Spread of Some Extended-Spectrum Beta-Lactamases in Clinical Isolates of Gram-Negative Bacilli in Najaf

A thesis

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آية 32

I certify that this thesis was prepared by **Fadhil Hussain Nasir Al-Muhannak** under my supervision at the College of Medicine, University of Kufa, as a partial requirement for the degree of Master of Science in Medical Microbiology.

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DEDICATION

TO MY LATE PARENTS

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Fadhil Hussain Nasir

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List of Abbreviations

Abbreviation	Key
β	Beta
AmpC	Class C β-Lactamases
ATCC	American Type Culture Collection
bp	Base Pair
CLSI	Clinical and Laboratory Standards Institute
CTX-M	Cefotaximase β-Lactamase
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Di-Amine Tetra Acetic Acid
ESBL	Extented-Spectrum β-Lactamase
GNB	Gram-Negative Bacteria
ICU	Intensive Care Unit
IS	Insertion Sequence
LPS	Lipopolysaccharide
MDR	Multidrug-Resistant
MDRGN	Multidrug-Resistant Gram-Negative
MHA	Muller Hinton Agar
MR-VP Broth	Methyl-red Voges-Proskauer Broth
NAG	N-acetylglucosamine
NAM	N-acetylmuramic Acid
NCCLS	National Committee for Clinical Laboratory Standards
OXA	Oxacillinase β-Lactamase
PBPs	Pinicillin Binding Proteins
PCR	Polymerase Chain Reaction
PG	Peptidoglycan
Ps	*Pseudomonas*
SDS	Sodium Dodecyl Sulfate
SHV	Sulphydryl Variable β-Lactamase
TBE	Tris-Borate-EDTA Buffer
TE buffer	Tris EDTA Buffer
TEM	Temoneira β-Lactamase
Tris-OH	Tris-(Hydroxymethyl) Methylamine

Summary

Extended-spectrum beta-lactamase (ESBL)-producing isolates of Gram-negative bacteria (GNB) have emerged as a major problem in hospitalized and community based patients. The objectives of this study were to determine the occurrence and molecular types of ESBLs causing resistance to extended-spectrum cephalosporins in clinical isolates of GNB.

A total of 613 clinical samples were collected from Al-Sadr Teaching Hospital and Tuberculosis and Chest Disease Clinic in Najaf city from November 2008 through February 2009. Those samples fell into two categories. The first included 390-swabs for patients with wound infections. The second category included 223 samples of sputum for patients experiencing symptoms of lower respiratory tract infections.

In order to detect types of Gram-negative bacilli, those samples were cultured on MacConkey agar medium to isolate the most common kinds of bacteria, the result was 203 isolates.

After investigating the appearance and biochemical tests of 203 isolates, it seemed that both *Pseudomonas aeruginosa* and *Klebsiella* spp. (62 isolates, 30.5% each) were the commonest, followed by *Escherichia coli* (56 isolates, 27.7%), and then *Proteus* spp. (23 isolates, 11.3%). Isolates of *Klebsiella* spp. were identified as having a sub-species level.

In order to detect the β-lactamase producing bacilli, those bacilli were tested for antibiotics sub-class aminopenicillin. The results revealed that 193 (95.1%) isolates were resistant to ampicillin and amoxicillin. 61 (31.6%) of which were *Klebsiella* spp., 59 (30.6%) were *Ps. aeruginosa*, 52 (26.9%) were *E. coli*, and 21 (10.9%) were *Proteus* spp.

To study the sensitivity of those isolates against general antibiotics, 22 types of different antibiotics were tested using Kirby-Bauer disc diffusion

method. 174 (85.7%) isolates were considered to be multi-drug resistant, because they were resistant to at least 3 classes of antibiotics. Those isolates showed high resistance to cefazolin (87.7%) and low resistance to imipenem (5.1%).

To detect extended-spectrum β-lactamase, phenotype methods were used depending on CLSI (2007). 189 isolates were ESBL producers according to initial screening method, whereas the confirmation method showed only 10 (5.2%) isolates carried ESBL enzymes. It is noteworthy that the difference in number was large. A possible cause for this disparity was its producing AmpC enzymes which as some researchers think, when it exists with high activity will act as a mask against ESBL enzymes, what supports this probability was the resistance of those isolates to cefoxitin.

On the other hand, the genotype method was used to detect the most common four kinds of ESBLs; TEM, SHV, CTX-M, and OXA. For this purpose, four kinds of species primers were used with the help of PCR and electrophoresis systems. The outcome showed that 51 (82.3%) of 62 potential ESBL producing Gram-Negative Bacterial isolates carried one or more genes of the four enzymes mentioned above; 17 (27.4%) isolates carried *bla* $_{TEM}$; 21 (33.9%) isolates carried *bla* $_{SHV}$; 24 (38.7%) isolates carried *bla* $_{CTX-M}$; and 17 (27.4%) isolates carried *bla* $_{OXA}$ genes. It appeared clearly that those isolates carrying ESBL genes were all multi-drug resistant.

In contrast to that, these isolates were highly sensitive to imipenem. Seeing those results, it seems that the existence ratio of ESBL enzyme is considered to be moderate although there was no previous study to compare the occurrence of CTX-M and OXA enzymes in the region of the study. The present results may be a guide for choosing an appropriate therapy, particularly when treating suspected ESBL-producing bacterial infections.

CHAPTER ONE

INTRODUCTION

Introduction

The β-lactam antibiotics are among the most important drugs used as a bactericidal to fight bacterial infections, mainly against Gram-negative bacteria (GNB). These drugs target the cell wall and typically do so by interfering with peptidoglycan synthesis. Peptidoglycan is essential to the structural integrity of the cell wall and its disruption allows the cell to succumb to osmotic lysis (Zapun *et al*., 2008). These drugs are the most widely used group of antibiotics, owing to their high effectiveness, low cost and minimal side effects (Wilke *et al*., 2005).

During the last five decades, the overuse and sometimes misuse of antimicrobials in both human and veterinary medicine has resulted in the emergence of resistance mechanisms, allowing pathogenic bacteria to survive antibiotic treatment (McDermott *et al*., 2003).

The various mechanisms of antimicrobial resistance in GNB include changes in the permeability of the bacterial cell wall to prevent antimicrobial agents to target sites, efflux of the antimicrobials out the bacterial cell, mutation in the target site, as well as the ß-lactamase enzymes which considered as a major mechanism of resistance in the Gram-negative bacteria (Piddock, 2006). ß-lactamases are enzymes that cleave the amide bond of the four-membered characteristic ß-lactam ring, rendering the antimicrobial ineffective (Babic *et al*., 2006).

Antibiotic resistance in pathogens is increasing worldwide in both outpatients as well as hospitalized patients, which are considered as a focus of infection. These resistances can be acquired by mutation or by the acquisition of resistance genes from other organisms (Tenover, 2006).

Extended-spectrum beta-lactamases (ESBLs) have emerged as an important mechanism of resistance to ß-lactam antibiotics in GNB, mostly in

Enterobacteriaceae, (Mendelson *et al.*, 2005). They constitute one of the major threats regarding pathogenic microorganisms because they are capable of hydrolyzing and inactivating a wide variety of β-lactam antibiotics especially third-generation cephalosporins and monobactams (Paterson, 2006). These enzymes are typically associated with multiple antibiotic resistances, leaving a few therapeutic options. It is important to know the occurrence of ESBL producing isolates as well as their antibiotic susceptibilities to newer agents to guide empirical therapy for various infections (Taneja *et al*., 2008).

In Iraq, researchers are now interested in the use of molecular techniques, especially in those genes that produce various enzymes in bacteria which play an important role in controlling the widespread and epidemiologic diseases that may get access to the Iraqi environment, particularly hospitals environment.

The genotypes of ESBL producing strains largely remain unknown in Iraq; hence, the present study aims to determine the occurrence of ESBLs in GNB, their molecular types and associated risk factors in a wound and lower respiratory tract infections.

The objectives of this study were to:

a) Determine the spread of various Gram-negative bacteria in wound and lower respiratory tract infections.

b) Identify the occurrence of ESBL production among Gram-negative bacteria isolated from wound and lower respiratory tract infections.

c) Describe the susceptibility patterns of the isolated Gram-negative bacilli.

d) Evaluate the dissemination of *bla* $_{TEM}$, *bla* $_{SHV}$, *bla* $_{CTX-M}$, and *bla* $_{OXA}$ genes among ESBL-producing isolates.

CHAPTER TWO

REVIEW OF

THE LITERATURES

2.1. Bacterial Cell Wall:

The cell wall is an essential multilayered and a complex structure located between the cytoplasmic membrane and capsule of most prokaryotic cells. The nature of the bacterial cell wall determines the classification of a bacterium as a Gram-positive and Gram-negative bacteria (GNB), because of fundamental differences in the structure of their cell walls. It consists of an inner layer of peptidoglycan (PG) (also called murein) in periplasmic space and an outer membrane consisting of lipopolysaccharides (LPS) and lipoproteins (Holt *et al.*, 1994).

The peptidoglycan layer is a polymer consisting of interlocking chains of identical peptidoglycan monomers. Each monomer consists of two joined disaccharides, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), with a pentapeptide chain attached to the latter. The monomers are synthesized in the cytoplasm of the bacterium while the final cross-linking is catalysed outside the cytoplasmic membrane by a group of membrane-anchored bacterial enzymes which are transglycosylases, joining the new monomer to the pre-existing peptidoglycan chain, transpeptidase (achieve transpeptidation step), and carboxypeptidase within the periplasm (Macheboeuf *et al.*, 2006). In the cross-linking reaction, a peptide bond is formed between the D-alanine on one chain and the free amino end of a diamino pimelic acid on the other chain (Chambers, 2001). A linkage is formed with the D-alanine, causing the terminal D-alanine to be cleaved (Wilke *et al.*, 2005). Completion of the cell wall or attaching to the penicillin binding proteins (PBPs) are suppressing the cell wall hydrolyses that in turn act to lyses the cell wall (Samaha-Kfoury and Arej, 2003). Once the new

peptidoglycan monomers are inserted, glycosidic bonds then link these monomers into the growing chains of peptidoglycan. The peptide cross-links introduce covalent connectivity to the meshwork, impart mechanical strength and provide the major structural barrier to osmotic pressure forces that could kill the bacterium (Walsh, 2003). Energy for transpeptidases to cross-link is produced when carboxypeptidase cleaves D-alanine from the glycopeptide. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as PBPs (Wise, 1996).

The peptidoglycan provides structural support, maintains the characteristic shape and rigidity of the cell, and protects the cell protoplast from mechanical damage and from osmotic lysis. The periplasmic space in Gram-negative bacterial cell is also containing transport, degradative and cell wall synthesis proteins (Meyer, 2006).

The outer membrane consists a lipid bilayer structure intercalated with proteins on the outside of the peptidoglycan sheet in Gram-negative bacteria. It acts as a permeability barrier to large molecules within which aqueous channels (porins) and outer membrane porins play a role in regulating the passage of small, hydrophilic molecules into the cell. Porin proteins form a trimer that acts, usually nonspecifically, as a channel to allow the entry of essential substances such as sugars, amino acids, vitamins, and metals, as well as many antimicrobial drugs such as penicillins. Antibiotics may be altered during passage through the periplasm (e.g. by enzymes) affecting their penetration and ultimate activity inside the cytoplasmic membrane (Meyer, 2006).

Lipopolysaccharides (LPS) of outer membrane consist of lipid A, core polysaccharide, and O antigen. LPS confers both antigenic properties from

the carbohydrate chains and toxic properties from the lipid A component. Endotoxin is not secreted by bacteria; it is considered to be one of the most injurious and fatal structures of GNB. The species-specific virulence factors effect on host response; when LPS is released from bacterial cell wall its toxic moiety, Lipid A that functions as endotoxin, is observed as potent inflammation inducer and the immune responses can cause tissue destruction, endotoxin shock, and can be lethal if encountered in too high doses (Amersfoort *et al.*, 2003).

2.2. ß-lactam Antibiotic:

The ß-lactam are family of antimicrobial agents which are considered the most widely used drugs for treatment of bacterial infections worldwide (Villanueva *et al*., 2003). The effectiveness of all ß-lactam antibiotics relies on their ability to inhibit bacterial growth by inactivation of the penicillin-binding-proteins (PBPs) and prevent them from cross-linking peptidoglycan via a transpeptidation reaction (Buynak, 2006; Zapun *et al*., 2008). The ß-lactam antibiotics are bactericidal cell wall synthesis inhibitors, owing to their high effectiveness, broad spectra and low toxicity, low cost, ease of delivery and minimal side effects (Wilke *et al*., 2005). These drugs consist of six major groups: penicillins, cephems (cephalosporins and cephamycins) monobactams, penems, and ß-lactam/ß-lactamase inhibitor combinations (CLSI, 2007).

The characteristic of the ß-lactam antibiotic structure is the four member lactam ring, a heteroatomic ring structure, consisting of three carbon atoms and one nitrogen atom. The β-lactam antibiotics are analogues of the

terminal amino acid (D-alanyl-D-alanine) residues on the precursor NAM/NAG-peptide subunits of the peptidoglycan layer. This family of antimicrobial agents differ from each other in the nature of one or two side chain and additional rings that determines the antibacterial spectrum of the antibiotic, for example a five-membered thiazolidine ring for penicillin, a six-membered dihydrothiazine ring for cephalosporins, and double ring structure for carbapenems (a methylene replacement for sulphur in the five-membered α-ring structure), whereas in monobactams only the ß-lactam ring is present (Correa, 2001).

2.2.1. Penicillins:

Penicillin was discovered by Sir Alexander Fleming in 1928. Several penicillins have been developed from the benzyl-linked compound that was first used (Livermore and Williams, 1996). They share features of chemistry, mechanism of action, pharmacologic, clinical effects, and immunologic characteristics with other β-lactam antibiotics. Penicillins are highly effective antibiotics with an extremely low toxicity (Murray *et al*., 2002).

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-penicillic acid (Miró *et al*., 2004). Penicillins are in three groups, penicillins (e.g. penicillin G) have little activity against GNB, antistaphylococcal penicillins (e.g. methicillin, oxacillin) are active against β-lactamases producing Gram-positive bacteria, but inactive against GNB, and broad-spectrum penicillins (e.g. ampicillin and antipseudomonal penicillins) retain the antimicrobial

spectrum of penicillin and have improved activity against GNB (Livermore *et al*., 2006).

