained from clinical samples showed positive PCR amplification for bla_{AmpC} gene.

Genes encoding AmpC β-lactamases are commonly found on the chromosomes of several members of the family *Enterobacteriaceae*. The *ampC* is the structural gene for the AmpC enzyme. Recently, this gene has moved from the chromosome into self-transmissible plasmids. Gram-negative bacteria that lack an chromosomal AmpC enzyme (such as *K. pneumoniae, Salmonella* spp. and *P. mirabilis*) may acquire plasmids resulting in a stably derepressed resistance phenotype. Obviously, the cefoxitin resistant and bla_{AmpC} positive *K. pneumoniae* isolates may be acquired plasmidic *ampC* gene. Pai *et al*., (2004) mentioned that 50% of *K. pneumoniae* isolates received resistance to cefoxitin by the transmissibility plasmidic bla_{AmpC} gene.

On the other hand, this study indicated that the remaining 72.6% of cefoxitin resistance isolates were bla_{AmpC} negative (Figure 4-12). Cefoxitin resistance may be due to some other resistance mechanisms. Hernandez-Alles *et al.* (1999) 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 *K. pneumoniae*.

Figure (4-12) points out that no bla_{AmpC} gene was detected in cefoxitin susceptible or intermediate resistant *K. pneumoniae* isolates.

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Whereas, Manchanda and Singh (2003) found that two (7.4%) of the 27 cefoxitin susceptible *K. pneumoniae* isolates carried bla_{AmpC} gene. Sometimes cefoxitin resistance may be caused by the production of certain carbapenemases and a few types of class A β-lactamases (Jacoby, 2009), rather than AmpC enzyme production. Additionally, a strain with a plasmid-mediated AmpC enzyme can also produce other β-lactamases, such as ESBL, which may complicate the detection of the AmpC phenotype (Sidjabat *et al.*, 2009; Corvec *et al.,* 2010).

Table (4-11) shows that out of 65 *K. pneumoniae* isolates recovered from clinical samples that were tested, 31.6% isolates exhibited positive results with modified three-dimension test, in compare with 19% isolates yielded positive results with bla_{AmpC} gene. Based on these findings, the isolates were not confirmed as a bla_{AmpC} positive by PCR could be phenotypically expressed carbapenemase and associated with outer membrane porine loosing (Bradford *et al.,* 1997). On the other hand, the performance of AmpC enzyme detection by confirmatory tests may be relatively high sensitivity and low specificity. Additionally, these isolates may be harboring plasmid mediated AmpC β-lactamases that unidentified yet (Palasubramaniam *et al.,* 2007). In this study, the emergence of bla_{AmpC} harboring *K. pneumoniae* isolates among clinical cases may be due to the over-use of non accurate antibiotic choice. However, the extended-spectrum cephalosporins had preferentially been used as first-line drugs at the hospital (Carattoli, 2008).

Results of table (4-11) also reveales that 2 (25.0%) of the 8 *K. pneumoniae* isolates obtained from hospital environmental samples carried bla_{AmpC} gene. The result was inequality with modified three-dimensional test; it was found that only one (12.5%) isolate was confirmed as AmpC producer. This isolate may has *ampC* gene but might

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be not expressed, or has low level expressed *ampC* gene (Yan *et al*., 2002).

5.9. Emergence of bla_{AmpC} Carrying *K. pneumoniae* Isolates

The epidemiology, clinical features, and clinical data associated with infections caused by AmpC β-lactamase-producing *K. pneumoniae* isolates have not been well described. In the present study, an attempted was made to evaluate the clinical characteristics of the patients infected with bla_{AmpC} positive *K. pneumoniae* isolates. Rate of the bla_{AmpC} carrying *K. pneumoniae* isolates was heigh in the both extremes of ages, 51-72 years (3.7%) and 2-10 years (2.5%) (Table 4-12). This data is more similar with singular studies, Pai *et al.* (2004) noticed that the patients aged 54±12.6 years were more susceptible to infect with AmpC-producing *K. pneumoniae*. However, Roh *et al*. (2008) stated that all isolates of AmpC positive *K. pneumoniae* isolates were coexisted among patients of 43-87 years old. A recent study, Obiajuru *et al*., (2010) stated that, adults group is more prevalent by AmpC *K. pneumoniae* producers than pediatric and youth groups. These patient populations are believed to have impaired respiratory and urinary systems defenses. However, the organisms may gain access after the host aspirates colonizing oropharyngeal microbes into the lower respiratory tract (Hirsch *et al*., 2005).

In spite of the previous studies reported by Pai *et al.* (2004) who mentioned that the gender has insignificant role in AmpC producing *K. pneumoniae* infection. Table (4-12) revealed that male patients were more susceptible to infected by bla_{AmpC} gene positive *K. pneumoniae* (2.7%) than females (1.5%). This result may refer to the differnce in personal healthier and educational factors.

Patients had one of the underlying diseases like diabetes mellitus, leukemia, solid tumor and liver cirrhosis were considered immune

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suppressed (Jacoby, 2009 ; Tsai *et al.,* 2009). The study presented here, found that all *bla*$_{AmpC}$ gene positive *K. pneumoniae* isolates (6.0%) were recovered only from the immune suppressed patients. These diseases may have been significantly (*P*<0.05) increased the chance of infection by nosocomial AmpC producing *K. pneumoniae.*

Present surveillance included all clinical samples from inpatients and outpatients (community sites). The study observed that in the Najaf hospitals, the *bla*$_{AmpC}$ gene positive *K. pneumoniae* isolates was occurred predominantly among inpatients (3.4%) as compare to outpatients (1.7%) (Table 4-12). The result is identical with other studies, Fortineau *et al.* (2001) observed that all *K. pneumoniae* isolates harboring plasmidic *bla*$_{DHA-2}$ were isolated from hospitalized childs in Paris hospital. Pai *et al.* (2004) noticed that 40.7% of patients infected with AmpC producing *K. pneumoniae* had stay in the hospital for more than two weeks. Accordingly, the prolong hospitalization of patients may heighten the exposure to resistant nosocomial isolates than community sittings.

Eventually, Table (4-12) revealed that all clinical samples were collected from patients had previously received some antibiotics within distinct periods. However, all *bla*$_{AmpC}$ gene positive *K. pneumoniae* isolates were distinguished from antibiotic received patients. This result was verified by Pai *et al.* (2004) who found that 77.7% of patients had received some antibiotics within 30 days prior to the onset of AmpC producing *K. pneumoniae* infection. Moreover, extensive use of broad-spectrum antibiotics in hospitalized patients has led to both increased carriage of resistant strains and, subsequently, the development of multidrug-resistant strains (Paterson *et al.,* 2000).

To date, the local studies on the frequency of *bla*$_{AmpC}$ gene positive *K. pneumoniae* isolates in non-clinical habitats have not been carried out yet. Table (4-13) indicates that the 245 samples collected from different

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hospital environmental sites. Only two (0.8%) isolates (from kitchen and bathrooms) yielded amplification products with AmpC-PCR specific primers.

The modest detection rate in kitchen and bathrooms, was expected because these samples provide an optimum growth conditions for these bacteria. Rather than, the ability of *K. pneumoniae* to colonization and aggregative adherence that lead to form excellent micro environment may be facilitate the transition of resistance (Podschun and Ullmann, 1998).

5.10. Antibiotic Susceptibility in bla_{AmpC} Positive *K. pneumoniae* Isolates

Therapeutic options for infections caused by *K. pneumoniae* expressing AmpC β-lactamases are limited because these organisms are usually resistant to all β-lactam antibiotics except for cefepime, cefpirome (fourth generation cephalosporins) and carbapenems (Li *et al.,* 2009). This emphasizes the need for detecting AmpC β-lactamase harbouring isolates in Iraqi hospitals, to avoid therapeutic failures and nosocomial outbreaks.

The present study demonstrated the percentage of resistance and sensitivity of bla_{AmpC} gene positive *K. pneumoniae* isolates to a range of common groups of antibiotics. Results of Figure (4-13) revealed that high degree of resistance to most antibiotics. All isolates were resistant to carbenicillin, piperacillin, ticarcillin, amoxi-clav, cefoxitin, cefazolin, cefotaxime, nalidixic acid and trimethoprime. Based on this observation, the ability of these isolates for fully resistant to antibiotics could be caused by either; predominate exposure of present isolates to suboptimal levels of antibiotic, prolong use of broad-spectrum antibiotics, exposure to isolates carrying resistant genes, lack of hygiene in clinical environments and usage of antibiotics in foods and agriculture. As the cefotaxime are used in large scale in the recent years in Najaf, however

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drug resistance of bacteria in nosocomial infection makes physicians headache. Compared to the results from Feizabadi *et al.* (2010); Khadri *et al.* (2007) and Nasim *et al.* (2004), the resistance rate in present survey is higher than the average levels in the Iran, India and Canada, respectively.