Carboxypenicillins that include carbenicillin and ticarcillin, differ from other penicillins in that they contain a carboxylate moiety in the side-chain of the molecule. These molecules are more effective against GNB, most probably because these antibiotics penetrate the outer cell wall more extensively than other penicillins (Forssten, 2009).

2.2.2. Cephems:

2.2.2.1. Cephalosporins:

The first isolation of cephalosporin compounds were from cultures of *Cephalosporium acremonium* in 1948. The cephalosporins contain an aminocephalosporanic acid nucleus consisting of a β-lactam ring fused to a dihydrothiazine ring. They are similar to the penicillins in structure, mechanism of action, toxicity, and act by binding to PBPs of susceptible organisms. They are more stable than penicillins to many bacterial β-lactamases (Correa, 2001).

The cephalosporins are chemically divided into two groups: oxyiminocephalosporins and methoxycephalosporins. They are classified by grouping into five generations based on their antibacterial activity:

2.2.2.1.1. First Generation of Cephalosporins:

They consist of narrow-spectrum drugs, like cephalothin and cefazolin. These drugs are very active against Gram-positive cocci, but against

methicillin-resistant strains are not. They are not active against GNB either. Wild type *E. coli* and *Klebsiella* spp. are susceptible to these drugs (Yao and Moellering, 2003).

2.2.2.1.2. Second Generation of Cephalosporins:

The members of this group are cefaclor, ceforanide, and cefamandole. They are stable to certain β-lactamases and have hence an increased activity against GNB. They are active against organisms affected by first generation drugs, and also have an extended Gram-negative coverage. These drugs are active against *H. influenzae* but not against *Serratia* spp. or *Bacterioid fragilis*. Second generation of cephalosporins may exhibit *in vitro* activity against *Enterobacter* species, but they should not be used to treat infection caused by these organisms because resistant mutants that constitutively express a chromosomal β-lactamase that hydrolyzes these compounds are readily selected (Doucet, 2006).

2.2.2.1.3. Third Generation of Cephalosporins:

Agents of this group are cefotaxime, ceftazidime, cefixime, ceftizoxime, cefpodoxime, cefoperazone, ceftriaxone and ceftibuten. The major traits of these agents, except cefoperazone, are their expanded Gram-negative coverage and the ability of some to cross the blood brain barrier. The third generation cephalosporins are much more active against *Enterobacteriaceae* than narrow-spectrum drugs. These antibiotics are more stable to β-lactamases and are able to pass through the outer cell envelopes of GNB.

Ceftazidime and cefoperazone are useful activity against *Ps. aeruginosa* (Chaudhary and Aggarwal, 2004).

2.2.2.1.4. Fourth Generation of Cephalosporins:

The fourth generation cephalosporins, like cefepime, developed in 1994, reduce the affinity and increase the stability for chromosomally but also some plasmid-mediated β-lactamase enzymes (Yao and Moellering 2003). It is similar to third generation cephalosporins agents but it is more resistant to hydrolysis by chromosomal β-lactamases and some ESBLs that inactivate many of the third generation cephalosporins. It has good activity against *Ps. aeruginosa* and *Enterobacteriaceae*, and highly active against *Haemophilus* and *Neisseria* (Hanson, 2003).

2.2.2.1.5. Fifth Generation of Cephalosporins:

Ceftobiprole is a broad spectrum cephalosporin with activity against Gram-positive and Gram-negative pathogens, it has been described as fifth generation cephalosporin. Several studies have demonstrated that ceftobiprole has a strong affinity for the penicillin binding protein of bacteria (Noel, 2007). It has powerful antipseudomonal characteristics and appears to be less susceptible to development of resistance (Queenan *et al*., 2007).

2.2.2.2 Cephamycins:

The members of cephamycins are like cefotetan, cefoxitin and cefmetazole which are closely related to cephalosporins but have an α-methoxy (-OCH_3) group called oxacephem group instead of the 7-

aminocephalosporanic acid in oxyiminocephalosporins. The cephamycins are highly resistant to various β-lactamases (Yao and Moellering, 2003).

Cephamycins are a heterogeneous group of drugs with marked individual difference in activity, pharmacokinetics, and toxicity. They have activity against anaerobes, *B. fragilis* and *Serratia* spp. strains but are less active against *H. influenzae* (Livermore and Brown, 2001).

2.2.3. Monobactams:

They are monocyclic ß-lactam ring compounds derived from the 3-aminomonobactamic acid. There major characteristic is the presence of the β-lactam ring alone and not fused to another ring (Marin and Gudiol, 2003), hence they lack the double ring structure found in traditional β-lactam antibiotics and they can easily be synthesised. The monocyclic nucleus of monobactams (e.g. aztreonam) has different side-chains (Forssten, 2009).

Monobactams are relatively resistant to ß-lactamases and active against Gram-negative rods, including *Pseudomonas* and *Serratia*. They have no activity against Gram-positive bacteria or anaerobes. Modification of monobactams has produced aztreonam, the only clinically useful. These drugs have a wide range of activity to aerobic GNB, including *Ps. aeruginosa* by binding to PBP3 of their cell wall (Bush, 1996).

2.2.4. Carbapenems:

The carbapenems are structurally very similar to the penicillins, but the sulphur atom in the position 1 of the structure has been replaced with a

carbon atom, and hence the name of the group is the carbapenems. These antibiotics are recognized by double ring structure (Correa, 2001). They have a side chain with a hydroxyethyl side chain in trans configuration at position 6 which confers stability toward most ß-lactamases, including the ESBL (Bonfiglio *et al*., 2002). The additional side-chains and its special disposition confer an important affinity towards the PBPs and make this group of antibiotics resistant to a great part of β-lactamases (Miró *et al*., 2004).

They are used against Gram-positive and GNB as well as anaerobic bacteria. They are active against many highly penicillin-resistant strains of pneumococci. Imipenem, meropenem, and ertapenem are available in treatment. A carbapenem is the antibiotic of choice for treatment of *Enterobacter* infections. Imipenem with or without an aminoglycoside may be an effective treatment for febrile neutropenic patients (Chambers, 2001).

2.2.5. ß-lactam/ß-lactamase Inhibitors:

They are designed to inhibit or destroy the effectiveness of β-lactamase enzymes. These drugs have a poor activity on their own against PBPs and are hence co-administered with β-lactam antibiotics. The β-lactamase inhibitors are so called suicide inhibitors; they form an irreversible acyl enzyme complex by a covalent bond during the catalysis reaction with the β-lactamase, which leads to activity loss of the enzyme. They are most active against Ambler class A β-lactamases, plasmid encoded TEM β-lactamases in particular, such as those produced by species of *E. coli* and

K. pneumoniae, but not good inhibitors of class C β-lactamases, which typically are chromosomally encoded, produced by *Enterobacter* and *Pseudomonas*. These compounds are divided into two groups: clavulanic acid and penicillanic acid sulfones. 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. The penicillanic acid sulfones (sulbactam and tazobactam) are structurally related, they have an oxidation of the sulphur present in the β-lactam ring (Miró *et al*., 2004).

Clavulanic acid acts synergistically with different penicillins and cephalosporins (e.g. amoxicillin) against GNB that produce β-lactamases. The sulbactam is combined with ampicillin, while tazobactam is combined with piperacillin which act against β-lactamase producing strains, since tazobactam does not inhibit the chromosomal β-lactamase (Koletar, 2000).

2.3. ß-lactam Drug Resistance:

Several studies have demonstrated an association between an increased antibiotic consumption and an increase in bacterial resistance to the drug (Steinke and Davey, 2001). The antibiotics or their action usually regulate the expression of resistance genes. Therefore, bacteria expend a considerable amount of energy and genetic space to actively resist antibiotics (Wright, 2005). Bacteria achieve active antimicrobial resistance develops through a number of different ingenious mechanisms. All of these promote the survival of antibiotic-resistant bacteria (World Health Organisation, 2001); and these

mechanisms require new genetic programming by the cell in response to the presence of antibiotics.

Bacteria may be intrinsically resistant to one class or more of antimicrobial agents that will not offer a target or adequate access etc., or resistance can be acquired by genetic alterations leading to protection of the bacteria from the action of an antibiotic drug, or by the acquisition of resistance genes from other organisms (Tenover, 2006; Courvalin, 2006). By their nature, intrinsic resistance and resistance due to chromosomal mutation pose a low risk for horizontal spread. Added resistance genes, especially those carried by mobile genetic elements, may be more easily transferred between bacteria. Acquired resistance can result from the acquisition of a mutation in the regulatory or structural genes and/or the acquisition of a foreign resistance gene (Harbottle *et al*., 2006).

2.3.1. Modification of PBPs Target:

Alteration of the drug target sites PBPs has been implicated in resistance to aminoglycosides, β-lactams, macrolides, fluoroquinolones, glycopeptides and sulphonamides (Waterer and Wunderink, 2001). Mutation of the gene whose product is targeted by the antibiotic may increase the level of resistance or expand the spectrum of resistance. The resistant organisms produce PBPs that have low affinity for binding β-lactam antibiotics and as a result, they are not inhibited except at relatively high drug concentrations (Chambers, 2001). Alterations may occur either by point mutations in PBP genes, by remodeling of PBP genes with foreign DNA, or by acquisition of a resistant PBP (Essack, 2001). For example the alteration of the antibiotic

target to produce a low affinity PBP (PBP5) mediates resistance to ampicillin and penicillin particularly in *Enterococcus faecium*. Vancomycin-resistant enterococci (VRE) produce altered peptidoglycan pentapeptide precursors that terminate not in the typical D-alanyl-D-alanine, but in either of D-alanyl-D-lactate (VanA, VanB, VanD) or D-alanyl-Dserine (VanC, VanE, VanG) which are unaffected by vancomycin (Woodford *et al*., 2005).

2.3.2. Impaired Penetration of Drug to PBPs Target:

The Gram-negative hydrophobic outer membrane contains a number of channel forming proteins (porins), which allow the passage of certain molecules into the bacterial cell with varying degrees of selectivity. Some of the ß-lactam antibiotics use porins to enter the microbe. Down regulation of porins or changes in their chemical structure prevents the antibiotic from exerting its effects (Wilke *et al*., 2005).

ß-lactam resistance in Gram-negative bacilli can occur by mechanism of alterations of porin proteins in the cell membrane causing reduced permeability. *Ps. aeruginosa* lacks the typical high permeability porins; instead, it has low-efficiency porins, which only allow the diffusion of small molecules at about one-hundredth the rate through classical porins (Ferguson, 2007).

2.3.3. The Presence of An Efflux Pump:

Most bacterial organisms must protect themselves from a variety of naturally occurring noxious compounds. Gram-negative bacteria commonly

produce proteins, which act as efflux pumps for antibiotics, which export a variety of compounds (Putman *et al*., 2000). The drug is pumped out faster than it can diffuse in, intrabacterial concentrations of the antibiotic are kept low and ineffectual. This acts as a protective mechanism for the microorganism and prevents it from being killed by its own chemical weapons (Walsh, 2000).

In addition to providing protection from noxious compounds, efflux pumps have been proposed to have roles in detoxification of intracellular metabolites, host pathogenesis, and intercellular communication (Fajardo *et al*., 2008; Martinez *et al*., 2009). Efflux pumps that are able to pump a variety of compounds are most often associated with MDR.

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 regulators can lead to high level expression and confer enhanced antibiotic resistance (Poole and Srikumar, 2001; Piddock, 2006).

2.3.4. Inactivation of Antibiotic by ß-lactamases:

Antimicrobial inactivation is another widespread resistance mechanism, where enzymatic modification or destruction of the antimicrobial abrogates its activity (Wright, 2005). Some of these enzymes inactivate the antibiotic

by hydrolysing susceptible bonds which are central to the antibiotics biological activities (β-lactamases), whereas others modify the antibiotic resulting in structural alterations that impair target binding. The genes encoding β-lactamases are found on chromosomes, in gene cassettes and in plasmids (Walsh, 2003).

β-lactamase produced by *Haemophilus* spp. and *E. coli* is relatively narrow in substrate specificity and will hydrolyze penicillins but not cephalosporins. *Ps. aeruginosa* and *Enterobacter* spp. produced another β-lactamase (broader in spectrum) that will hydrolyze penicillins and cephalosporins. Carbapenems, which are highly resistant to hydrolysis by penicillinases and cephalosporinases, are hydrolysed by a metallo-β-lactamase (Bush, 2001).

β-lactamases are the most common cause of bacterial resistance to the medically important β-lactam antibiotics in Gram-negative bacteria (Babic *et al*., 2006). ß-lactamase enzymes in GNB are either secreted to the outside membrane environment as exoenzymes or remain in the periplasmic space, where they attack the antibiotic before it reaches to its receptor site (PBPs) (Samaha-Kfoury and Arej, 2003). Susceptibility to ß-lactams in *E. coli* depends on the concentration of antimicrobial agents available in periplasmic space and on the amount of the antimicrobial agent capable of binding to penicillin binding proteins (Oliver *et al.*, 2002).

Different classifications based on phenotype, gene or amino acid protein sequences and function have been attempted since the beginning of the 1970s (Hall and Barlow, 2005). 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 (Ambler *et al.*, 1991) (Table 3-2).

2.3.4.1. Structure and action of β-lactamases:

The β-lactamases are divided into two classes; serine and metallo β-lactamases that do not share sequence or structural homology. However, both β-lactamase classes are a major mechanism of resistance in the Gram-negative bacteria which include ß-lactamases by hydrolyse the amide bond of the four-membered β-lactam ring.

The serine β-lactamase classes A, C and D share similarity at the protein structure level, which proves that they descended from a common ancestor (Hall and Barlow, 2004). Its reactions are in three steps. At first, formation of the non-covalent Michaelis-Menten complex by associating non-covalently the enzyme with the drug (binding step). The lactam bond of ß-lactam drugs nucleophilic is attacked by the free hydroxyl on the side chain of a serine residue at the active site of the enzyme, yielding a covalent acyl ester (acetylation step) (Pratt, 2002). The acyl intermediate of the ß-lactamases undergoes deacylation much more rapidly and is therefore very

efficient at destroying the antibiotic and protecting the peptidoglyacan synthesis (Sun *et al.*, 2003). Then finally in hydrolysis step, the ester liberates active enzyme and hydrolysed inactive β-lactam antibiotic (Bonomo and Rice, 1999). The ability ß-lactam drugs to be efficiently

Table (2-1): Functional and molecular characteristics of the major groups of ß-lactamases (Bush, 2001)

Functional group	Major subgroups	Molecular class	Attributes of ß-lactamases in functional group
1		**C**	Often chromosomal enzymes in Gram-negative bacteria but may be plasmid-encoded. Confer resistance to all classes of ß-lactams, except carbapenems (unless combined with porin changes). Not inhibited by clavulanic acid.
2		**A, D**	Most enzymes responsive to inhibition by clavulanic acid (unless otherwise noted).
	2a	**A**	Staphylococcal and enterococcal penicillinases included. Confer high resistance to penicillins.
	2b	**A**	Broad-spectrum ß-lactamases, including TEM-1 and SHV-1, primarily from gram-negative bacteria.
	2be	**A**	Extended-spectrum ß-lactamases conferring resistance to oxyimino-cephalosporins and monobactams.
	2br	**A**	Inhibitor-resistant TEM (IRT) ß-lactamases; one inhibitor-resistant SHV-derived enzyme.
	2c	**A**	Carbenicillin-hydrolyzing enzymes.
	2d	**D**	Cloxacillin-(oxacillin)–hydrolyzing enzymes; modestly inhibited by clavulanic acid.
	2e	**A**	Cephalosporinases inhibited by clavulanic acid.
	2f	**A**	Carbapenem-hydrolyzing enzymes with active site serine, inhibited by clavulanic acid.
3	**3a, 3b, 3c**	**B**	Metallo–ß-lactamases conferring resistance to carbapenems and all ß-lactam classes except monobactams. Not inhibited by clavulanic acid.
4		**? b**	Miscellaneous unsequenced enzymes that do not fit into other groups.

hydrolyzed by the ß-lactamase depends upon the composition and structure of attached R groups. Certain R groups prevent or hinder entry of ß-lactam drugs into the active site (**Figure 2.1**) (Garau *et al*., 2005).

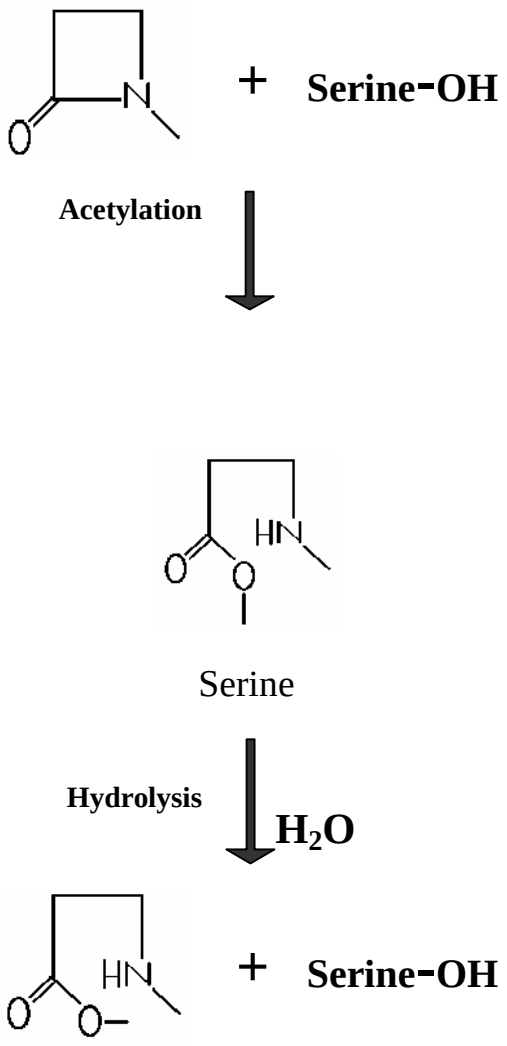

Figure (2.1): Schematic representation of the activity of a serine β-lactamase (Ambler classes A, C, and D).

The metallo β-lactamases need a bivalent cation, usually zinc ion, to be able to hydrolyse the β-lactam ring (**Figure 2.2**) (Garau *et al*., 2005).

Figure (2.2): Schematic representation of the activity of a metallo β-lactamase (Ambler class B).

2.3.4.2. Types of ß-lactamases:

2.3.4.2.1. Narrow Spectrum ß-lactamases:

They are also called broad-spectrum ß-lactamases which are the chromosomal or plasmid-encoded β-lactamases TEM-1, TEM-2, and SHV-1. These enzymes are recognized to confer resistance to penicillins and cephalosporins, but not to the newer cephalosporins (Poole, 2004). These β-lactamases are the most prevalent among clinical isolates of *Enterobacteriaceae* worldwide. The TEM-1 β-lactamase encoded by the bla_{TEM-1} gene is present in Tn2 and Tn3 transposons.

2.3.4.2.2. ESBL:

The extended spectrum ß-lactamases (ESBLs) are so called because of their increased spectrum of activity (Bradford, 2001). Generally, ESBLs evolved from class A, TEM variant (from TEM-1 or TEM-2) and SHV variant (from SHV-1), class D ESBLs (the OXA enzymes), as well as CTX-M and VEB enzymes which remain the most prevalent types of ESBLs. OXA, and CTX-M are non-TEM and non-SHV plasmid mediated ESBLs that have been reported (Bonnet, 2004; Jacoby and Munoz-price, 2005). The majority of ESBLs contain

a serine at their active site and attack the amide bond in the ß-lactam ring of antibiotics causing their hydrolysis (Chaudhary and Aggarwal, 2004).

ESBLs enzymes are capable of hydrolyzing and inactivating a wide variety of therapeutic ß-lactam antimicrobials, including third generation cephalosporins (such as ceftazidime and cefotaxime), penicillins and monobactam (aztreonam) except carbapenems and cephamycins (e.g., cefoxitin) (Chaudhary and Aggarwal, 2004). They are generally inhibited by ß-lactamase inhibitors, (e.g. clavulanic acid) (Al-Jasser, 2006; Pfaller and Segreti, 2006). However, it has been reported that ESBL producing strains can become resistant to cephamycins when loss of outer membrane porin protein occurs (Ananthan and Subha, 2005). They are found in a range of Gram-negative bacteria.

They exceed 200, ESBLs are encoded by chromosomes and generally encoded by plasmid-borne genes, usually plasmid-borne and involves multiple resistance determinants, and is often indicative of ESBL production (Potz *et al.*, 2006). Therefore, they are commonly resistant to different antibiotic families including (besides ß-lactams) fluoroquinolones, aminoglycosides and trimetoprim-sulfametoxazole, which is considered as multidrug-resistant (Weldhagen *et al.*, 2003).

2.3.4.2.3. AmpC ß-lactamases:

AmpC is the main representative of Class C β-lactamases which have active site serine (Majiduddin *et al.*, 2002). They are clinically significant because they confer resistance to a variety of penicillins, broad and extended spectrum cephalosporins, aztreonam and ß-lactamase inhibitors. (e.g. clavulanic acid) (Hanson, 2003); however, they are inhibited by cloxacillin. These enzymes are typically associated with multiple antibiotic resistances including

ceftazidime and cefotaxime (Livermore and Brown, 2001). Cefepime and carbapenems resist hydrolysis by AmpC and can hence be used as a possible screening agent. Almost 100 different AmpC enzymes exist today and are commonly isolated from extended-spectrum cephalosporin-resistant Gram-negative bacteria (Jacoby and Bush, 2009).

2.3.4.2.4. Carbapenemases:

Carbapenemases are responsible for resistance to carbapenems antibiotics as well as the other β-lactams so called carbapenem-hydrolyzing β-lactamases. These enzymes have been classified to the class B (metallo-ß-lactamases), which has cations of zinc, the class A (clavulanic acid-inhibited carbapenemases like KPC), and the class D (oxacillinases), which both are serine β-lactamases.

2.4. The Spread of Antibiotic Resistance by Gene Transfer Systems:

Transferable β-lactamase genes can be spread from a donor to a recipient on plasmids, transposons, insertion sequences and integrons, by conjugation, transduction or transformation. The result cell has a genome different from the donor or the recipient (Courvalin, 2006).

2.4.1. Conjugation:

During conjugation, the donor bacterium extends a rod-like conjugation pilus that connects with the recipient bacteria. It is the unidirectional transfer of bacterial genetic material from donor to recipient. This relation occurs mainly in

Gram-negative bacteria. But in many Gram-positive bacteria, transmissible plasmids differ in the mechanisms used to establish cell to cell contact. They do not appear to use pili as an initiator of conjugation; instead, transfer appears to be by aggregation of bacterial cells mediated by cell surface structures (Grohmann *et al.*, 2003).

Conjugation has long been considered the most important mechanism for the dissemination of antibiotic resistance genes. A number of different DNA elements have played a main role in the development of resistance in bacteria (Normark and Normark, 2002).

2.4.2. Transformation:

It is a physiological process characteristic of several bacterial species in which a recipient cell takes up free DNA directly from the environment. After death or cell lysis, some bacteria release their DNA into the environment. Other bacteria can come into contact with these fragments, take them up and incorporate them into their DNA by recombination. There is no requirement for contact between a cell and another. Although competence occurs throughout the bacterial cell cycle, the early exponential growth phase and at the beginning of the stationary phase, has the highest transformation rate (Licht and Wilcks, 2006).

2.4.3. Transduction:

Transduction is mediated by bacteriophage which are extrachromosomal genetic elements (DNA or RNA) termed bacterial viruses due to their ability to

infect bacterial cells and to transfer independently. To enter the host cell, the bacteriophage must attach to specific receptors on the surface of the bacteria and may therefore only infect bacteria carrying these receptors. Once inside the cell, they may integrate into the host genome without killing the host or replicate in large numbers causing the cell to lyse. When exiting the host genome, pieces of host DNA that may contain resistance determinants may also be transferred by chance, along with the bacteriophage to a recipient bacterium and inserted into the recipient chromosome (McDermott *et al.*, 2003).

2.5. Mechanisms of Transfer of Resistance:

Genetic elements include plasmids, transposons, genomic islands, phage, integrons and gene cassettes.

2.5.1. Plasmids:

Plasmids are extra-chromosomal that replicate independently of the chromosome and play a crucial role in the sharing of genetic information (Madigan and Martinko, 2006). Most are circular, double stranded DNA molecules that have been found in bacterial genera of medical importance. They vary in size from 2 kbp to more than 100 kbp. They encode the mechanism for their own mobilization and are therefore excellent vehicles for transferring genes, not only to their progeny but also from one bacterium to another. Plasmids generally carry genes that play a role in the bacterium's adaptation to a change in its environment, such as those involved in resistance to antibiotics, disinfectants and heavy metals (Dobrindt *et al.*, 2004). However, they also encode metabolic properties such as metabolism of carbohydrates and amino acids, virulence factors and conjugal properties such as sex pili production and mobilization function. Plasmids may be integrated in part or in total into the

chromosomal DNA or may represent vectors for transposons via conjugation or transformation (Schwarz and Nobel, 1999). Some plasmids carry as many as six or seven genes that confer resistance to different antibiotics (Black, 2002).

2.5.2. Transposons:

Transposons are short linear DNA segments, although some form a circularized intermediate. They can insert more or less at random into plasmids or bacterial chromosomes by recombination, which is mediated by transposes encoded within the transposon (Masterton, 2003). They do not replicate independently and are usually incorporated into the bacterial chromosome (Mullany, S. 2002). They vary in size from 1 kbp to more than 60 kbp (Schwarz and Nobel, 1999). Transposons carry one or more additional genes most of which are genes conferring resistance to antibiotics, which can then 'jump' between the bacterial chromosome and a plasmid and vice versa.

Composite transposons consist of two insertion sequences (IS) flanking intervening DNA that often includes a genetic determinant for antibiotic resistance, and this structure moves as a unit (Galimand *et al.*, 2005).

2.5.3. Integrons:

Integrons are mobile genetic elements that are capable of acquiring and expressing genes in the form of gene cassettes which antibiotic resistance determinants can be passed on from one bacterium to another. 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 (Weldhagen, 2004; Wilke *et al.*, 2005).

Recruiting exogenous genes represents a rapid adaptation against antimicrobial compounds, and the integron functional platform is perfectly suited for capturing the genes that enable bacteria to survive during multiple antibiotic treatment regimes (Mazel, 2004).

Integron structure consists of two conserved regions (*int*I region and *sul*I region) flanking a variable region (gene cassette) that contains one or more resistance genes. The *int*I region encodes integrase, the *sul*I region confers resistance to sulphonamides, the gene cassette is mobile and can also exist in free circular form. There are several classes of integrons, each with a distinct integron-specific integrase. Class 1 integrons and integron-borne gene cassettes are the most common class found in clinical isolates and are widespread amongst *Enterobacteriaceae* and *Ps. aeruginosa* encompassing several classes of β-lactamases which are Ambler classes A (except TEM and SHV types), B and D β-lactamase enzymes, giving rise to widespread β-lactam resistance (Nordmann and Poirel, 2002).

2.6. Dissemination of Antimicrobial Resistance:

Antimicrobial resistance is the ability of a microorganism to withstand an antimicrobial compound. Antibiotic resistance traits are distributed in bacteria in vertical (clonal) dissemination when it passed to daughter cells during replication of a bacterial strain (clone), or in horizontal transmission by providing a bacterial host of a different strain, species or even genus with genetic information that can be transiently needed, such as antibiotic resistance

genes (Courvalin, 2006). Most bacterial genomes that have been sequenced contain DNA segments that have played a main role in the development of resistance in bacteria (Normark and Normark, 2002). Molecular-based epidemiological studies have shown that the mechanisms of ESBL spread include clonal strain dissemination and therapeutic selection among epidemic strains (DiPersio *et al*., 2005). The DNA mobile elements possess genetic determinants for several different antimicrobial resistance mechanisms and may be responsible for the rapid dissemination of resistance genes among different bacteria (Perez *et al*., 2007).