Furthermore, the majority (85-95%) of the test isolates were found to be resistant to cefexime, ceftazidime, ceftriaxone, cefepime, aztreonam, gantamicin, tetracyclin, nitrofurantoin (Figure 4-13). Simultaneously, the resistance of isolates to chloramphenicol, tobramycin and Amikacin is relatively high (70%). This could be explained by the fact that plasmid-mediated AmpC β-lactamases have a broad substrate profile. Plasmids carrying genes for AmpC β-lactamases often carry multiple other resistances including genes for resistance to aminoglycosides, floroquinolones, and other antibiotic classes. Thus plasmid-mediated AmpC β-lactamases are typically associated with multiple antibiotic resistances, leaving few therapeutic options (Jacoby, 2009).

Athgoug resistance to fluoroquinolones included: ciprofloxacin, levofloxacin, gatifloxacin and norofloxacin has emerged among bla_{AmpC} carrying *K. pneumoniae* isolates, 35-55% of all isolates tested were resistant to these drugs. The same finding has been reported in many other studies in different parts of the world (Sheng *et al.,* 2002; Kesteman *et al*., 2010).

Figure (4-13) also reveales that imipenem and meropenem were considered the most potent antibiotics, which was found to be effective against 80.0% of the bla_{AmpC} harboring *K. pneumoniae* isolates, This may be also resulted from the fact that these antibiotics are not common, and have restricted use in medication and prescribed in argent cases. Thus, the present study recommended that these two types of drug are the most

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successful in treating nosocomial infections caused by such isolates in Najaf hospitals.

5.11. Appearance of Multi-drug Resistance among *bla*$_{AmpC}$ Positive *K. pneumoniae* Isolates

Multi-drug resistance (MDR) clinically, is the ability of disease causing microorganism to withstand a wide variety of antimicrobial compounds (Mooij, 2009). Hence, a strain is considered a MDR if an isolate is resistant to representatives of three or more classes of antibiotics. The lack of standard definition of MDR makes it difficult to compare different studies. However, table (4-14) revealed that 55% of *bla*$_{AmpC}$ harboring *K. pneumoniae* isolates were resistant to at least five different antibiotics classes. Genes that encode AmpC β-lactamases are often found on plasmids together with genes encoding resistance to aminoglycosides, sulphonamides, tetracyclines and fluoroqinolones (Jacoby, 2009), and therefore AmpC β-lactamases are often MDR producers. 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. However, Roh *et al.* (2008) was recorded the first outbreak of clonal spread MDR *bla*$_{DHA}$ in Korean hospitals. The indiscriminate use and abuse of antibiotics appear to be more common amongst people. Furthermore, the exposure to multiple antibiotics including substandard drugs increases chances of mutation and multiplication of resistant bacterial isolates. This view agrees with earlier reports (Hoyle *et al.,* 2004; Obiajuru *et al.,* 2010) which stated that exposure to antibiotics increases the level of resistance observed in the normal bacterial flora of both animals and human.

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Unfortunately, in same table, 45% of *bla*$_{AmpC}$ positive *K. pneumoniae* isolates were found to harbor an extensive drug resistant (XDR). The XDR resistance is mainly caused by MBL strains (Souli *et al.,* 2008), but also by strains with carbapenemases, e.g. KPC. Accordingly, Forssten, (2009) found that out of 58 *K. pneumoniae* isolates, only two (3.4%) had an elevated MIC to meropenem (4µg/ml) were considered as XDR. The reason for this high occurrence of multiple resistant isolates perhaps, that these isolates have *bla*$_{AmpC}$ gene can be incorporated into different backbones on different plasmids. However, transference of resistance determinants by mobile genetic elements including plasmids, transposons, and gene cassettes in integrons between and across different bacterial species are important factors that can contribute to the increase in multiresistant strains (Livermore, 2007). This study suggested that the new clonal spread of XDR of *bla*$_{AmpC}$ positive *K. pneumoniae* isolates had been recorded as first occurrence among Najaf hospitals.

Although, the pandrug resistance isolates (PDR) is not detected in this study, but the occurrence of alarming MDR and XDR isolates may be threat to arise of PDR isolates in the future. The prevalence of PDR resistance in *Enterobacteriaceae* is also rare in Europe (Souli *et al.,* 2008) and other countries (Khadri *et al.,* 2007).

5.12. Minimum inhibitory concentrations (MICs) for *bla*$_{AmpC}$ Positive *K. pneumoniae*

Minimum inhibitory concentration is the lowest concentration on an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). The two-fold dilution MIC antibiotic susceptibility test was chosen because it overcomes several of

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the limitations of the disk diffusion test. MIC is a quantitative determination of the degree of susceptibility, not dependent on the subjective interpretation and measurement of zones of growth inhibition and interpolation of zone sizes to MIC values. The MIC_{50} is the validated *in vitro* biomarker for antibacterials. The actual number used is MIC_{90} (the concentration required to inhibit the growth of 90% of the strains likely to be encountered). However, in this method the actual amount of antimicrobial agent required to inhibit bacterial growth can be determined and not estimated (Fales *et al.,* 1989). In the present study, the MIC values of 20 bla_{AmpC} positive *K. pneumoniae* isolates against six β-lactam antibiotics (ampicillin, piperacillin, cephalothine, cefotaxime, ceftazidime, and meropenem) were arranged cumulatively.

Results in table (4-15) shows that all bla_{AmpC} positive *K. pneumoniae* isolates were highly resistant for ampicillin, cephalothine and piperacillin with concentrations reached beyond the break point values if compared with *E. coli* ATCC 25922 as a negative control. The break point represents the optimum concentration of the drug that can reach the serum and provide high level of therapy. The microorganism was considered sensitive if the estimated MICs were less than the break point. However, the MICs values of ampicillin ranged from 64 to 128 μg/ml, and the ampicillin MIC_{50} and MIC_{90} values of these isolates were alarmingly high, 128 μg/ml. In a local study, Hadi (2008) reported that the MICs values of ampicillin for all *K. pneumoniae* isolates collected from patients with significant bacteriuria were >128 μg/ml. In Russian epidemiological study, Reshedko *et al.* (2004) mentioned that the MIC_{50} and MIC_{90} values of ampicillin for *K. pneumoniae* clinical isolate were 256 μg/ml. It was also shown by other studies that TEM or SHV enzymes were detected in 80% of ampicillin resistant Gram-negative bacteria with

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MICs exceeding 256 µg/ml compared to 1-4 µg/ml in non- β-lactamases producer isolates (Livermore, 1995; Amador *et al.,* 2009).

Through the study, the MIC of piperacillin for the isolates harbouring bla_{AmpC} gene was more or equal to the break point (≥128 µg/ml). However, piperacillin showed lower efficiency, the MIC_{50} and MIC_{90} were 256 µg/ml. In a laboratory surveillance study, Fortineau *et al.* (2001) found that MIC value of piperacillin for clinical isolates of *K. pneumoniae* carrying bla_{DHA} gene was >512 µg/ml. Whereas, Reshedko *et al.* (2004) mentioned that the MIC_{50} and MIC_{90} values of piperacillin for *K. pneumoniae* clinical isolate were 256 µg/ml. It was also shown that TEM or SHV enzymes mostly confer resistance to penicillin group like piperacillin, ticarcillin, and carbenicillin, with MICs exceeding 256 µg/ml (Livermore, 1995).

For cephalothine, the MICs values were 16 to 64 µg/ml. It was also showed that MIC of 50% and 90% of isolates were 32 µg/ml and 64 µg/ml, respectively. These rates are in agreement with those reported by Al-Charrakh (2005) who tested 8 ESBL-producing *K. pneumoniae* isolates, and found that MICs values of cephalothine were ranged from 32 to >128 µg/ml. Notably, the resistance to first generation cephalosporins in Gram-negative enteric rods may be mediated by TEM-1, TEM-2, SHV-1 β-lactamases (Chiangjong, 2006).

For cefotaxime, the MIC values ranged from 8 to 128 µg/ml. This results correspond to those stated by Coudron *et al*. (2003) who found that MIC value of cefotaxime for all isolates of AmpC-producing *K. pneumoniae* reached to resistant ranges (64-128 µg/ml). In this study, MIC_{50} and MIC_{90} of cefotaxime were 64 and 128 µg/ml. In the United States, Jett *et al*. (1995) found that of 83 ESBL-producing *Klebsiella* spp. isolates, the MIC values for 90% of the isolates were 64 µg/ml.

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Table (4-15) also indicats that the MIC value of ceftazidime for tested isolates ranged from 16 to 128 µg/ml. Furthermore, MIC value of ceftazidime for 50% and 90% of isolates were more than the break point value (64 and 128 µg/ml, respectively). However, these values were much higher than Reshedko *et al.* (2004) reported that the MIC_{50} and MIC_{90} values of ceftazidime for *K. pneumoniae* clinical isolate were 32 µg/ml and 256 µg/ml, respectively. Furthermore, these values are in agreement with Roh *et al.* (2008), who found that the MICs of ceftazidime for multidrug *K. pneumoniae* harbored bla_{DHA-1} and bla_{SHV-12} ranged between ≥128 µg/ml.

Present investigation found that meropenem still retained good *in vitro* activity against the majority of bla_{AmpC} positive *K. pneumoniae* isolates with a modal MIC ranged 2 to 32 µg/ml. This investigation showed that 20.0% of the isolates were resistant to meropenem. Overall, the MIC_{50} of meropenem was in zone of susceptible (2 µg/ml), while the MIC_{90} (16 µg/ml) was equal to the break point value. Based on a finding the meropenem was the most efficient antibiotic against the bla_{AmpC} positive *K. pneumoniae* isolates. This result may be in accordance with those reported by Babini *et al.* (1998) who found the MIC value of meropenem was ≤15 µg/ml for hyperproduced AmpC β-lactamase *E.coli* isolates. In spite of, the present study discovered that two isolates had MIC values for meropenem overcome the break point (32 µg/ml), the resistance of *K. pneumoniae* to carbapenems is still extremely rare. However, studies performed to ascertain the mechanism responsible for this unusual resistance. Even though, resistance to carbapenem can be achieved by the occurrence of a single permeability mutation in a population that producing high levels of AmpC β-lactamase (Ehrhardt *et al.,* 1993).

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Eventually, in this study the elevated values of both MIC_{50} and MIC_{90} parameters for ampicillin, piperacillin, cephalothin, cefotaxime and ceftazidime (in zone of resistance) with positive correlation, indicated to the low efficiency of these β-lactam antibiotics against the bla_{AmpC} harbouring *K. pneumoniae* isolates. Moreover, MIC_{50} and MIC_{90} of meropenem were in zone of susceptible, suggesting that the meropenem was the most active tested against the bla_{AmpC} carrying *K. pneumoniae* isolates. However, the MIC_{50} and MIC_{90} are good parameters for periodically measurement of antibiotics efficiencies in the same bacterial population.

5.13. Occurrence of Plasmid-Mediate AmpC β-lactamases

Since AmpC β-lactamases were discovered, two stages possibly have been divided. The first stage; from the 1970's to 1990's, the AmpC β-lactamases demonstrated or presumed to be chromosomally mediated, which were found in local areas, the second stage; since 1990, the plasmid-mediated AmpC β-lactamases have been found in succession and caused the prevalence worldwide (Li *et al.,* 2009). Bobrouski *et al.* (1976) first reported a plasmid-mediated AmpC β-lactamase name. Until 1989, Bauernfeind *et al.* (1989) described an enzyme named CMY-1 in *K. pneumoniae* from South Korea. Since then, plasmid-mediated AmpC β-lactamases keep the pace of about 1-3 novel types per year in the world. Up to December 2008, more than 45 plasmid-mediated AmpC β-lactamases have been recorded in GenBank (Li *et al.,* 2009). The reaserchers considered that plasmid encoded *ampC* gene was one of the most important factors, which caused the prevalence of resistant strains worldwide in the past 10 years (Li *et al.,* 2009).

In Iraq, many clinical laboratories are not fully aware of the importance of plasmid-mediated AmpCs and how to detect them; laboratories may also lack the resources to curb the spread of this

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resistance mechanism. The consequence has been avoidable therapeutic failures (sometimes fatal) in patients who received inappropriate antibiotics and outbreaks of multidrug-resistant, Gram-negative pathogens that required expensive control efforts.

Methods for detecting plasmid-mediated AmpCs are technically demanding for Najaf clinical laboratories. Although nonsusceptibility to the cephamycins (e.g. cefoxitin) suggests increased production of AmpC β-lactamases, organisms that produce these types of enzymes often go undetected and have been responsible for several nosocomial infections. Previous studies noticed an increase in cefoxitin-resistant *K. pneumoniaie, E. coli, P. aeruginosa* isolates in Najaf and Hilla during 2009 and 2010 (Al-Hilali, 2010; Al-Muhannak, 2010, Belal, 2010; Al-Hilli, 2010, Shamkhi, 2011). To the best of the investigators knowledge, until now no published research has described the distribution of plasmid mediated *ampC* genes within Gram-negative isolates in Iraqi hospitals. However, six families of plasmid-mediated AmpC β-lactamases have been identified, but no phenotypic test can differentiate among them, a fact which creates problems for surveillance and epidemiology studies. Present study describes the use of monoplex PCR for the purpose of identifying family-specific AmpC β-lactamase genes within isolates. The study was conducted to determine the distribution and diversity of plasmid mediated *ampC* genes among all clinical and hospital environmental isolates of *K. pneumoniae* that showed carried bla_{AmpC} gene in Najaf, using PCR protocol for distinguishing family specific *ampC* genes. Five families of *ampC* genes namely, bla_{FOX}, bla_{CIT}, bla_{DHA}, bla_{EBC}, bla_{ACC} were analyzed. However, MOX-type AmpC was not included in this analysis (Figures (4-14)-(4-18)).

Plasmid mediated AmpC β-lactamases were detected in many countries, but in contrast to the findings for ESBLs, they are still rare;

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recent reports from the United States, United Kingdom and China showed that only 13.2%, 49% and 17%, respectively of *K. pneumoniae* strains contained this enzyme type (Moland *et al.,* 2002; Woodford *et al.,* 2007 and Li *et al.,* 2009). In table (4-16), the data were obtained by monoplex PCR revealed that, out of 20 isolates (yielded amplification products with AmpC-PCR specific primers), 11 (55%) had a bands compatible with *bla*$_{FOX}$ gene. This table shows that FOX family (FOX 1-5 and FOX5b) was the most prevalent AmpC-type enzyme. The FOX-1 was first isolated from *K. pneumoniae* in Argentina at 1994, as plasmid mediated AmpC β-lactamases. However, *Aeromonas caviae* is likely source of FOX derivative *ampC* gene (Conzalez Leiza *et al.,* 1994). The present study documented that *K. pneumoniae* isolates harboring plasmids coding for FOX AmpC-type β-lactamase are established in Najaf hospitals. In similar study, Black *et al.* (2005) pointed that among 44 isolates of AmpC positive enteric bacteria, *K. pneumoniae* was the most microorganism expressed FOX enzyme (63.6%). In a recent study, Tenover *et al.* (2009) found that *bla*$_{FOX}$ gene was detected in 2 (14.3%) of the 14 AmpC-PCR positive *K. pneumoniae* isolates. The origin of FOX enzymes still remains unknown (Li *et al.,* 2009), and the strains with *bla*$_{FOX}$ can not only cause nosocomial cross-infection, but also disseminate drug-resistant bacteria among countries (Bauernfeind *et al.,* 1998). As well as it was found that *bla*$_{FOX-5}$ gene captured with transition genetic elements typically class1 integrons may increased genes mobilization (Jacoby, 2009).

Results of table (4-16) shows that the CIT family (CMY2-7, LAT1-4 and BIL-1) was presented in 40% of *bla*$_{AmpC}$ positive *K. pneumoniae* isolates. This prevalent rate is in agreement with those reported by Tan *et al.* (2009) who found that 74/119 (62.2%) of plasmid mediated AmpC β-lactamase positive isolates of enteric bacteria were belonged to CIT enzyme type. Several studies mentioned that CIT-type enzymes appear to

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be prevalent in Asia and Canada (Li *et al.,* 2009; Koh *et al*., 2007). Hence, in this study, the bla_{CIT} gene harbored *K. pneumoniae* isolates appeared to be relatively prevalent in Najaf hospitals. These data are significant because the occurrence of CIT β-lactamases in Gram-negative pathogens may pose additional challenges in identifying appropriate antibiotic therapy. However, the CIT family comprised from 11 subtypes with high degree of similarity. The CIT family (CMY-2, LAT-2, and BIL-1) was first identified in *K. pneumoniae* isolates from Greece in 1990. They can be grouped based on their chromosomal origin that is similar to *ampC* gene of *C. freundii* (Perez-Perez and Hanson, 2002). However, CMY-2 was the most prevalent and most geographically distributed plasmid-mediated AmpC β-lactamase with reports from Africa, Europe, India, Taiwan, and the United States (Philippon *et al.,* 2002). Other genes of CIT group are found adjacent to an insertion sequence common region (IS*CR1*) involved in gene mobilization into (typically) complex class 1 integrons (Toleman *et al.,* 2006).