CHAPTER THREE

MATERIALS

and

METHODS

3-1 Materials:

3-1-1 Instruments and Equipment: The instruments and equipment used in this study are listed below:

Type of equipment	Manufacture (Origin)
Autoclave	Arnold and Sons (USA)
Bench centrifuge	Hettich (Germany)
Burners	Humboldt
Centrifuge	Memmert (Germany)
Cold centrifuge	Hettich
Cold incubator	Memmert
Compound light microscope	Olympus (Japan)
Digital camera	Sony (Japan)
Disposable cotton swab	BDH (England)
Distillator	GFL (Germany)
Electric oven	Memmert
Electrophoresis	Labnet International (Taiwan)
Incubator	Memmert
Laminar flow cabinets	Cruma (Spain)
Magnetic stirrer/ hot plate	Witag (Germany)
Micropipette	Slamed (Germany)
Millipore filter (0.22µm)	Difco (USA)
PCR system	Singapore
pH-meter	LKB (Sweden)

Type of equipment	Manufacture (Origin)
Refrigerator	Kiriazi (Egypt)
Sensitive balance	Memmert (Germany)
Shaker incubator	Gallen Kamp
Shaker water bath	Kottermann (Germany)
Spectrophotometer	Spectronic 20
Standard loop 0.01 ml	Hi media (India)
UV-transilluminator	Taiwan
Vortex mixer	Memmert
Water bath	Memmert

3-1-2 Biological and Chemical Materials: The biological and chemical materials used in this study are listed below:

Biological and chemical type	Manufacturer (Origin)
Acetic acid (CH_3COOH)	Difco (USA)
Agarose	Promega (USA)
L-arabinose	Rwdel Dchaenag
Barium chloride ($BaCl_2.2H_2O$)	Fluka (Switzerland)
Beef extract	Difco
Boric acid (H_3BO_3)	Fisher S. I. Co. (U.K.)

Biological and chemical type	Manufacturer (Origin)
Bromophenol blue	Difco (USA)
Cloroform	BDH
Dextrose	Difco
Disodium hydrogen phosphate ($Na_2H PO_4$)	BDH
Ethanol (96%)	BDH
Ethidium bromide	Sigma (USA)
Ethylenediaminotetraacitic acid (EDTA) ($C_{10}H_{14}N_2Na_2O_8.2H_2O$)	BDH
Glucose ($C_6 H_{12} O_6$)	Difco
Glycerol ($C_3H_8O_3$)	Fluka
Hydrochloric acid (HCl)	BDH
Hydrogen peroxide (H_2O_2) 30%	SID (Iraq)
Iodine	Mast Diagnostic (USA)
Iso-amylalcohol	BDH (England)
Isopropyl alcohol	Mast Diagnostic
Kovac's reagent	BDH
Maltose	Difco
D-mannose ($C_6H_{12}O_6$)	Difco
Methyl red	BDH
α-naphthol ($C_{10}H_8O$)	BDH

Biological and chemical type	Manufacturer (Origin)
Peptone	Difco (USA)
Phenol crystal	Merck (Germany)
Phenol red	BDH
Potassium dihydrogen phosphate (KH_2PO_4)	BDH
Potassium hydroxide (KOH)	BDH
Potassium iodide (KI)	Mast Diagnostic
D-raffinose ($C_{18}H_{32}O_{16}$)	Difco
Safranin	BDH
Sodium chloride (NaCl)	BDH
Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	BDH
Sodium dodecyl sulfate (SDS)	AppliChem (Germany)
Sodium hydroxide (NaOH)	BDH
Soluble starch	Difco
Sucrose ($C_{12}H_{22}O_{11}$)	Difco
Sulfuric acid (H_2SO_4)	Difco
Tris-(hydroxymethyl) methylamine ($NH_2.(CH_2OH)_3$ (Tis-OH)	BDH

Biological and chemical type	Manufacturer (Origin)
Urea solution	Mast Diagnostic
D-xylose	Difco
Yeast extract	Difco

3-1-3 Culture Media: The following media were used in this study:

Medium	Manufacturer (Origin)
Brain heart infusion broth and agar	Mast Diagnostic (UK)
Decarboxylase broth base	Mast Diagnostic
Eosin methylene blue agar	Hi media (India)
MacConkey agar	Biolife (Italy)
Mueller Hinton agar	Oxoid (UK)
MR-VP broth	Oxoid
Nutrient broth	Biolife
Nutrient agar	Biolife
Peptone water	Biolife
Simmons citrate agar	Mast Diagnostic
Tryptic soy broth	Biolife
Tryptic soy agar	Biolife
Triple sugar iron agar (TSI)	Biolife
Trypton (pancreatic digest of casein)	Oxoid
Urea agar base	Biolife

3-1-4 Antibiotics:

3-1-4-1 Antibiotics Powders:

Antimicrobial class	Antimicrobial subclass	Agents included generic names	Assembly	Manufacturer (origin)
penicillins	aminopenicillin	amoxicillin	**AMX**	A.P.m. (Jordan)
		ampicillin	**AMP**	Ajainta (India)

3-1-4-2 Antibiotics Discs:

Antimicrobial class	Antimicrobial subclass	Agents included generic names	Assembly	Content	Manufacturer (origin)
penicillins	ureidopenicillin	piperacillin	Pc	100 µg	Hi Media (India)
β-lactam/ β-lactamase inhibitor combinations		amoxicillin-clavulanic acid	A/C	30 µg	Hi Media
Cephems (parenteral)	Cephalosporin I	cefazolin	Cz	30 µg	Hi Media
	Cephalosporin III	cefotaxime	CTX	30 µg	Bioanalyse
		ceftazidime	CAZ	30µg	Bioanalyse
		ceftriaxone	CRO	30µg	Bioanalyse
	Cephalosporin IV	cfepime	FEP	30 µg	Bioanalyse
	Cephamycin	cefoxitin	FOX	30 µg	Hi Media

Antimicrobial class	Antimicrobial subclass	Agents included generic names	Assembly	Content	Manufacturer (origin)
Cephems (oral)	Cephalosporin	cefixime	FIX	5 µg	Hi Media
monobactams		Aztreonam	ATM	30µg	Bioanalyse
penems	carbapenem	imipenem	IPM	10 µg	Bioanalyse
aminoglycosides		gentamicin	GM	30 µg	Hi Media
		Tobramycin	TO	30 µg	Hi Media
ansamycins		rifampin	RA	5 µg	Bioanalyse
quinolones	quinolone	Nalidixic acid	NA	30 µg	Bioanalyse
	fluoroquinolone	Ciprofloxacin	CIP	10 µg	Hi Media
		levofloxacin	Le	5 µg	Hi Media
		norfloxacin	Nx	10 µg	Hi Media
Folate pathway inhibitors		trimethoprim	TR	25 µg	Hi Media
		Co-trimazine	Cm	25 µg	Hi Media
phenicols		Chloramphenicol	C	30 µg	Bioanalyse
tetracyclines		tetracycline	TE	10 µg	Hi Media

3-1-5 Standard Bacterium:

Standard strain	Serotype	Laboratory identifier	Key characteristics	Source
Escherichia coli	O6:H1	ATCC 25922	Susceptible to AMP^S, $Ceph^s$, and GM^s	American Type Culture Collection

3-1-6 Primers Used in PCR Technique (Alpha DNA, Montreal):

Primer name	DNA Sequence 5′- 3′	Amplicon size	References
TEM (F)*	ATGAGTATTCAACATTTCCG	858	Bedenic *et al.* (2001)
TEM (R)**	CCAATGCTTAATCAGTGAGG		
SHV (F)	CGCCGGGTTATTCTTATTTGTCGC	1016	Bedenic *et al.* (2001)
SHV (R)	TCTTTCCGATGCCGCCGCCAGTCA		
OXA (F)	ATATCTCTACTGTTGCATCTCC	619	Svärd (2007)
OXA (R)	AAACCCTTCAAACCATCC		
CTX-M (F)	GCTTTATGCGCAGACGAGTG	668	Yu *et al.* (2002)
CTX-M (R)	TCATTGGTGGTGCCGTAGTC		

* F: forward

** R: Reverse

3-1-7 Master Mix Used in PCR Technique:

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

3-2 Methods:

3-2-1 Preparation of Common Buffers and Solutions:

In the present study, the following solutions and reagents were used and sterilized by autoclaving. Millipore filters (0.22 µm) were used for sterilization of heat-sensitive solutions like antibiotics and urea. The pH of the solution was adjusted using 1M NaOH (**3-2-1-3**) and 1M HCl (**3-2-1-4**).

3-2-1-1 McFarland 0.5 Turbidity Standard:

It is composed from mixed of 0.05 ml of 1.175% $BaCl_2$ solution and 9.95 ml of 1% (0.36N) H_2SO_4 solution, the turbidity of a $BaSO_4$ solution was adjusted at 625 nm to be 0.08- 0.10, and stored in closely sold dark tube at room

temperature. It is used to approximate the turbidity of bacterial suspension (NCCLS, 2003a).

3-2-1-2 Saline Solution:

It was prepared by dissolving 0.85 gm of NaCl in 100 ml of D.W, autoclaved at 121˚C for 15 min, and stored at 4˚C until used (Collee *et al*., 1996).

3-2-1-3 Sodium Hydroxide Solution (1 M):

Sodium hydroxide (4 gm) was dissolved in 100 ml of D.W. (Collee *et al*., 1996).

3-2-1-4 Hydrochloric Acid Solution (1 M):

Hydrochloric acid (8.6 ml) was added to 91.4 ml of D.W. (Collee *et al*., 1996).

3-2-2 Preparation of Reagents:

Prepared as in MacFaddin (2000):

3-2-2-1 Oxidase Reagent:

Tetra methyl 1, 4-phenylenediamin dihydrochloride 0.1 gm was dissolved in 10 ml of D.W. and stored in a dark bottle; this reagent was used as an indicator in oxidase test.

3-2-2-2 Catalase Reagent:

Hydrogen peroxide H_2O_2 (3%) was prepared from stock solution and stored in a dark bottle. The reagent was used for the detection of bacterial ability to produce catalase enzyme.

3-2-2-3 Voges-Proskauer Reagents:

It consists of two solutions:

Solution A: 5 gm of α-naphthol in 100 ml of absolute ethanol.

Solution B: 40 gm of KOH in 100 ml of D.W.

These solutions were used as an reagent in VP test.

3-2-2-4 Methyl Red Reagent:

It was prepared by dissolving 0.1 gm of the dye in 300 ml of ethanol and made up to 500 ml with D.W. This reagent was used as an indicator in methyl red test.

3-2-3 Preparation of Culture Media:

3-2-3-1 Ready Prepared Culture Media:

According to manufacturer instructions, the culture media were prepared and sterilized in the autoclave at 121˚C for 15 minutes.

3-2-3-2 Maintenance Medium:

It consists of nutrient broth as a basal medium supplemented with 15% glycerol that was added to test tubes (5 ml in each one) and sterilized by autoclave. This medium was used to store the bacterial isolates in – 20 ˚C for a long time. (Collee *et al*., 1996).

3-2-3-3 Carbohydrates Fermentation Medium:

According to MacFaddin (2000), this medium was prepared as follows:

a) Basal medium:

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

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

b) Carbohydrates:

The solution of the following carbohydrate (sucrose, lactose, maltose, glycerol, arabinose, xylose, and starch) were sterilized separately by Millipore

filters (0.22µm) and added to the basal medium, the final concentration of each was 1%. These media were used to test the ability of bacterial isolates to ferment a specific carbohydrate incorporated in a basal medium.

3-2-3-4 Decarboxylase Broth Medium:

Decarboxylase Broth (as a basal medium) was autoclaved to 56˚C in water bath, 1% L-ornithine hydrochloride and L-ornithine were added separately after filtrated each one by 0.22 Millipore filter. The medium was circulated in 5 ml in sterile tubes, 1 ml of sterile liquid paraffin was added to provide a thick layer above the medium, and then kept at 4˚C until used. This medium was used to determine the ability of bacteria to lysine Decarboxylase or ornithine to amine with resulting alkalinity (MacFaddin, 2000).

3-2-3-5 Motility Medium:

The medium consists of 1% tryptose, 0.5% NaCl, and 0.5% agar, the pH was adjusted to 7.2, dispensed approximately 5 ml per tube, autoclaved at 121˚C for 15 minutes, allowed to cool in an upright positive, and refrigerated (4˚C) for storage (MacFaddin, 2000).

3-2-3-6 β-lactam Resistance Media:

Muller-Hinton agar was prepared as in manufacturer instruction, after cooled, ampicillin and amoxicillin were added separately, from stock solution (their concentrations 100 and 50 µg/ml, respectively), the medium was poured

into sterilized petri dishes, and stored at 4˚C. This medium was used to primary screening of β-lactam Resistant bacteria (NCCLS, 2003b).

3-2-4 Solutions Used in DNA Extraction:

The preparations of the following solutions were described by Pospiech and Neumann (1995) with some modifications:

3-2-4-1 Tris-HCl (0.5 M) pH (8.0):

It was prepared by dissolving 30.275 gm of Tris-HCl in 400 ml of D.W; pH was adjusted to 8.0, then completed to 500 ml with D.W, and sterilized by autoclaving.

3-2-4-2 Tris-HCl (0.1 M) pH (8.0):

Tris-HCl (6.055 gm) was dissolved in 400 ml of D.W; pH was adjusted to 8.0, completed to 500 ml with D.W, sterilized in autoclave and stored at 4˚C.

3-2-4-3 Tris-EDTA Buffer (TE buffer):

Tris-OH 250 ml (10 mM) pH (8.0) was mixed with equal volume of EDTA (1 mM), autoclaved at 121˚C for 15 minutes, and stored at 4˚C.

3-2-4-4 STE Buffer:

It was prepared by dissolving 20 mM of Tris-OH, 250 mM of EDTA solution, and 75 mM of NaCl in 750 ml of D.W., pH was adjusted to 8.0 and

completed to 1000 ml by D.W, then autoclaved at 121˚C for 15 minutes, and stored at 4˚C.

3-2-4-5 SDS Solution (25%):

Sodium dodecyl sulfate (2.5 mg) was dissolved in 10 ml of D.W, then sterilized by autoclaving, and stored at 4˚C.