DHA plasmid-borne *amp*C gene was first described for *Salmonella enterica* serovar Enteritidis in Saudi Arabia in 1997 (Gaillot *et al.,* 1997). A detailed study demonstrated that bla_{DHA-1} was located on an integron that originated from *Morganella morganii* (Barnaud *et al.,*1998). Plasmid-borne bla_{DHA} genes have also been found in *K. pneumoniae* in France (Fortineau *et al.,* 2001), Taiwan (Yan *et al.,* 2006), and the United States (Moland *et al.,* 2002). This investigation revealed that among the AmpC β-lactamase producers, 4 (20.0%) showed the presence of bla_{DHA} (DHA-1 and -2). This represents the first report of bla_{DHA} in the Najaf. In accordance with Lee *et al*. (2009) who observed that DHA group genes were predominantly in *K. pneumoniae* 102/105 (97.1%) followed by in *E. coli* 8/14 (57.1%). However, Yum *et al.* (2005) had been reported that 17/40 (42.5%) of plasmid-mediated AmpC β-lactamase, *K. pneumoniae*

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positive isolates were carrying *bla*$_{DHA}$ gene. Although, in this study the *bla*$_{DHA}$ gene was not highly disseminated among AmpC-expressed *K. pneumoniae* isolates, the valuable spread of *bla*$_{DHA}$ was due to dissemination of endemic clones and horizontal transfer of the resistance gene. Table (4-16) also reveales that 2/4 (50%) of *bla*$_{DHA}$ borne *K. pneumoniae* isolates were previously confirmed as inducible AmpC-βlactamase-producing by ceftazidim-imepenem antagonisim test.

Furthermore, the counterpart of *bla*$_{DHA-1}$ gene is closely related to the chromosomal *ampC* of *M. morganii*, and is associated on the same plasmid with the regulator *ampR* gene, which is responsible for its inducibility. However, the present study suggests that the intergenus or interspecies exchange of plasmids may play an important role in the occurrence of *bla*$_{DHA}$ gene among isolates. This suggestion is verified by Kim *et al*. (2004) in Korea, who found that same *bla*$_{DHA}$ gene variant of DHA-1 producing *Salmonella montevideo*, was found in *E. coli* and *K. pneumoniae*. In conclusion, the present study has demonstrated that plasmid-mediated DHA β-lactamase has emerged in Najaf. However, the occurrence of DHA, an inducible type of enzyme, raises clinical concerns.

Additionally, a new plasmid-mediated AmpC β-lactamase, which was designated EBC (ACT-1 and MIR-1) family, was found in Najaf hospitals. Among the 20 *K. pneumoniae* isolates with *bla*$_{AmpC}$ gene, 4 (20%) had a *bla*$_{EBC}$ type gene (Table 4-16). In comparison with other studies, Cocks and Vaughan (2009) reported that EBC enzyme was the most common gene (21/57, 36.3%) among plasmid mediated β-lactamases producing enteric bacteria. While, Tenover *et al.* (2009) found that out of 53 isolates, only one (1.9%) of *E. coli* isolate carried *bla*$_{EBC}$ gene type, and non of *K. pneumoniae* isolate had been EBC positive during the molecular screening test. Reports mentioned that homology of

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bla$_{ACT-1}$ is 91.4% in comparison with the gene of MIR-1, ACT-2, and ACT-3 has been found in China (Hurata *et al.,* 1998). However, the MIR-1 and ACT-1 are originating from *Enterobacter cloacae*. Reisbig and Hanson (2002) suggested that the importance of arising EBC genes family among AmpC producing *K. pneumoniae* isolates may be resulted from the expression of MIR-1 bases on promoter *prA* and mixed promoter *prB* is responsible for high-level expression required for resistance to the extended-spectrum cephalosporins, ceftazidime, and cefotaxime, as well as cefoxitin and ampicillin.

Table (4-16) indicats that that only one (5%) of *bla*$_{AmpC}$ β-lactamase positive *K. pneumoniae* was successfully analyzed by ACC-PCR positive with *bla*$_{ACC}$ specific primers. This result is in agreement with Cocks and Vaughan (2009) who found that only 1/57 (1.8%) isolate expressed ACC enzyme in enteric bacteria. In 1999, a novel ACC plasmid-borne AmpC β-lactamase was isolated from *K. pneumoniae* in Germany; the authors proposed the name Ambler class C1(ACC-1). It was subsequently shown that ACC-1 was derived from the chromosome-borne AmpC of *Hafnia alvei*. ACC-1 has so far been isolated only in Germany, France, Spain, and Tunisia (Ohana *et al.,* 2005). The ACC enzyme group (ACC1- and -2) was extremely rare among plasmid mediated AmpC β-lactamases.

In particular, AmpC β-lactamases provide resistance to the third generation cephalosporins, cephamycins, except for plasmid mediated ACC-1, which is sensitive to cefoxitin (Philippon *et al.,* 2002). In this study, ACC-producing isolate (*K. pneumoniae* K20) was resistant to cefoxitin by the disc diffusion method. This indicates the probable presence of other β-lactamases enzymes. However, the imipenem-EDTA disk test confirmed that *K. pneumoniae* K20 isolate were metallo-β-lactamases producer. This isolate was recovered from patient with significant bacteriuria. Based on this unique property, the cefoxitin

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susceptible isolates may be neglected in plasmidic AmpC molecular detection, that leads to miss diagnosis of *bla*$_{ACC}$ gene in these isolate have been confer with treatment failure.

However, *bla*$_{MOX}$ gene was not detected in any *bla*$_{AmpC}$ β-lactamases carrying *K. pneumoniae* isolates. This could be the *bla*$_{MOX}$ gene variant may disseminated in other geographic area. Moreover, the *bla*$_{MOX}$ gene serves as a genetic variant of the same gene in the analysis. However, the percent similarities among the all family members within these clustered groups were 94.3, 94.2, 89.4, 95.7, 98.6, and 84.2% for the ACC, FOX, MOX, DHA, CIT, and EBC groups, respectively (Perez-Perez and Hanson, 2002). This may increase the detection complexity.

Eventually, based on this finding, the present study has a warning for an epidemic status in local geographic region by AmpC plasmidic genes among *K. pneumoniae* isolates. However, nothing is known about the distribution of plasmid-mediate AmpC β-lactamases in the Iraqi hospitals and community; however, it is not unlikely that isolates producing these enzymes could spread in a population where antibiotics are available without prescription. A more comprehensive survey of plasmid-mediate AmpC β-lactamases from Iraq is therefore urgently needed not only for the hospital setting but also for the community.

5.14. Multiple Plasmid-Mediated *ampC* Genes

Multiplex polymerase chain reaction is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. This method has been successfully applied in many fields of DNA testing (Henegariu *et al.,* 1997). Several phenotypic tests can distinguish resistance mechanisms but are unable to differentiate the different types or families of plasmid-mediated AmpC β-lactamases. In addition, the use of routinely phenotypic methods, as adequate for less complicated

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bacteria, is not adequate for the newer generation of antibiotic-resistant pathogens that express multiple resistance mechanisms and produce multiple β-lactamases (Perez-Perez and Hanson, 2002). None of the encoded enzymes can be distinguished from another by phenotypic testing. Hence, this study is designed to use multiplex PCR for distinguishing family-specific *ampC* genes in all 20 bla_{AmpC} β-lactamases positive *K. pneumoniae* isolates.

However, the data in table (4-17) and figure (4-19) for *ampC* multiplex PCR reveale that three *ampC* genes pattern, bla_{FOX}, bla_{CIT}, bla_{EBC} were detected in two (10.0%) isolates. It has been mentioned that few studies, reported that more than one *ampC*-gene were detected by multiplex PCR in *K. pneumoniae* iaolates. Kim *et al.* (2004) stated that 14/61 (22.9%) isolates of *K. pneumoniae* had both bla_{CMY} and bla_{DHA} genes. Whereas, Tan *et al.* (2009) found that only three isolates of *K. pneumoniae* able to express CIT and DHA enzymes together. In this study, the repeatedness of triple pattern of bla_{FOX}, bla_{CIT}, bla_{EBC}, may refer to the phylogenetic relationship among these genes. As would be expected, *ampC*-genes may arise from either organisms belonging to the same genus, could be clustered together (Hall and Barlow, 2004), or the AmpC β-lactamases of *Enterobacteriaceae*, are more convergence related. In this regard, further discriminatory power of multiplex PCR, may be distinguish the presences of known transferable *ampC* genes in certain isolate. In addition, *ampC* multiplex PCR also discriminated between transferable *ampC* genes coding for inducible AmpC β-lactamases as long as they were not of the same origin (Perez-Perez and Hanson, 2002).

Simultaneous appearance of bla_{FOX} and bla_{CIT} was observed in four 20.0%) *ampC*-gene positive *K. pneumoniae* isolates, separately. That led

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to confirmed that both bla_{FOX} and bla_{CIT} were highly repetitive genes among plasmids sequence of study isolates. This may due to these genes can be incorporated into different backbones of different plasmids (Jacoby, 2009). Furthermore, a variety of genetic elements has been implicated in the mobilization of *ampC*-genes onto plasmids; moreover, *K. pneumoniae* does not possess chromosomal *ampC* (Bauernfeind *et al.,* 1998). Therefore, detection of plasmid-mediated *ampC* in *K. pneumoniae* is straightforward.

The molecular analysis by *ampC* multiplex PCR showed the presence of 14 (70%) isolates of *ampC*-gene harbored *K. pneumoniae* had one type of plasmid *ampC*-genes (Table 4-17). Of these, 5 isolates contained the bla_{FOX} gene, 4 isolates contained the bla_{DHA} gene, 2 isolates contained bla_{CIT} gene, 2 isolates contained bla_{EBC} gene and one isolate contained bla_{ACC} gene. These isolates may express more than one plasmid-mediated AmpC β-lactamase, but have not been identified. Thus, the *ampC*-gene positive *K. pneumoniae* isolates may not be able to express two or more plasmid-mediated *ampC* genes. However, if multiple plasmid-mediated *ampC* genes can be expressed in a single organism, the *ampC* multiplex PCR technique can be used to differentiate them. Finally, multiplex PCR is a rapid and convenient screening assay in both the clinical and the research laboratory and this technique is introduced as a mean of shortening the amount of time required for analysis compared with monoplex PCR, moreover, it is cost-effective (Henegariu *et al.,* 1997; Bartllet and Stirling, 1998).

5.15. ESBL among *K. pneumoniae* Isolates Carrying bla_{AmpC} Genes

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Extended spectrum β-lactamase producing *Enterobacteriaceae* are now an increasing problem worldwide. ESBLs are mostly plasmid-mediated β-lactamase enzymes capable of hydrolyzing and inactivating a wide variety of β-lactam antibiotics, including third-generation cephalosporins, penicillins and aztreonam (Aggarwal and Chaudhary, 2004). Notable, the detection of bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{OXA}, bla_{VEB}, bla_{PER}, and bla_{GES} genes were performed with PCR assay. In fact, coexistence of broad-spectrum β-lactamases with ESBLs, ESBLs with AmpC β-lactamase, multiple ESBLs with metallo-β-lactamase has become common in multi-resistant *K. pneumoniae* (Hanson and Sanders, 1999; Yan *et al.,* 2001; Essack *et al.,* 2004; Al-Hillali, 2010, Shamkhi, 2011). Of these enzymes, ESBLs were the most prevalent in *K. pneumoniae* (Bradford, 2001).

Through the study, a coexistence of AmpC β-lactamase and ESBLs was detected in 16 (80%) of the 20-plasmid *ampC*-gene positive *K. pneumoniae* isolates (Table 4-18). This could be explained by the dissemination of plasmid-mediated AmpC β-lactamases among *K. pneumoniae*, sometimes in combination with ESBLs, suggesting a clonal spread of multi-enzyme producers *K. pneumoniae* at Najaf hospitals.

Of the 16 ESBLs and plasmid mediated AmpC β-lactamase producer isolates, 10 (50%) isolates confirmed as molecular analysis positive ESBL, but were identified as phenotypic negative. In nationwide surveillance study, Jeong *et al.* (2009) found that all enteric bacterial isolates that harbored ESBL and AmpC enzymes exhibited false-negative for ESBL using phenotypic methods. Although, the isolates supposed as false-negative for ESBL phenotypicaly, due to misleading or masking results in phenotypic tests, it was found that the CLSI disk confirmatory tests yielded false negative results in 14% of *K. pneumoniae* isolates coproducing ESBLs and plasmid mediated AmpC β-lactamase (Song *et*

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al., 2007). This may occur if the AmpC activity is larger than the ESBL activity. Accordingly, Yu *et al.* (2006) mentioned that the isolate producing CTX-M-14 and high-level expression of CMY-2, may have given phenotypically a false negative results with ESBL.

Results of table (4-18) shows that 6 (30.0%) *ampC*-genes harbored *K. pneumoniae* isolates were verified as ESBL in both phenotype and molecular analysis. Moreover, the phenotypic positivity for ESBL in *ampC*-gene possessed isolates may be resulted from equally enzymes expressed, suggesting a possible high copy number of *bla*-genes carrying plasmids.

The most commonly identified ESBL gene was $bla_{CTX\text{-}M}$ type (14, 70%) (Table 4-19). The first CTX-M in Europe was described in 1989 in Germany in an *E. coli* isolate and almost simultaneously in *E. coli* in France (Bauernfeind *et al.,* 1990). In Najaf, CTX-M enzymes were detected in 38.7% of enteric Gram-negative clinical isolates obtained from wound and sputum samples (Al-Muhannak, 2010), In the Middle East area, reports from Lebanon and Kuwait pointed out that CTX-M is the predominant ESBL in *Enterobacteriaceae* (Moubareck *et al.,* 2005; Poirel *et al.,* 2005). Despite the recent local studies indicated to the high occurrence of ESBL producer isolates. It is unclear why the spread of CTX-M has been more extensive than others in Najaf hospitals. Even though this may be due to the different antibiotic, policies exist in various hospitals with excessive use of third generation cephalosporins. However, some risk factors of the acquisition of $bla_{CTX\text{-}M}$ gene may be pressure from the surroundings by antimicrobial agents. Furthermore, a selective pressure is created, by the use of antibiotics as feed additives in animal farming and agriculture (Woodford *et al.,* 2004).

The results of table (4-19) indicats that 10 (50%) of *ampC*-genes positive *K. pneumoniae* isolates carried bla_{SHV} gene. The recent rates of

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bla$_{SHV}$ in Gram-negative clinical isolates obtained from Najaf hospitals were 54% (Belal, 2010) , and 100% (Al-Hilali, 2010). The present result was much higher than reported by Pongpech *et al.* (2008) who found that, 3 (8%) of the 37 confirmed ESBL producing *E. coli* in Thailand had SHV types. Most *K. pneumoniae* isolates have chromosomally or plasmid-mediated SHV-1 β-lactamase, which is a narrow-spectrum β-lactamase with activity against penicillins (Bush *et al.,* 1995). However, Ford and Avison (2004) proposed an evolutionary model that explain the association between the flanked insertion sequence IS26 and *bla*$_{SHV}$ gene as well as the vector for *bla*$_{SHV}$ which responsible for mobilization from chromosomal loci to plasmide and interspecies dissemination. Nevertheless, the results of this study agree with the hypothesis that most ESBL *bla*$_{SHV}$ are plasmid-mediated and are present in high copy numbers in bacteria (Forssten, 2009).

Table (4-19), shows that five (25%) isolates of *ampC*-genes positive *K. pneumoniae* yielded amplification products with TEM-PCR specific primers. However, this result is in accordance with those reported by Hadi (2008) in Najaf who found that two isolates (15.4%) of *K. pneumoniae* subsp. *pneumoniae* harbored *bla*$_{TEM}$ gene and Shamkhi (2011) in kut found the similar results. While,, Tasli and Bahar (2005) found that 14 (31.1%) of the 45 *K. pneumoniae* strains isolated from clinical samples in Turkey gave TEM-PCR- positive. Accordingly, the transfer of *bla*$_{TEM}$ gene on mobile plasmid, lead to spread rapidly to members of the same species or organisms of different genera (Lartigue *et al*., 2007).

The results in table(4-19) also reveales that, only 2 (10%) *ampC*-genes positive *K. pneumoniae* isolates harboured a *bla*$_{OXA}$ β-lactamase gene as determined by PCR. In conducted studies, Al-Hilli (2010) and Shamkhi (2011) found that all *E. coli* and *Klebsiella* spp. isolates from

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Merjan teaching hospital in Hilla city were negative in OXA-PCR. In 2007, Bhattacharjee *et al.* reported that out of the 361 ESBL-positive enterobacteria isolated from different clinical specimens in India, only one isolate harboured the OXA-10. However, OXA β-lactamases represented one of the most prevalent plasmid-encoded β-lactamase. These β-lactamases are classified into class D in the Ambler classification. They had been identified mainly in the *P. aeruginosa* (Harada *et al.,* 2008). Therefore, this study suggested that the phenotypic detection of ESBL may miss class D oxacillinases with exception of the OXA-18, which is strongly inhibited by clavulanic acid. However, most of OXA genes are plasmid and integron located and extremely rare in *K. pneumoniae* with ESBL isolates (Bhattacharjee *et al,*. 2007).