3-2-4-6 NaCl Solution (5 M):

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

3-2-4-7 Phenol Solution:

One hundred ml of phenol (melting in waterbath at 68˚C), 0.1 ml of 8-Hydroxyquinoline, and 100 ml of Tris-HCl (0.5 M), were mixed by vortex mixture. The solution was left to be separated in 2 layers, the upper layer was discarded, and 100 ml of Tris-HCl (0.1 M) were added, mixed well, and discarded the upper layer again, these steps were repeated until the pH of solution became 8.0.

3-2-4-8 Chloroform: Isoamyl Alcohol Solution (24:1):

It was prepared by adding 240 ml of chloroform to 10 ml of isoamyl alcohol.

3-2-4-9 Ethanol 70%:

Absolute ethanol 70 ml was added to 30 ml of D.W.

3-2-5 Solution Used in Gel Electrophoresis:

3-2-5-1 Bromophenol Blue:

A bromophenol blue powder (25 mg), and sucrose (4 mg) were dissolved in 10 ml of D.W, then the pH was adjusted to 4.6 and stored at 4˚C (Ferguson, 2007).

3-2-5-2 Ethidium Bromide Solution:

Ethidium bromide (0.05 gm) was dissolved in 10 ml of D.W and stored in a dark tube. This dye forms a brightly fluorescent band of separated DNA fragments that can be photographed on gels (El-Enbaawy and Yousif, 2006).

3-2-5-3 Tris-Borate-EDTA Buffer (TBE):

Tris-OH (0.089 M), boric acid (0.089 M), and 0.002 M of EDTA were dissolved in 980 ml of D.W, then the pH was adjusted to 8.0, was completed the volume to 1000 ml, autoclaved, and stored at 4˚C (Sambrook *et al*., 1989).

3-2-6 Biochemical Tests:

3-2-6-1 Oxidase Test:

Small pieces of filter paper were soaked in 1% oxidase reagent. A fresh

young culture was scraped with a sterile wooden stick and rubbed on the filter paper. A positive result was indicated by an intense deep-purple color which appeared within 5-10 sec (MacFaddin, 2000).

3-2-6-2 Catalase Production Test:

With an inoculating needle, an 18-24 hr pure colony was picked and placed on a clean glass slide. A drop of 3% H_2O_2 was placed on the organism with a dropper. The production of gas bubbles indicates a positive reaction (MacFaddin, 2000).

3-2-6-3 Indole Production Test:

Peptone water was inoculated with a young agar culture and incubated at 37 ˚C for 48 hr Kovacs reagent (0.5 ml) was added. A red color in the alcohol layer indicated a positive reaction (MacFaddin, 2000).

3-2-6-4 Simmons Citrate Test:

Simmons citrate slant was inoculated with a young agar culture and incubated at 37˚C for 48 hr. A blue color and streak of growth appearance indicated a positive test (MacFaddin, 2000).

3-2-6-5 Voges-Proskauer Test:

Methyl Red-Voges-Proskauer broth was inoculated with a young agar culture and incubated at 37˚C for 48 hr then added to each tube one ml of

solution B (40% KOH solution) and three ml of solution A (5% α-naphthol solution). After 15 min, the positive result is represented by the color change to pink red which indicates partial hydrolysis of sugar and production of acetyl methyl carbinol (MacFaddin, 2000).

3-2-6-6 Methyl Red Test:

Methyl Red-Voges-Proskauer broth was inoculated with a young culture and incubated at 37˚C for 24 hr, five drops of methyl red reagent were added, mixed and the result was read immediately, a bright red color indicated that the pH was reduced to 4.5 or less and complete hydrolysis of sugar (MacFaddin, 2000).

3-2-6-7 Urease Test:

Urea agar slant tube was inoculated heavily over the entire slant surface with a young culture and incubated at 37˚C. The result was read after 24 hr, and every day for 6 days. Changing the color of medium to purple-pink indicates a positive result (MacFaddin, 2000).

3-2-6-8 Triple Sugar Iron Agar Test:

By needle, bacterial colony was inoculated over the surface of slope (slant) and stabbed into the bottom of TSI iron agar. Then incubated at 37˚C for 18 hr.

The results were according to the color of top-slant and bottom, as well as the gas production of bottom and formation of hydrogen sulfate as a black deposit (MacFaddin 2000).

3-2-6-9 Carbohydrate Fermentation Test:

Young bacterial colonies were inoculated in carbohydrate fermentation broth and incubated at 37˚C for 1-5 days. The yellow color media with or without gas production indicates positive test (MacFaddin, 2000).

3-2-6-10 Ornithine Decarboxlase Test:

Ornithine decarboxlase broth was inoculated through the paraffin layer by a straight wire and incubated at 37˚C for 2 days. The positive test was detected by changing indicator color to violate (MacFaddin, 2000).

3-2-6-11 Motility Test:

Motility medium tubes were inoculated with a young agar culture by stabbing by a straight wire vertically into the center of the agar butt to a depth of approximately 2 cm and incubated for 24-48 hr formation of cloudy growth out of line of stab indicates a positive result (MacFaddin, 2000).

3-2-6-12 Growth at 10˚C:

Nutrient agar plate was streaked by bacterial isolates and incubated at 10˚C for 24 hr The colonies of bacteria were appeared that mean the ability of grow in this low temperature.

3-2-7 Preservation and Maintenance of Bacterial Isolates:

The bacterial isolates were preserved according to Collee (1996) as follows:

a) Preservation for a short time: The bacterial isolates were inoculated on nutrient agar slant, incubated at 37˚C for 24 hr and preserved at 4˚C. The isolates were maintained monthly during the study by subculture on new nutrient agar medium.

b) Preservation for long time: The bacterial isolates were inoculated in nutrient broth supplemented with 15% glycerol and maintained frozen (-20˚C) for several months.

3-2-8 Sample Collection and Processing:

A total of 613 samples were recorded, these samples were obtained from patients suffering from wound infections (390 samples) and lower respiratory tract infections (223 samples), during the period from November 2008 to February 2009. Samples were collected from patients attending to Al-Sadr Teaching hospital and Tuberculosis and Chest Disease Clinic / Najaf city. The wound samples were collected by sterile swabs while sputum samples were collected into sterile containers.

All samples were investigated for the presence of *Ps. aeruginosa, E. coli, Klebsiella* spp., and *Proteus* spp. Swab from wound infection and a loopful

from the sputum were cultured on the MacConkey agar plates and incubated at 37˚C under aerobic condition for 24 hr.

The study population included all age groups. For each patient, a questionnaire was filled in about his age, gender, and address.

3-2-9 Identification of Microorganisms:

The isolates were identified to the species level by their characteristic appearance on the media, Gram stain reaction and the patterns of biochemical reaction (listed in **3-2-7**), according to the methods of Cowan (1985), Holt *et al*. (1994), and MacFaddin (2000). Isolates obtained either in pure culture or as predominant growth were included in this study, the isolates were stored at maintenance media (listed in **3-2-8**), until further workup.

3-2-10 Screening Test for β-lactam Resistance:

Bacterial isolate was inoculated on Muller-Hinton agar with ampicillin (100 µg/ ml), and Muller-Hinton agar with amoxicillin (50 µg/ ml) prepared in (**3-2-3-6**), and incubated at 37˚C for 18 hr isolates with resistance to ampicillin and/ or amoxicillin were selected for further study. Results showed preliminary isolates resistance to β-lactam antibiotics (NCCLS 2003b).

3-2-11 Antibiotics Susceptibility Testing:

Isolates that are resistant to β-lactam antibiotics were selected and screened for susceptibility to antibiotics shown in Table (**3-1-4-2**). Antibiotics

susceptibility were done on Muller-Hinton agar plates with the disc diffusion technique according to the recommendation of the disc manufacturer and based on the method of Kirby and Bauer (Bauer *et al*., 1966). The culture were incubated at 37˚C for 18 hr under aerobic conditions, and then bacterial growth inhibition zone, around the discs were measured by caliper and compared with National Community for Clinical Laboratory Standard (CLSI, 2007).

The implied 22 antibiotics were chosen according to that commonly used for the treatment of bacterial infection in our region and according to CLSI (2009), these are listed in (**3-1-4-1, 2)**. *E. coli* ATCC 25922 reference strain from the American collection was used as control.

3-2-12 Screening of ESBL-producers:

Two methods for detection of ESBL were used:

3-2-12-1 Initial Screen Test:

ESBL production by β-lactam resistant isolates was initially screened by using disc diffusion of cefotaxime, ceftazidime, ceftriaxone, and aztreonam (30 µg each) placed on inoculated plates containing Muller-Hinton agar according to the CLSI recommendations (CLSI, 2009). After 18 hr incubation at 37˚C, the diameters of the inhibition zones around the antibiotics were measured by caliper. The isolates which showed inhibition zone $\leq$ 27 mm for cefotaxime, $\leq$ 22 mm for ceftazidime, $\leq$ 25 mm for Ceftriaxone, and $\leq$ 27 mm for aztreonam were suspected for ESBL production.

3-2-12-2 Phenotypic Confirmatory Test:

The suspected isolates for ESBL production were further investigated through a second confirmatory method [double disc synergy test (Jarlier *et al*., 1988)]. Muller-Hinton agar was inoculated with suspension of bacterial colonies (equivalent to a McFarland 0.5 standard) by sterile swab and within 15 min distributed antibiotic discs. Susceptibility disc containing amoxicillin/ clavulanate (20/10 µg) was placed in the center of Muller-Hinton agar plate and disc containing cefotaxime, ceftazidime, ceftriaxone, and aztreonam (30 µg each) were placed 20 mm (centre to centre) from amoxicillin-clavulanate disc. Incubation followed for 18 hr at 37˚C. The isolate showing enhancement of the zone for one or more of the tested discs on the side facing the amoxicillin-clavulanate disc was considered ESBL producer (Coudron *et al*., 1997). Strain of *E. coli* ATCC 25922 was used as negative control for ESBL production.

For all ESBL-producing isolates, the susceptibility test was reported as resistant to all penicillins, cephalosporins, and aztreonam, irrespective of the individual in vitro test result, as recommended by the (NCCLS, 2002).

3-2-13 Template DNA Preparation:

DNA used as a template for PCR amplification was prepared by two methods:

3-2-13-1 Wizard Genomic DNA Purification Kit (Promega):

Plasmid DNA from the isolates were used to prepared using the wizard genomic DNA purification kit and according to the manufacturer's instructions.

3-2-13-2 Alkaline Lysis Procedure:

Plasmid DNA was extracted from β-lactam resistant isolates by employing the method of Pospiech and Neumann (1995) with some modifications. Cells from 5 ml of an overnight nutrient broth culture were pelleted by centrifugation (4000 rpm for 10 minutes), washed 3 times in TE buffer and recentrifuged. Then the pellet was resuspended in 5 ml TE buffer and mixed. A volume of 600 µl of freshly made SDS (25%) and 2 ml NaCl (5 M) solutions were added, mixed by inversion the cell suspension, incubated at 55˚C for at least 10 minutes (until be clear), mixed thoroughly by inversion, and let to be cooled at room temperature. Thereafter, proteins were extracted by mixing the lysate with 3.5 ml of phenol and equal volume of chloroform: isoamylacohol solution, mixed by inversion at room temperature for 30 minutes and then centrifuged at 6000 rpm for 10 minutes. The aqueous phase (which contains nucleic acid) was retained and transferred to a fresh tube, added 0.6 volume of isopropanol and mixed by inversion. Then DNA spooled on to a sealed pasture pipette, transferred to 5 ml ethanol (70%), air dried, and dissolved in 1 ml TE buffer at 55˚C, finally it was kept in -20˚C until used.

3-2-14 PCR Amplification of ESBL Genes:

The bacterial genomic DNA extracted from β-lactam resistant isolates was used as a template in specific PCRs for detection of bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{OXA} genes.

The oligonucleotide PCR primers specific for the β-lactamase genes, and melting temperature were listed in (**3-1-6**). Negative controls included in the assay (No DNA template amplification) will also monitor contamination during the PCR (Espy, 2006). As well as *E. coli* ATCC 25922 was used as a negative control.

Amplification was carried out in a total volume of 50 µl containing 25 µl Go *Taq* Green Master Mix (400µm dATP, 400µm dGTP, 400µm dCTP, 400µm dTTP, and 3mM $MgCl_2$), 2 µl each primer (upstream primer and downstream primer), 5 µl DNA template, and 16 µl Nuclease-Free Water as shown in Table (3-1).

Table (3-1): Concentration of each reagent in a PCR mixture (total volume 50 µl) for the *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, and *bla*$_{OXA}$ genes

Component	Volume (µl): 1 sample	Final concentration
Go Taq Green Master Mix.	25	1X
Upstream Primer	2	0.5 µM
Downstream Primer	2	0.5 µM
DNA Template	5	200 ng
Nuclease-Free Water	16	

An eppendorf thermal cyclar was used for gene amplification. PCR conditions for *bla* genes were shown in Table (3-2).

Table (3-2): PCR conditions for *bla* genes

Step of PCR	PCR condition*			
	TEM	SHV	OXA	CTX-M
Predenaturing	**94ºC**, 5 min.	**94ºC**, 5 min.	**94ºC**, 5 min.	**94ºC**, 5 min.
Cycles of	30 **Cycles**	35 **Cycles**	30 **Cycles**	35 **Cycles**
Denaturing	**94ºC**, 1 min.	**94ºC**, 30 sec.	**94ºC**, 45 sec.	**94ºC**, 30 sec
Annealing	55**ºC**, 1 min.	68**ºC**, 30 sec.	55**ºC**, 45 sec.	53**ºC**, 30 sec
Extension	72**ºC**, 1 min.	72**ºC**, 50 sec.	72**ºC**, 1 min.	72**ºC**, 40 sec
Final extension	72**ºC**, 10 min.	72**ºC**, 10 min.	72**ºC**, 5 min.	72**ºC**, 5 min.
Final hold step	4**ºC**	4**ºC**	4**ºC**	4**ºC**

* according to the manufacturer's instructions of Promega company

3-2-15 Agarose Gel Electrophoresis:

Agarose (1.5 gm) in 1X TBE buffer (10X TBE buffer, pH 8.0 containing 10 g Tris base, 55 g boric acid, 4.3 g EDTA, and 1 liter D.D. water) was dissolved by boiling the solution in water bath, allow to cool to 50˚C, and 3 µl of ethidium bromide (0.5 mg/ml) was added for staining the DNA molecules. The agarose-ethidium bromide solution was poured into the gel tray of the electrophoresis apparatus containing the combs and allow to set at room temperature for 30 minutes. After removing the comb gently, the tray was fixed in electrophoresis chamber and filled with 1X TBE buffer to cover the surface of the gel. 5µl of each PCR product was loaded into the gel well, and the electrophoresis was run at 70 volts for approximately 2 hr. Bands were visualized under ultraviolet light by using UV transilluminator (320 nm) and photographed.