Among the 20 bla_{AmpC} carrying *K. pneumoniae* isolates, the molecular analysis of ESBL genes revealed that the bla_{VEB} was detected in only one (5%) isolate. However, this is the first report of its occurrence in Najaf. In similar study in China, Wei-feng *et al.* (2009) found that bla_{VEB} gene not detected among 18 isolates of ESBL-producing *K. pneumoniae*. Whereas, in a study done in Thailand, Chiangjong (2006) found that 11/30 (36.6%) of ESBL-producing *K. pneumoniae* isolates carried $bla_{\mathrm{VEB\text{-}1\text{-}like}}$ gene. This enzyme is class A β-lactamase and was named VEB-1 (for Vietnamase extended-spectrum β-lactamase). The latter confers high-level resistance to amoxicillin, ticarcillin, piperacillin, cefotaxime, ceftazidime, and aztreonam, which is inhibited by clavulanate (Poirel *et al.,* 1999). In this study, the low detection rate of bla_{VEB} gene may be the VEB enzyme is currently replacing mutant in Najaf hospitals, and typically plasmid-borne in *Enterobacteriaceae* but chromosomal in *P. aeruginosa* and *A. baumannii* (Carbonne *et al*., 2005).

The present study revealed that no ESBL genes, including bla_{PER} and bla_{GES} were identified in 20 *ampC*-genes positive *K. pneumoniae*

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isolates, which could be either due to the absence of *bla*$_{PER}$ and *bla*$_{GES}$ genes or the presence of other subtypes of genes that could not be targeted by the primers used in this study. These enzymes have been most frequently associated with single occurrences in *P. aeruginosa* strains caused a small nosocomial outbreak in Korean patients (Jeong *et al.,* 2005).

The present study found a large number of ESBL producing *K. pneumoniae*, also produced AmpC β-lactamases. Microbiology laboratories in all hospitals should proceed to detect multiple β-lactamases in individual isolates, which are already designated as ESBL producers, so that the appropriate therapy can be chosen for patient management, and sound data can be generated on resistance mechanisms and hospital infection epidemiology.

5.16. Carbapenemase Production

Carbapenems are group of β-lactams consists of (imipenem, meropenem, doripenem, ertapenem, panipenem and biapenem). This group has the widest spectrum of antibacterial activity of the antibiotic agents that are currently available. Carbapenems have provided a highly effective treatment option for serious infections with ESBL and/or AmpC-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 (Queenan and Bush, 2007).

Figure (4-2) reveales that most β-lactam resistant *K. pneumoniae* isolates were susceptible to both imipenem and meropenem. Only four (3.9%) of screened isolates were resistant to both imipenem and meropenem by standard disk diffusion method. These isolates was

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recovered from clinical samples with clear significant difference ($P<0.05$). In the same manner, the study results were in agreement with a Turkish study conducted by Bicmen *et al.* (2004) who pointed that resistance to carbapenems among Gram-negative bacteria recovered from various specimens of hospitalized patients was 24 % for imipenem and 21.3% for meropenem.

Table (4-20) shows that, PCR technique had been confirmed that all carbapenem-resistant isolates (K6, K13, K16, and K20) were harbored plasmid mediated *ampC*-genes. Three methods were used to confirm the carbapenemase production in these isolates. Imipenem-EDTA disk test was less significant effect ($P<0.05$) in comparison with other methods (confirmed only 50% of carbapenems disk resistant isolates as phenotypic carbapenemase producers). There are two isolates gave negative results with EDTA disk synergy test. This is means using EDTA may not inhibit the activity of all β-lactamases, suggesting the absence of a class B1 enzyme (like IMP and VIM), or these isolates may produce other enzymes (like IMI, GES and KPC) that were not inhibited by EDTA. Moreover, the resistance to carbapenems may involve several combined mechanisms other than carbapenemases, include modifications to outer membrane permeability and up-regulation of efflux systems associated with hyperproduction of AmpC β-lactamases and cephalosporinases (ESBLs) (Nordmann *et al.,* 2009).

Recently, modified Hodge test (Figure 4-25) became the most acceptable confirmatory test to detect all type of carbapenemases for infection control and epidemiological purpose. Table (4-20) shows that all carbapenems resistant *K. pneumoniae* (4, 100%) gave positive test. In a related study, Al-Hilli (2010) stated that 1(2.4%) of *Klebsiella* spp. isolates recovered from hospital environment in Hilla was confirmed as

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carbapenemase producer using modified Hodge test, whereas the same isolate gave negative result with imipenem-EDTA synergy test.

Besides, KPC CHROMagar technique was used to select the carbapenemase-producers *K. pneumoniae* on medium that enhanced by KPC supplement, this medium allows to the growth of *K. pneumoniae* carbapenemase-producers only and inhibit the KPC negative bacteria. Because the originality of this method, no available studies used this technique previously. However, KPC CHROMagar technique (Figure 4-26) revealed that all carbapenems resistant *K. penumoniae* isolates were successful gave overnight heavy growth. This result interpreted that possibility of these isolates to carry serine type carbapenemase KPC or OXA carbapenemase.

In same table, the identity between modified Hodge test and KPC CHROMagar in enzyme detection may be due to the modified Hodge test which has >90% sensitivity and specificity in detecting of KPC and variable sensitivity and specificity to detect the other metallo-β-lactamases. Howvere, there is no significant difference (P<0.05) between modified Hodge test and KPC CHROMagar. Moreover, this investigation found low efficiency of EDTA synergy test to confirm all type of carbapenemase production in compare with CHROMagar technique and modified Hodge test. This may be because, metallo-β-lactamase producers isolates may remain almost fully susceptible to carbapenems in vitro (Livermore and Brown, 2005).

Carbapenemases production was also detected in bla_{AmpC} harbouring *K. pneumoniae* isolates by PCR experiments. Using most common carbapenemases specific primers for bla_{IMP}, bla_{VIM}, and $bla_{KPC.}$ The results were negative among all isolates. One limitation of the carbapenemase-specific assay for carbapenems resistant isolates surveillance in this study, is that it does not detect all carbapenems

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resistant genotypes. The results in this investigation indicated that the four isolates were negative in PCR, which could be due to either the absence of bla_{IMP}, bla_{VIM}, and bla_{KPC} genes or the presence of other type of gene variants that could not be targeted by the primers used in this study. On the contrary, many reports from Japan, Singapore, and Taiwan (Arakawa *et al.,* 2000 ;Yan *et al*., 2001) and recently in Lebanon (Daoud *et al.,* 2008) indicated that IMP, VIM varieties have been detected in most Gram-negative bacilli isolated from nosocomial infections.

5.17. Emergence of Multiple *bla* Genes Carrying *K. pneumoniae* Isolates

Plasmids carrying genes for AmpC β-lactamases often carry multiple other resistances including genes for other β-lactamases such as TEM, CTX-M and SHV varieties (Jacoby, 2009). Table (4-21) reports the finding of a plasmid containing multiple resistance genes, including genes encoding the plasmid-mediated AmpC β-lactamases as well as those encoding ESBLs. The study findings showed that 80.0% of the bla_{AmpC} harbouring *K. pneumoniae* isolates carried at least one type of ESBL *bla*-genes. These results strongly suggest that there is coexistence of ESBL and AmpC in these isolates. However, the present results corroborates to that reported elsewhere by previous workers, in Iran and Korea, TEM-, SHV-, and CTX-M-type ESBLs and CMY-, DHA-type AmpC β-lactamases have been reported in *E. coli* and *K. pneumoniae* isolates from a few individual institutions (Yum *et al*., 2005; Feizabadi *et al.,* 2010). Manchanda and Singh, (2003) reported that 69% of Gram-negative bacteria exhibited both ESBL and AmpC β-lactamase production in India. This indicates that co-production of ESBL and AmpC β-lactamase which cause antibiotic resistance is a worldwide problem.

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Among 16 plasmid-mediated AmpC β-lactamase and ESBL producers, 2 isolates carried 2 gens, 11 isolates carried 3 genes, 2 isolates carried 4 genes, and 2 isolates carried 6 genes (Table 4-19). Present study suggests a clonal spread of multidrug-resistant *K. pneumoniae* at Najaf hospitals. In related studies, Jeong *et al.* (2009) found that among 27 isolates of *K. pneumoniae*, 3 (11.1%) isolates carried SHV-2 and DHA-1, 5 (18.5%) isolates carried SHV-12 and DHA-1, 1 (3.7%) isolate carried SHV-12 and ACT-1 and 1(3.7%) isolate carried SHV-12, CTX-M-14 and DHA-1. This may be due to the *ampC*-genes which is usually part of an integron and bla_{AmpC} genes. They are found adjacent to an insertion sequence (Jacoby, 2009). Hence, the study noted that the same bla_{AmpC} gene could be incorporated into different sequence on different plasmids. On the other hand, such isolate is alarming to causes limitations in the selection of antimicrobial agents for optimal treatment of patients. Moreover, the coexistence of both plasmid mediated AmpC β-lactamase and ESBL in the same isolate is the most important cause of false-negative results in ESBL confirmatory tests (Coudron, 2005).