CHAPTER FOUR

RESULTS

and

DISCUSSION

4.1 Source of Gram-negative Bacilli Isolates:

During the study period from November, 2008 to February, 2009, a total of 203 (33.1%) non-repetitive Gram-negative cultures (on MacConkey agar) obtained from 390 patients with wound infections and 223 sputa of patients with lower respiratory tract infection were included in the analysis. However, 410 (66.9%) samples showed no growth on MacConkey agar probably due to previous antibiotic therapy or prior present other microorganisms such as Gram-positive cocci and Gram-positive bacilli that could not grew on MacConkey agar (Table 4-1).

Table (4-1): Sources of GNB Isolates

Sample source	Sample No.	GNB growth	Yielded no growth
		No. (%)	No. (%)
Wound	390	134 (34.4)	256 (65.6)
Sputum	223	69 (30.9)	154 (69.1)
Total (%)	**613**	**203 (33.1)**	**410 (66.9)**

Samples source in terms of inpatients and outpatients were determined. The GNB isolates were more commonly isolated from inpatients (124, 61.1%) as compared to outpatients (79, 38.9%) (Table 4-2). Out of inpatients isolates, all GNB 92.5% were obtained from wound samples. Out of outpatients isolates, 7.5% were from wound samples, and 100% were from sputum samples. GNB have emerged as serious nosocomial pathogens throughout the

world. Nosocomial infections inpatients occur via transmission from other patients through health care workers (Jamal *et al*., 2009). The isolates can survive in the hospital environment and can be transmitted from patients to patients, through hands of hospital staff and are usually found in those areas of hospitals where patients condition is critical (Navaneeth *et al*., 2002).

Table (4-2): Distribution of GNB isolates in different samples according to the type of patients

Sample source	Total	Inpatients	Outpatients
	No. (%)	No. (%)	No. (%)
Wound	134 (66.0)	124 (92.5)	10 (7.5)
Sputum	69 (34.0)	0.0 (0.0)	69 (100)
Total (%)	203 (100)	124 (61.1)	79 (38.9)

A study by Spencer *et al*. (1987) showed that, more than half of the patients were colonized after 3 days stay in the hospital. However, a part from intensive care units (ICUs), ESBL-producing Gram-negative isolates have been isolated from patients in general ward and nursing homes (Luzzaro *et al*. 2006).

In all isolates, patients gender was recorded, 108 (53.2%) were males and 95 (46.8%) were females. Out of GNB isolates in males, 65 (60.2%) were from wound isolates, and 43 (39.8%) were from sputum isolates. Out of GNB isolates in females, 69 (72.6%) were from wound isolates, and 26 (27.4%) were from sputum samples (Table 4-3).

Table (4-3): Gender distribution of GNB isolates in different samples

Sample source	Total No.	Male	Female
		No. (%)	No. (%)
Wound	134	65 (48.5)	69 (51.5)
Sputum	69	43 (62.3)	26 (37.7)
Total (%)	203	108 (53.2)	95 (46.8)

The patients ages with GNB infection varied from 7 to 75 years old (mean = 40.557). More isolates were obtained from young and middle age patients (18 to 60 years, 169, 83.3%).

Out of 134 wound samples, GNB were most frequent in 31-40 years of ages (28, 20.9%), followed by 11 to 20 and 41-50 years old of age groups, whereas none was cultured from patients between 1 month to 10 years of age groups.

More isolates of lower respiratory tract infections were obtained from 21-30 years old of age groups (17, 24.6% each), followed by 51- 60 years old of age groups (15, 21.7%), while none was cultured from patients between 71 to 80 years of age groups (Table 4-4).

4.2 Identification of Bacterial Isolates:

The primary aim of this study was to determine the incidence of GNB including *Pseudomonas aeruginosa*, *Klebsiella* spp., *E. coli*, and *Proteus* spp. in wound and lower respiratory tract infections and the sensitivity pattern to commonly used antibiotics. The bacterial isolates obtained as a pure or predominant growth from samples were only considered for the present study.

Table (4-4): Overall prevalence of GNB at different age groups

Age group (year)	No. and % of patients (Frequency)		Sample source (%)*			
			wound		sputum	
	No.	%	No.	%	No.	%
1 month – 10	2	1.0	0	0.0	2	2.9
11 – 20	30	14.8	25	18.7	5	7.2
21 – 30	36	17.7	19	14.2	17	24.6
31 – 40	38	18.7	28	20.9	10	14.5
41 – 50	32	15.8	25	18.7	7	10.1
51 – 60	39	19.2	24	17.9	15	21.7
61 – 70	20	9.9	7	5.2	13	18.8
71 – 80	6	3.0	6	4.5	0	0.0
Total	203	(100%)	134	60.96**	69	39.04**

* Percentage from each sample source.

** Percentage from a total of 203 patients.

Ps. aerugenosa, *E. coli*, *Klebsiella* spp., and *Proteus* spp. pathogens were isolated and identified according to the standard microbiological techniques of Bergey's Manual of Systemic Bacteriology (Holt *et al*., 1994) and MacFaddin (2000). All *Klebsiella* isolates were identified and classified at the level of subspecies using the traditional biochemical tests (Appendix 1).

Conclusions and Recommendations

Out of the 62 isolates of *Klebsiella* spp., 56 were speciated a *K. pneumoniae* (37 isolates from wound infections and 19 isolates from sputum samples) which included 19 isolates of *K. pneumoniae* subsp. *pneumoniae*, 15 isolates of *K. pneumoniae* subsp. *ozaenae*, 14 isolates of *K. pneumoniae* subsp. *rhinoscleromatis*, and 8 isolates of *K. pneumoniae* subsp. *aerogens*, whereas 6 isolates were identified as *K. oxytoca* (4 isolates from wound infection and 2 isolates from sputum samples) Table (4-5).

Table (4-5): Species and numbers of *Klebsiella* isolated from wound and sputum samples of patients

Species and subsp. of *Klebsiella*	Wound	Sputum	Total
K. pneumoniae			
1. *pneumoniae*	16	3	19
2. *ozaenae*	9	6	15
3. *rhinoscleromatis*	8	6	14
4. *aerogens*	4	4	8
K. oxytoca	4	2	6
Total	41	21	62

In the present study, of the 203 GNB recovered, *Ps. aeruginosa* and *Klebsiella* spp. were the most prevalent pathogens (30.5% each) followed by *E. coli* (27.6%), and *Proteus* spp. (11.3%) [*P. mirabilis* (18, 8.9%) and *P. vulgaris* (5, 2.5%)] as seen in Figure (4-1).

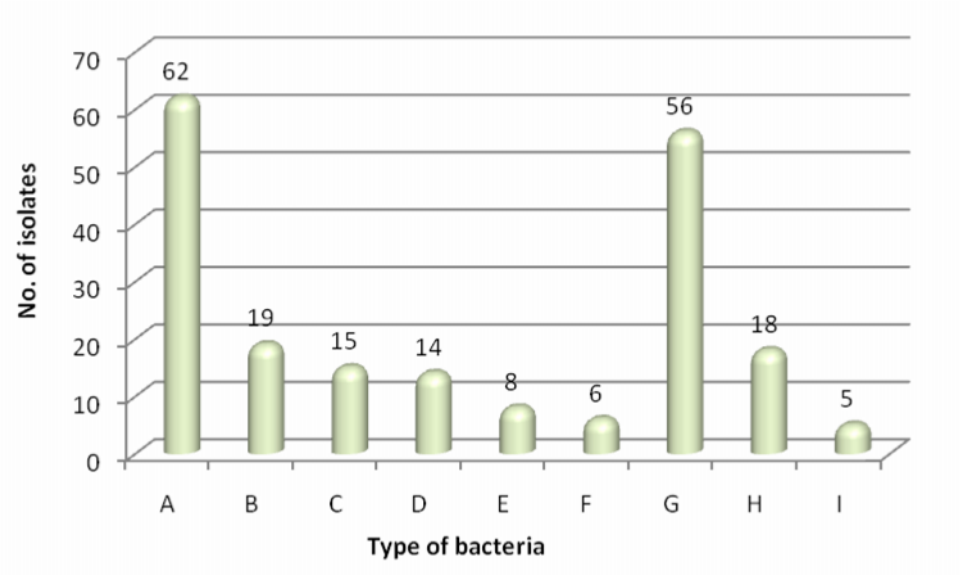

Figure (4-1): Types and numbers of bacterial isolates recovered from wound infection and lower respiratory tract infection patients.

A= *Pseudomonas aeruginosa*
B= *Klebsiella pneumoniae* subsp. *pneumoniae*
C= *Klebsiella pneumoniae* subsp. *ozaenae*
D= *Klebsiella pneumoniae* subsp. *rhinoscleromatis*
E= *Klebsiella pneumoniae* subsp. *aerogens*
F= *Klebsiella oxytoca*
G= *Escherichia coli*
H= *Proteus mirabilis*
I= *Proteus vulgaris*

A wound is a break in the skin and exposure of subcutaneous tissue following loss of skin integrity that provides a moist, worm, and nutritive environment that is conductive to microbial colonization and proliferation (Bowler *et al*., 2001). In the present study, a total of 134 GNB isolates were obtained from 390 wound cultures, of which 22 (16.4%) from males and 21 (15.7%) from females (Table 4-6). However, in all cases, cultures were monomicrobial, but no bacterial isolates were obtained in 256 cases. *Ps. aeruginosa* was predominant microorganisms (43, 32.1%) followed by *Klebsiella* spp. (41, 30.6%), *E. coli* (33, 24.6%), and *Proteus* spp. (17, 12.7%). *Ps. aeruginosa* is an opportunistic pathogen found along with other

Conclusions and Recommendations

Pseudomonas species as part of the normal flora of the human skin. When the host is immunocompromized, as in the case of a thermal burn or surgical wound, this opportunistic bacteria can quickly colonize and infect the burn and wound sites (Deplano *et al.*, 2005; Page and Heim, 2009). In cases studies of burn patients who developed *Ps. aeruginosa* septicemia, the mortality rate > 75% (Roderic *et al*., 2004).

Table (4-6): Occurrence of GNB in wound infection in relation to gender

Isolates	Male		Female		Total	
	No.	%	No.	%	No.	%
Ps. aeruginosa	22	16.42	21	15.67	43	32.09
K. pneumoniae* subsp. *pneumoniae	7	5.22	9	6.72	16	11.94
K. pneumoniae* subsp. *ozaenae	2	1.49	7	5.22	9	6.72
K. pneumoniae* subsp. *rhinoscleromatis	4	2.99	4	2.99	8	5.97
K. pneumoniae* subsp. *aerogenes	2	1.49	2	1.49	4	2.98
K. oxytoca	2	1.49	2	1.49	4	2.98
E. coli	17	12.69	16	11.94	33	24.63
Proteus mirabilis	6	4.48	6	4.48	12	8.96
Proteus vulgaris	3	2.24	2	1.49	5	3.73
Total	65	48.51	69	51.49	134	100

Conclusions and Recommendations

The increasing incidence of *Ps. aeruginosa* in wound infections was observed by several scientists especially in recent years. Masaadeh and Jaran (2009) found that most causative agent of post operative infections in Irbid, Jordan was *Ps. aeruginosa*, 32 isolates (27.8%), following *E. coli*, 18 isolates (15.6%). In another study, Kaur *et al*. (2006) demonstrated that *Ps. aeruginosa* (19%) was the commonest isolate from wound infections followed by *S. aereus* (15%), *E. coli* (10.5%), and *Klebsiella* species (7.5%). Anupurba *et al*. (2006) showed that prevalence rate of *Ps. aeruginosa* was 32% of all pathogens isolated from wound infections in India. It is thus clear that the prevalent rate of *Ps. aeruginosa* record in this study is in agreement with that obtained in other studies.

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 40-50 per 1000. Since the etiology agents of lower respiratory tract infections cannot be determined clinically, microbiological investigation is critical for both treatment and epidemiological purpose (Ozyilmaz *et al*., 2005). In a study reported herein, 223 lower respiratory tract infected patients were evaluated. Sputum samples were obtained when the patients were able to expectorate. In this study, sputa were considered acceptable for culture if they contained > 25 polymorphonuclear cells and < 25 epithelial cells per low-power field as recorded by Srifuengfung *et al*., 2005.

In the present study, a total of 69 (30.9%) GNB isolates were obtained. Of these 43 (62.3%) from males and 26 (37.7%) from females. The bacterial isolates from the patients in the study are summarized in Table (4-7).

Conclusions and Recommendations

The National Nosocomial Infected Surveillance (NNIS) of CDC of United State of America reports 60% of nosocomial lower respiratory tract infections to be caused by aerobic Gram-negative bacteria (Veena Kumari *et al*., 2007).

Table (4-7): Occurrence of GNB in lower respiratory tract infection in relation to gender

Isolates	Male		Female		Total
	No.	%	No.	%	
E. coli	**14**	**20.4**	**9**	**13.0**	**23**
Ps. aeruginosa	**10**	**14.5**	**9**	**13.0**	**19**
K. pneumoniae* subsp. *ozaenae	**5**	**7.2**	**1**	**1.5**	**6**
K. pneumoniae* subsp. *rhinoscleromatis	**5**	**7.2**	**1**	**1.5**	**6**
K. pneumoniae* subsp. *aerogenes	**2**	**2.9**	**2**	**2.9**	**4**
K. pneumoniae* subsp. *pneumoniae	**3**	**4.3**	**0**	**0.0**	**3**
K. oxytoca	**1**	**1.5**	**1**	**1.5**	**2**
Proteus mirabilis	**3**	**4.3**	**3**	**4.3**	**6**
Total	**43**	**62.3**	**26**	**37.7**	**69**

However, French and Ransjö (2006) found 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 equipment or the hand of staff (Dancer, 2004). Results show that *E. coli* was the most frequently isolated organism (23, 33.3%) (Table 4-7). *E. coli* considered as one bacteria that causes pneumonia which transferred from a genitourinary tract or gastrointestinal source (Packham, 2007). However, data on the presence of *E. coli* in lower respiratory tract infected patients in different parts of the world are variable. In Thailand, 1 (1.4%) of the 53 sputum of the patients under 5 years of age were found to be *E. coli* positive (Ekalaksananan *et al*., 2001). Out of 383 sporadic cases with lower respiratory tract infections in Bangladesh, *E. coli* was found only in 2 (0.5%). The investigators show that *E. coli* is not a frequent care of lower respiratory tract infections (Amin *et al*., 2009). However, the report detection rate of this pathogen in China's population was 4% (Ding *et al*., 2009). In Bankok, Thailand, *E. coli* infection is emerging as one of most important lower respiratory tract infections in HIV-positive patients (Srifuengfung *et al*., 2005). In the study of Okesola and Ige (2008), the most common bacterial types present in endotracheal tubes were *Klebsiella* spp., *Ps. aeruginosa*, and *E. coli*.

The present study has found that *Klebsiella* spp. was the second frequently isolated organisms (21, 30.4%) (Table 4-7). Numerous *in vitro* studies have shown that *Klebsiella* spp. as the most common causes of lower respiratory tract. In an Indian study carried out by Veena Kumari *et al.* (2007) on bacterial isolates from lower respiratory tract of intensive care unit patients, the percentage isolation rate for *Ps. aeruginosa*, *Klebsiella* spp., and *Enterobacter*

spp. have been reported 21.5, 19.8, and 6.2% respectively. Additionally, in Russian town, Reshedko *et al*. (2004) found that the most common Gram-negative bacteria isolated from sputum of lower respiratory tract infected patients were *Ps. aeruginosa* (35.3%), *K. pneumoniae* (17.2%), *Acinetobacter baumannii* (15.9%) and *E. coli* (8.0%). In some parts of Asia, the prevalence of respiratory isolates of *Klebsiella* spp. varied from 37.1% in India (Taneja *et al*., 2009) to 15% in China (Ding *et al*., 2009) and upto 10.8% in Thailand (Srifuengfung *et al*., 2005).

In the present study, out of all the lower respiratory tract cultures positive, 19 (27.5%) showed *Ps. aeruginosa* isolates (Table 4-7). This organism has the unique ability to infect all body system especially the respiratory tract which *Ps. aeruginosa* is the major leading cause of infection (Savas *et al*., 2005). Some strains synthesizes two lectins LecA and LecB, strains that produce high levels of these virulence factors exhibit an increased virulence potential in chronic respiratory tract infections (Tielker *et al*., 2005), High incidence of *Ps. aeruginosa* in lower respiratory tract infections were observed by other scientists, Reshedko *et al*. (2004) record prevalent rate of 35.3% in Russian intensive care unit patients. In a study on the epidemiology of respiratory tract bacterial pathogens carried out by Varotto *et al.* (2001), *Ps. aeruginosa* has been reported as the most prevalent organism (24%) in Tehran, Iran followed by *Streptococcus pyogenes* (18%), *Staphylococcus aureus* (17%), and *K. pneumoniae* (8%). However, in study by Veena Kumari *et al*. (2006), the most common Gram-negative bacteria pathogens isolated from lower respiratory tract infection in India were *Ps. aeruginosa* (21.5%), followed by *Klebsiella* spp. (19%) and *E. coli* (12.4%). In other study, pharyngeal colonization with *Ps. aeruginosa* were found in 18.2% cases admitted to the respiratory tract unit in Bangkok (Chayakulkeeree *et al*., 2005). However, the result of present study is

variable (lower and higher) with the reported by others, this could be attributed to difference in geographical location and hygienic measures.

In this investigation, it was observed that *Proteus* spp. had lower occurrence (6, 8.7%) in the 69 sputum samples analyzed than other GNB (Table 4-7). This finding is in consonance with the workers of Reshedko *et al*. (2004) and Kumari *et al*. (2004).

4.3. Survey of β-lactam Resistance in Clinical Isolates:

Resistance to β-lactam antibiotics is now a problem in patients throughout the world. The prevalence of β-lactamases among clinical isolates vary greatly worldwide and in geographic areas and are rapidly changing over time. However, the occurrence of β-lactam resistance in GNB clinical isolates (n = 203) were screened on Muller-Hinton agar supplemented with ampicillin and amoxicillin (each alone) at final concentrations of 50 and100 µg/ml, respectively. Such two β-lactam antibiotics were selected because they are the most commonly used antibiotics in the therapy of bacterial infections, compared to other β-lactam antibiotics. As part of their therapeutic usage, these antibiotics can provide a comprehensive primary survey of β-lactam resistant isolates, because the isolates, that is resistance to new generations of cephalosporins and carbenicillin, is already resistant to ampicillin and amoxicillin (Bush *et al*., 1995).

During the entire study period, 193 (95.1%) GNB isolates grew normally with ampicillin and amoxicillin (Table 4-8). This rate agrees with studies of Patel *et al*. (2009) and Javiya *et al*. (2008) which demonstrated that more than

Table (4-8): β-lactam resistance of Gram-negative bacterial isolates

Isolate	No. of isolates	No. (%) of isolates resistant to β-lactam
Ps. aeruginosa	**62**	**59** (95.2%)
E. coli	**56**	**52** (92.9%)
K. pneumoniae subsp. *pneumoniae*	**19**	**19** (100%)
K. pneumoniae subsp. *ozaenae*	**15**	**15** (100%)
K. pneumoniae subsp. *rhinoscleromatis*	**14**	**14** (100%)
K. pneumoniae subsp. *aerogenes*	**8**	**7** (87.5%)
K. oxytoca	**6**	**6** (100%)
P. mirabilis	**18**	**16** (88.9%)
P. vulgaris	**5**	**5** (100%)
Total	**203**	**193** (95.1%)

90% *Enterobacteriacae* species were resistance to ampicillin and amoxicillin. Hence the isolates were confirmed β-lactam resistant. Such resistance in present study may be emerged because of the wide spread use of these drugs in Iraqi

hospitals. In this investigation, the reason of β-lactam resistance of GNB isolates is probably due to the production of β-lactamases, which may be genetically localized on the chromosome or on a plasmid. Other resistance reasons may be due to decrease the affinity of target PBPs or decrease permeability of the drug into the cell (Jacoby and Munoz-Price, 2005). Amyes (2003) mentioned that there are three further resistance mechanisms include conformational changes in PBPs, permeability changes in the outer membrane, and active efflux of the antibiotic. Other studies reported that *qnr* genes (integron-associated) are associated with resistance to several classes of antibiotics including β-lactam (Paterson, 2006).

As shown in Table (4-8), 59 (95.2%) *Ps. aeruginosa* isolates were resistant to both ampicillin and amoxicillin. In an Indian study, *Ps. aeruginosa* of tracheal and bronchial samples showed 97.8% and 100% resistance to ampicillin, respectively (Veena Kumari *et al*., 2006). In another study, Gad *et al*. (2008) found that all *Ps. aeruginosa* isolates obtained from respiratory tracts and skin infection were resistant to ampicillin and amoxicillin. However, it is important to stress that *Ps. aeruginosa* infection are difficult to treat because of the bacteria's intrinsic resistance to many antibiotics, owing to its low outer membrane permeability and its ability to acquired new resistance mechanisms during antibiotic treatment. *Ps. aeruginosa* broad spectrum of resistance relies on a wide range of different mechanisms, which are either encoded in its own chromosome or on a plasmids. Resistance to most penicillins is produced by firstly constitutive or inducible β-lactamases. Secondly, *Ps. aeruginosa* is also capable of modifying the PBP. Lastly, it can use an efflux system "Mex-AB-Opr M", which is expressed constitutively to expulse the β-lactam molecule out of the cell (Julio *et al*., 1999).

Conclusions and Recommendations

In a study reported herein, 52 (92.9%) of *E. coli* isolates were resistant to β-lactam antibiotics (Table 4-8). This rate was higher than that obtained by Hadi (2008) who found that 82.6% of *E. coli* isolates collected from patient with significant bacteriuria in Najaf were resistant to both ampicillin and amoxicillin. While it was lower than that reported in Babylon (Flaih, 2005) and in India (Veena Kumari *et al*., 2006). The production of β-lactamases is considered the predominant mechanism of β-lactam resistance in *E. coli* (Pearson *et al*., 2007). The first β-lactamase was identified in *E. coli* prior to the release of penicillin for use in medical practice (Al-Jasser, 2006). However, the reason of β-lactam resistance *E. coli* isolates is may be due to the production of TEM and/or SHV β-lactamase, which probably chromosomal origin or plasmid mediated. Up to 90% of ampicillin resistance in *E. coli* is due to production of TEM-1. This β-lactamase enzyme is able to hydrolyze penicillin and early cephalosporin (Bradford, 2001).

The results in this study also showed that 100% *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae*, *K. pneumoniae* subsp. *rhinoscleromatis*, and *K. oxytoca* were resistance to ampicillin and amoxicillin, while seven (87.5%) of the eight *K. pneumoniae* subsp. *aerogenes* were resistance to both antibiotics (Table 4-8). The study of Flaih (2005) in Babylon University showed that all *Klebsiella* isolates were resistant to both ampicillin and amoxicillin. In other studies, Veena Kumari *et al*. (2006) found that 100% of *Klebsiella* isolates in India were resistant to ampicillin. The SHV-1 β-lactamase is the most commonly found enzyme in *Klebsiella* species and is mainly responsible for ampicillin resistance in this bacteria (Tzouvelekis and Bonomo, 1999).

In this study, it has been found that all (100%) *Proteus vulgaris* and 88.9% of *Proteus mirabilis* were resistant to ampicillin and amoxicillin (Table 4-8).

The prevalence of β-lactam resistance *Proteus* spp. isolates in this study was comparable to those reported by Datta *et al*. (2004) who found that 93.5% of *Proteus* spp. were resistant to ampicillin.

In the present study, although the β-lactamase undoubtedly plays a major role in the resistance to β-lactam antibiotics, the high ratio of resistance to ampicillin and amoxicillin was not only attributable to the production of β-lactamase enzymes, it has been found that some bacteria have two types of mechanisms for β-lactam resistance, enzymatic and that related to permeability, which provide a strong defense mechanisms for these bacteria, making their therapy a difficult task (Philippon *et al*., 2002; Jacoby and Munoz-Price, 2005).

4.4. Susceptibility Patterns of Gram-negative Bacilli:

In Gram-negative bacilli, β-lactamases remain the most important contributing factor to β-lactam resistance and their increasing prevalence and evolution represent a serious challenge for clinical microbiology laboratories. In addition to increasing resistance to β-lactam antibiotics, resistance to other commonly used antibiotics is increasing. The present study demonstrated the percentage of resistant and sensitive isolates to a range of common groups of antibiotics for the 193 β-lactam resistance isolates of *Ps. aeruginosa* (n = 59), *E. coli* (n = 52), *Klebsiella* spp. (n = 61), and *Proteus* spp. (n = 21) tested. In this investigation, the susceptibility of the isolates to various antimicrobial agents were determined by Kirby-Bauer disc diffusion method (Ratna *et al*., 2003) and recommended by the Clinical and Laboratory Standards Instituted (CLSI, 2009).

A summary of resistance rates for all antibiotics against the β-lactam resistance GNB is shown in Figure (4-2). The highest resistants were found for

each of cefazolin and gentamicin (87.7%), piperacillin (86.7%), cefoxitin (84.6%), augmentin (84.1%), cefotaxime (76.9%), cefixime (76.9%), ceftazidime (73.8%), cefepime (72.3%), tetracycline (68.7%), and trimethoprim (65.1%). Intermediate resistance rates were obtained for aztreonam (60.5%), each of ceftriaxone and levofloxacin (59.0%), nalidixic acid (56.4%), co-trimazine (53.8%), rifampicin (46.7%), chloramphenicol (43.6%), tobramycin (40.0%), norfloxacin (32.8%), and ciprofloxacin (32.3%). The lowest resistance rates were observed for imipenem (5.1%). The high-level resistance to many antibiotics in the present study may be as a result of both intrinsic and acquired mechanisms. This resistance is widespread and constitutes serious clinical threats (Mathur *et al*., 2002).