5.18. Purification and Characterization of AmpC β-Lactamase

In the present study, *K. pneumoniae* isolate K19 was chosen as a source of AmpC β-lactamases for enzymatic study. Depending on phenotype and genotype investigations, the study suggests that this isolate was testifying ApmC producer (carried bla_{FOX} and bla_{CIT}). However, K19 isolate was confirmed as negative for ESBLs and carbapenemase in both phenotype and genotype detection (Table 4-22). AmpC β-lactamase was extracted from the sonicated cells after adequate incubation with 1/4 MIC

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of meropenem (to avoided the effecting of other enzymes if present). In general, carbapenems are a strong inducer for AmpC β-lactamase production, but much more stable for hydrolysis (Jacoby, 2009). Yang *et al*. (1988) stated that imipenem is a strongest inducer at low concentrations, giving 200-300-fold induction when used below 1 mg/L, in comparing with cefoxitin, which is a strong inducer, giving 100-fold induction at 10 mg/L, while the other β-lactams were very poor inducers.

On the other hand, in the case of Gram-negative bacteria, β-lactamase synthesized within the cell and secreted into the periplasmic region. However, the main part (66%) of the β-lactamase was found intracellularly (Hedberg *et al.* 1995). In this study, the AmpC β-lactamase activity in 40 ml of crude extract was low (1.1 U/ml). Hence, most of the β-lactam compounds remained stable to hydrolysis under such conditions, probably because (after sonication) β-lactamase was diluted 10,000-fold relative to its actual concentration in the periplasmic space. It is known that dilution may cause rapid decrease in the stability and activity of β-lactamases (Bellido *et al.*, 1991).

Table (4-23) reveales that the specific activity of AmpC β-lactamase in crude extract was 0.18 U/mg of protein. In similar studies, Yang and Livermore (1988) stated that the specific activity of β-lactamase in sonicated extract in presence and absence of inducer was varied from 5 to 170 U/mg. However, Hedberg *et al.* (1995) found that the specific activity of new β-lactamase in the supernatant of disrupted cells was 4.8 U/mg of protein. Although, sonication is a cell disruptive technique was used in this study for enzyme releasing, NIIR (2006) published that 20 fold increase in purification of enzymes that located in periplasmic region may be obtainable by sonication compared to other techniques. Bellido *et al.* (1991) suggested that a concentrated solution of purified β-lactamase

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from sonicated lysate was probably the best available model to mimic the actual conditions of the periplasm.

Through the study, the sonicated crude extract was then precipitated by ammonium sulfate with (30%-75%) saturation. However, the use of ammonium sulfate in enzyme purification has been described by many authors, and has fulfilled the dual purpose of both purification and concentration. β-lactamase activity was assayed after precipitation step, the measurement results revealed that relatively increasing in both activity and specific activity of AmpC β-lactamase to 3.4 U/ml and 0.32 U/mg of protein, respectively. Whereas, the purification fold was more than one and half time and enzymatic yield was 19.3% (Table4-23). However, this study is in agreement with Castillo *et al.* (2001), who found that an indicative increment in β-lactamase activity up to 6.8 U/ml and purification fold 2.44, when the supernatant was precipitated by solid ammonium sulfate (75%) saturation. Moreover, NIIR (2006) published that the using of ammonium sulfate (20% w/v) in purification of penicillinase from *E. coli* strain W3310 is fairly typical. Furthermore, ammonium sulfate most commonly used, since its high solubility, lack of toxicity to most enzymes, cheapness, and its stabilizing effect on enzymes. Accordingly, the precipitation by ammonium sulfate based on the "salting out" process, in which; the salts of high valence produce higher ionic strength to neutralize the charged protein molecules. This may cause disturbance in surrounding watery film. This process could be more efficient in protein solubility and precipitation (Janson and Ryden, 1998). However, in electrolyte solutions, proteins are more soluble at temperatures above zero centigrade. Moreover, the solubility of protein may be depending on its configuration and the presence of other non-ionic compounds (Scopes, 1987).

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Subsequently, AmpC β-lactamase dialyzed sample (2.5ml) obtained from previous step was further purified by passing through the sephadex G75 which was able to separate protein molecule within (3-70) kDa. Gel filtration (size exclusion) separates molecules according to difference in size. In this purification step, the absorbance of eluted fractions was measured at 280 nm and the results of separation yielded one peak of protein, which decreased gradually. However, the active fractions were pooled and the enzyme activity was assayed. Results of figure (4-27) reveales that two peaks of AmpC enzyme activity were focused in more than one fraction. Those results are in correspondance with the study of Conzalez Leiza *et al.* (1994) who found that the semi-purified FOX-1β-lactamase from *K. pneumoniae* that fractionated on a sephadex G75 column gel filtration, exhibited two β-lactamase activities in different fractions with *pI*s of 6.8 and 7.2 and molecular sizes of approximately 37 and 35 kDa, respectively. Considerably, the present study suggested that present of two homologous (monomeric enzymes) AmpC β-lactamases (extracted and purified from tested isolate) had approximate two molecular weights. Notably, this probability was confirmed previously by multiplex PCR technique. It was found that K19 tested isolate harbored two types of plasmid-mediated AmpC β-lactamases (FOX and CIT). However, FOX group (FOX-1, 2, 3, 4, 5, 6) and CIT group (CMY-1, 8, 9) genes homology of different subtypes are very similar. The amino acid sequences homology within the FOX group is 95% and within the CMY-1, CMY-8, and CMY-9 are more than 97% (Li *et al.,* 2009). Nevertheless, the sequences of *ampC* genes from the same family show slight variations (genetic changes). These variations can lead to an amino acid substitutions resulting in the individual family member (Perez Perez and Hanson, 2002). In the same way, the interpretation of two homologous enzymes of AmpC β-lactamases

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activity may belong to oligomeric enzyme which contains at least two or more subunits (Koolman and Roehm, 2005). Therefore, the present study suggests that this separated protein may undergo processing to form more than one final enzyme.

Furthermore, gel filtration step by sephadex G75, proved the highest values of enzyme activity (2.2, 0.54 U/ml) and specific activity (7.8, 2.7 U/mg) for the first and second peaks respectively. To be present with noticeable decrease in protein concentration (0.2 mg/ml), in compare with the previous precipitation steps. However the purification fold and enzyme yield values were 43.3, 15 and 60%, 11%, for first and second peaks respectively (Table 4-23). In a similar study, Hedberg *et al.* (1995) reported that gel filtration step achieved higher purification fold (16 times) than anion exchange step with elevated specific activity (75 U/mg) for purified new β-lactamases extracted from *Bacteroides uniformis*. The high value of AmpC β-lactamases specific activity with reduce total protein concentration in certain sample size, denoted high purity of enzyme. However, the data were obtained from gel filtration curve demonstrated that coincidence peaks of protein absorption with AmpC β-lactamases activity was a further evidence for enzyme purity.

5.19. Physical Kinetic Properties of AmpC β-Lactamase

Virtually, the temperature has a dual effect on enzyme reaction, however, the reaction velocity increases with temperature until a peak velocity is reached. In this study, the results of figure (4-28) shows that the AmpC enzyme catalyzed reaction started at room temperature 25°C and the activity was 0.03 U/ml. The increasing in rate of reaction may result from the requirement to the starting point temperature as a function to increase number of molecules having sufficient energy to pass over the

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energy barrier (Koolman and Roehm, 2005). Obviously, the present study revealed that the optimum temperature for highest activity of AmpC β-lactamases was 37°C. This result is consistent with that previously reported by Flayeh (2005) who found that the optimum temperature for β-lactamase activity produced by *K. pneumonia*e was 37°C.

The study reported herein has also pointed that β-lactamases were remaining slightly active at elevated temperature until 50°C. While, the enzymes were inactivated at 80°C and the activity dropped down to 0.01 U/ml. These results are correlated with those observed by Vanhove *et al.* (1997) who found that the mutant TEM-β-lactamase from *E. coli* was inactivated within a few minutes at 55C^{o}. Because of the protein nature of AmpC enzyme, the further elevation of the temperature results in a decrease of the affectivity and consequently decreases the reaction rate, a result of thermal-induced denaturation of the enzyme.

Figure (4-29) also shows that the maximum activity (0.06 U/ml) of AmpC β-lactamases at pH value 7.5. In a similar study, Flayeh (2005) cited that the best activity of β-lactamases extracted from *K. pneumonia* at pH 7.0. However, Laraki *et al.* (1999) found that β-lactamases produced by *P. aeruginosa, E. coli* and *K. pneumoniae* exhibit a maximum activity at pH 6.5 and 7.5. Present results demonstrated that AmpC enzymes activity were diminished (< 0.05 U/ml) at alkaline pH (8.0-10.0), and the activity was also decreased to 0.02 U/ml at pH value 4.0. These findings are corresponding to that reported by Castillo *et al.* (2001) who illustrated that the optimum pH for β-lactamase was more than 7.2 and the activity having lost 50% at pH more than 8. However, extremes of pH can profoundly lead to denaturation of the enzyme, because the structure of the catalytically active protein molecule depends

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on the ionic character of the amino and carboxy acid side chains (Koolman and Roehm, 2005).

The rate of hydrolysis is determined by the activity of the enzyme on the specific antibiotic (K_m and V_{max}) and the concentration of the enzyme (Livermore, 1995)

The Lineweaver-Burk reciprocal plot method was adopted to determine the kinetic parameters K_m, V_{max} and hydrolysis efficiency (relative V_{max}/K_m) (Figure 4-30). The kinetic parameters were studied for the partial purified AmpC β-lactamase using two-fold concentration of penicillin G, cefotaxime, and meropenem as substrate. The K_m and V_{max} values were calculated by the use of the Michaelis-Menten equation, as previously described.

However, in table (4-24), the AmpC β-lactamases marked greater affinity (K_m, 2.8 mM) and more rapidly hydrolyzed (V_{max} 0.02 µmol/min) for penicillin G. This behavior confirmed that AmpC β-lactamases have a strong hydrolysis activity against β-lactams. However, these values are similar to those reported by Marchese *et al.* (1998) who found that the K_m and V_{max} values of FOX-3 type AmpC enzyme produced by *K. oxytoca* were 0.036 mM and 0.013 µmol/min, respectively. Moreover, Babini *et al.* (1998) stated that AmpC β-lactamases from *E. coli* isolates was most efficient against benzylpenicillin (K_m 0.143 mM) than other β-lactams. The high catalytic efficiency of AmpC β-lactamases for β-lactams may be due to certain characteristics of enzyme-substrate complex such as: specificity, the conformation of complex protein molecule, the uniqueness of enzyme active site and the structural configuration of substrate molecule (Koolman and Roehm, 2005). However, AmpC β-lactamases may exhibit substrate group specificity (more than one compound may serve as enzyme substrate). Thus, when an enzyme

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attacks penicillin it may lead to serial attacks of homologous β-lactams antibiotics.

Besides, the kinetic values of AmpC β-lactamase towered cefotaxime were higher than penicillin G. The present study revealed that AmpC enzyme exhibited relatively high values of K_m (3.36 mM) and V_{max} (0.045 µmol/min). At one time, this study showed that the MIC test revealed that *K. pneumoniae* isolate (K19) appeared to be resistant to cefotaxime (128 µg/ml). These results were in agreement with values reported by Bellido *et al.* (1991) who calculated K_m and V_{max} for cefotaxime at $2x10^{-3}$ mM and $5x10^{-4}$ µmol/min, respectively. However, Beceiro *et al.* (2009) found that AmpC enzyme has very low K_m value toward cefotaxime in AG3 isolates of *Acinetobacter* genomic species. Although the moderate affinity and high reaction rate of AmpC β-lactamase toward cefotaxime (relatively to penicillin G), and the hydrolysis efficiency of enzyme for cefotaxime was very high (191.32%). However, this rate, termed physiological efficiency, reflected the capability of β-lactamase to hydrolyze a given β-lactam. These results may reflect the weak stability of enzyme-cefotaxime complex.

In this study, the kinetic parameters AmpC β-lactamase toward meropenem (K_m 9.3mM, V_{max} 0.03 µmol/min) were much higher than that of penicillin G and cefotaxime, indicating to the low affinity with slow reaction rate (Table 4-24). However, MIC test results revealed that the K19 test isolate was meropenem sensitive (2 µg/ml). Even though, all the previous studies mentioned that meropenem and imipenem were stable to hydrolysis by AmpC β-lactamase, despite the hydrolysis of meropenem by AmpC β-lactamase was too slow (46.08% relatively with penicillin G). The results obtained from the kinetics parameters of AmpC β-lactamase were very similar to those published by Yang and Livermore (1988) who observed that the imipenem hydrolysis rate was 20 to 25% of

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its initial value and thereafter remained almost constant. Thus Yang *et al.* (1988) mentioned that the β-lactamase afforded very low-level protection against imipenem. This may be resulted from a non-hydrolytic mechanism, and the enzyme-meropenem binding was a relatively stable complex.

Conclusions:

1- High prevalence of ESBL-producing *K. pneumoniae* isolates was detected.The $bla_{\text{CTX-M}}$ and bla_{SHV} were the predominant genes.

2- Phenotypic characterization of AmpC β-lactamases provide information concerning the correlation with molecular analysis.

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3- The vast majority of AmpC enzymes were encoded by plasmid genes.
4- FOX and CIT enzymes were the most common types of AmpC β-lactamases in Najaf hospitals.
5- The co-existence of multiple plasmid-genes (AmpC and ESBL) in *K. pneumoniae* isolate is frequently accompanied with the multidrug resistance.
6- Carbapenem was the drug of choice for AmpC producer *K. pneumoniae*, but carbapenemase production is threaten the antimicrobial treating program in Najaf hospitals.
7- The kinetic behavior of partially purified AmpC β-lactamases was highly related with the phenotypic expression and molecular analysis.

Recommendations:

1- Phenotypic confirmatory tests of ESBL, AmpC β-lactamases and carbapenemase production should be applied routinely in all hospital laboratories .

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2- The therapeutic option of all third generation cephalosporins should be avoided against pathogenic isolates that appear to be AmpC producers.

3- It is recommended to use recent techniques like PCR/*NheI*, pulsed-field gel electrophoresis to determine the dissemination source and studying the epidemiology of AmpC β-lactamases- producing isolates.

4- Attempting to complete purification of AmpC β-lactamases by other methods of chromatography to study their effect on infected tissues and immune system.

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Test	***K. pneumoniae* (n=130)**

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	subsp. *pneumoniae* (n=111)	subsp. *ozaenae* (n=19)
Gram stain	-	-
Catalase	+	+
Oxidase	-	-
H_2S production	-	-
Indole	-	-
Methyl red	-	+
Voges-Proskaures	+	-
Citrate utilization	+	+
Urease	+	-
Gelatin liquifaction	-	-
TSI	A/A	A,K/A
Motility	-	-
Phenylalanine deaminase	-	-
L-Ornithine decarboxylase	-	-
L-Arginine decarboxylase	-	-
Glucose	+	+
Lactose	+	V*
Sucrose	+	-
Maltose	+	+
D-Mannitol	+	+
D-Mannose	+	+
D-Sorbitol	+	V
D-xylose	+	+
L-Arabinose	+	+

Appendices:

Appendix (1): Morphological and biochemical tests of *K. pneumoniae* isolates recovered from clinical and hospital environmental samples in Najaf.

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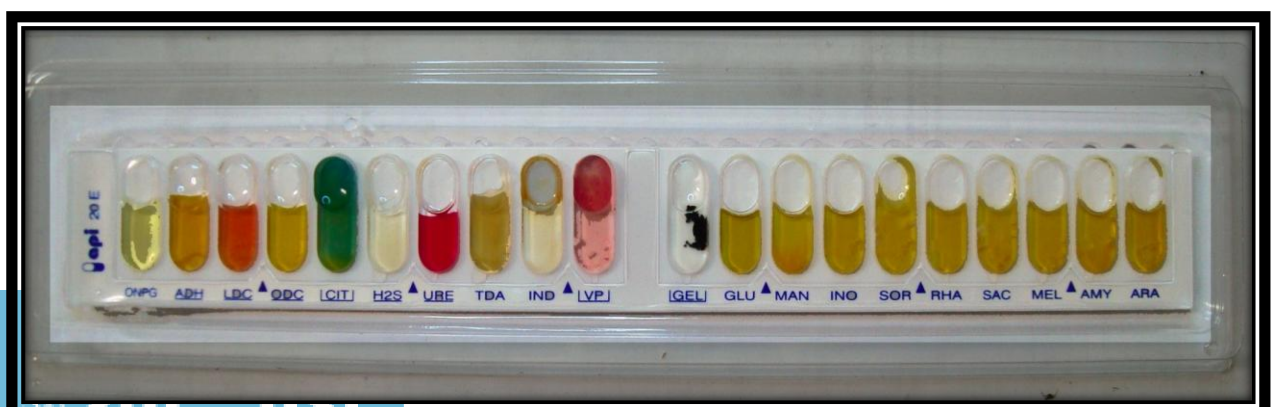
***V: Variable reaction**



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(A)

(B)

Appendix (2): Excellent identification level of API 20E system according the analytical profile index. (A): *K. pneumoniae* subsp. *pneumoniae* isolate (K1) with 7 digit number (521577 3), (B): *K. pneumoniae* subsp. *ozaenae* isolate (K76) with 7 digit number (5204543).

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