Results from Figure (4-2) revealed that GNB isolates were highly resistant to cefoxitin (84.6%). This antibiotic is stabile to broad spectrum β-lactamases mediated hydrolysis as compared with other cephalosporins. The resistance to cefoxitin may be as a result of the development of porine-deficient mutants (Manchanda, and Singh, 2003). In addition, increasing numbers of GNB strains express different types of β-lactamases including inducible and/or plasmid-mediated AmpC type of enzymes may also increase the chance for resistance to cefoxitin (Siu *et al.*, 2003). Many GNB species like *E. coli* and *Ps. aeruginosa* possess β-lactamases of the AmpC types. The product of AmpC gene is an enzymes, that are broadly active against cephalosporins but is not inhibited by clavulanate. Furthermore, migration of chromosomal AmpC gene into plasmids poses a serious threat (Komatsu *et al.*, 2000). As shown in Figure (4-2), all bacteria isolates were highly resistant to cefotaxime (76.9%) and ceftazidime (73.8%). On the other hand moderately

Tetracycline (TE)

Chloramphenicol (C)

Co-trimazine (Cm)

Trimethoprim (TR)

Norfloxacin (NX)

Levofloxacin (LE)

Ciprofloxacin (CIP)

Nalidixic acid (NA)

Rifampicin (RA)

Tobramycin (TO)

Gentamicin (GM)

Imipenem (IPM)

Aztreonam (ATM)

Cefixime (FIX)

Cefoxitin (FOX)

Cefepime (FEP)

Ceftriaxone (CRO)

Ceftazidime (CAZ)

Cefotaxime (CTX)

Cefazolin (CZ)

Amoxicillin/clavulanate(A/C)

Piperacillin (Pi)

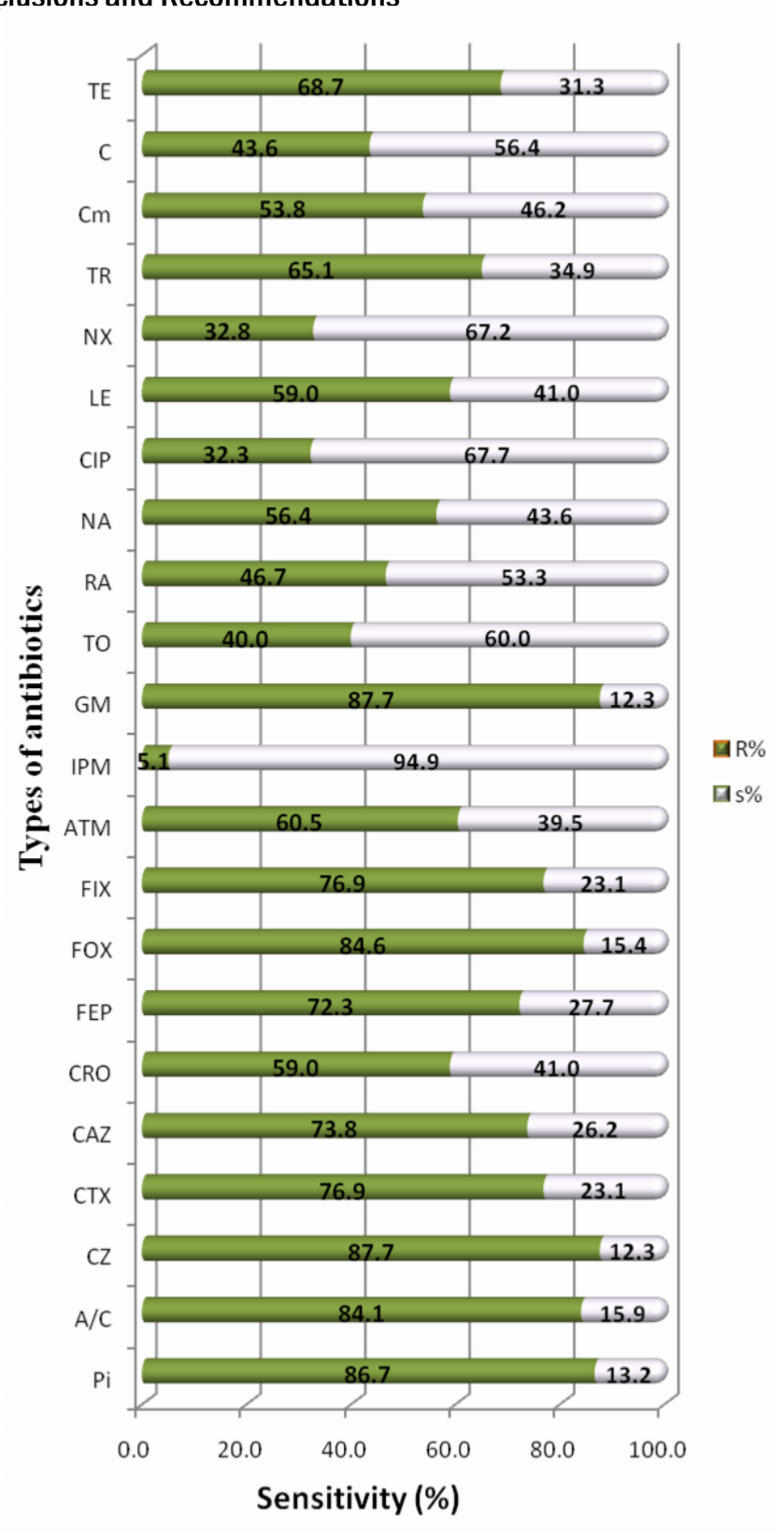

Figure (4-2): Sensitivity patterns of 193 GNB clinical isolates against different antibiotics.

resistant to aztreonam (60.5%) and ceftriaxone (59.0%). These resistances, mainly, are an important indicator for the presence of ESBLs. ESBL-producing Gram-negative rods are undoubtedly one of the most important etiological agents of many severe and life-threatening nosocomial infections (Kang *et al.*, 2005). The genes encoding ESBLs are usually localized on large, transferable plasmids that can easily become widespread in Gram negative bacilli (Franiczek *et al.*, 2007).

In the present study, there was a high resistance level to β-lactam/β-lactamase inhibitor combination (amoxicillin/clavulanic acid) (84.1%). This is likely to be due to the heavy selection pressure from overuse of this antibiotic and seem to be losing the battle. This result is accordant with the results being reported by Al-Zahrani and Akhtar (2005). In previous study, Taneja *et al.* (2008) found that 93.4% of GNB isolated from patients with UTI were resistant to amoxicillin/clavulanic acid, this may be due to also the production of AmpC β-lactamases.

Also there was a reduce activity of quinolones including levofloxacin, norfloxacin, and ciprofloxacin (resistance rates were 59.0%, 32.8%, and 32.3%, respectively) (Figure 4-2). Quinolone-resistance is typically encoded chromosomally. This resistance against fluoroquinolones in this study may reflect significant antibiotic pressure in the environment rather than co-carriage of this resistance gene on plasmids. Quinolone resistant is usually caused by various chromosomal mutations that alter the target enzymes, such as DNA gyrase and topoisomerase IV, or activate efflux systems (Jeong *et al.*, 2005). Plasmid-mediated quinolone-resistant has only recently discovered. The plasmid genes responsible for quinolone-resistance, termed *qnr* (Shigemura *et al.*, 2008).

In the present study, β-lactam resistance GNB isolates showed highest susceptibility to imipenem (94.9%). It appears that imipenem are the drug of

choice for serious infection with broad spectrum β-lactamase producing organisms as has been recommended earlier (Pongpech *et al.*, 2008). However, these should not be administrated as empirical therapy for Gram-negative infections that are not life treating because their overuse can pose a significant problem (Al-Zahrani and Akhtar 2005).

Among the most commonly used antibiotics discs, a striking feature of this study is the large number of antibiotic resistance found in *Ps. aeruginosa* isolates. *Ps. aeruginosa* were showed highest resistance against both cefoxitin and cefazolin with resistance rate of 100%. The next most resistant to antibiotics (with resistance rate between 98.3% to 91.9%) were amoxicillin/clavulanic acid, cefotaxime, nalidixic acid, trimethoprim, piperacillin, cefexime, cefepime, and tetracycline. While these isolates showed the highest sensitive to imipenem (72.9%) (Figure 4-3), these results considered in high levels because this microorganisms may acquire more enzymatic agents in an environment of the hospitals, *Ps. aeruginosa* possesses greater potential for enzymatic modification and degradative drug resistance mechanisms (Stover *et al*., 2000). The recently researches revealed different genes encoded different enzymes, MexXY-OprM has been reported to confer tetracycline resistance in *Ps. aeruginosa* (Jeannot *et al*., 2005). Therefore, the bacteria which tetracycline resistance may have MexXY-OprM genes. AAC(6')-IIa is commonly found in *Ps. aeruginosa*, it confers resistance to gentamicin, and tobramycin (but not to amikacin), whereas AAC(6')-Ib

Tetracycline (TE)

Chloramphenicol (C)

Co-trimazine (Cm)

Trimethoprim (TR)

Norfloxacin (NX)

Levofloxacin (LE)

Ciprofloxacin (CIP)

Nalidixic acid (NA)

Rifampicin (RA)

Tobramycin (TO)

Gentamicin (GM)

Imipenem (IPM)

Aztreonam (ATM)

Cefixime (FIX)

Cefoxitin (FOX)

Cefepime (FEP)

Ceftriaxone (CRO)

Ceftazidime (CAZ)

Cefotaxime (CTX)

Cefazolin (CZ)

Amoxicillin/clavulanate(A/C)

Piperacillin (Pi)

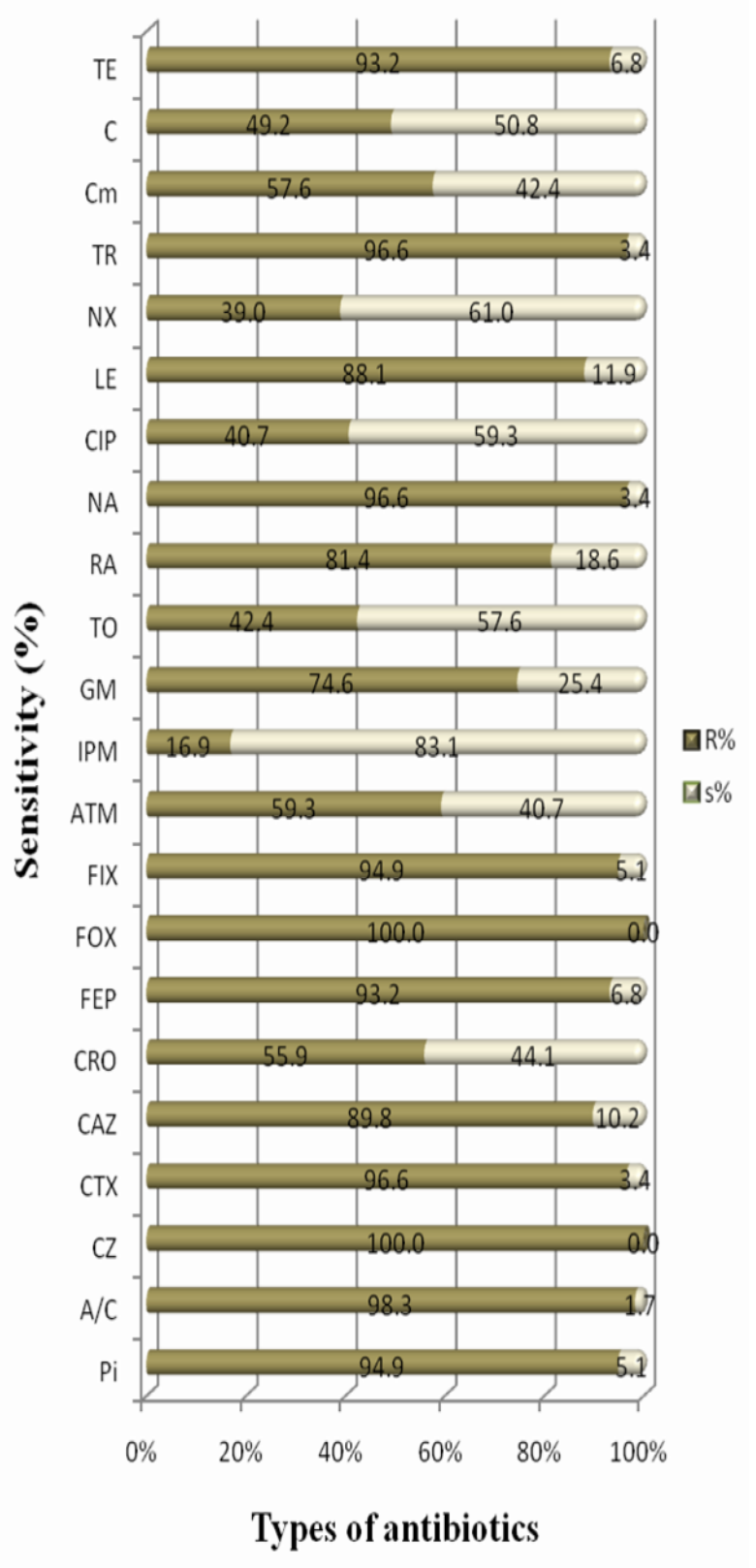

Figure (4-3): Resistivity patterns of 59 *Ps. aeruginosa* against different antibiotics.

confers resistance to amikacin, tobramycin, and kanamycin (but not to gentamicin). Almost all *aac(6')*-Ib genes were found in the gene cassette within the integron in *Ps. aeruginosa* (Casin *et al*., 2003).

Other studies in different places were reported to have similar results, Veena Kumari *et al*. (2006), from India showed high rate the resistance of *Ps. aeruginosa* to cefazolin (100%), ceftriaxone (80.5%), gentamicin (73.6%), ciprofloxacin (69%), cefotaxime (62.1%), ceftazidime (59.8%), and piperacillin (52.9%). In another study of Rashid *et al*. (2007) who reported that susceptibility of *Ps. aeruginosa* was 93.3% to cefixime, 86.1% to ceftriaxone, 80% to gentamycin, and 86.8% to ceftazidime. From Egypt, Gad *et al*. (2008), found the resistance of *Pseudomonas* spp. to amoxicillin/clavulanic acid was 100% and aztreonam was 59%, whereas resistance to imipenem was 70.5%, ciprofloxacin was 29.5%, and ceftriaxone 68%. Masaadeh and Jaran, (2009) (in Jordan), *Ps. aeruginosa* isolates were found to be high resistant gentamycin (72%), tobramycin (69%), ciprofloxacin (66%). The reason behind these results is that the intrinsic antibiotic resistance of *Ps. aeruginosa* may be due to the expression of chromosomally encoded efflux pumps (Li and Nikaido, 2004).

In the present study, among the 52 *E. coli* isolates that were tested for antimicrobial susceptibility, all these isolates were resistance to gentamicin, while the vast majority of the isolate were resistant to piperacillin 80.8%. Resistance to cefazolin was shown in 78.8% of these isolates. Moreover, 69.2% and 63.5% of *E. coli* isolates showed susceptibility to chloramphenicol

Tetracycline (TE)

Chloramphenicol (C)

Co-trimazine (Cm)

Trimethoprim (TR)

Norfloxacin (NX)

Levofloxacin (LE)

Ciprofloxacin (CIP)

Nalidixic acid (NA)

Rifampicin (RA)

Tobramycin (TO)

Gentamicin (GM)

Imipenem (IPM)

Aztreonam (ATM)

Cefixime (FIX)

Cefoxitin (FOX)

Cefepime (FEP)

Ceftriaxone (CRO)

Ceftazidime (CAZ)

Cefotaxime (CTX)

Cefazolin (CZ)

Amoxicillin/clavulanate(A/C)

Piperacillin (Pi)

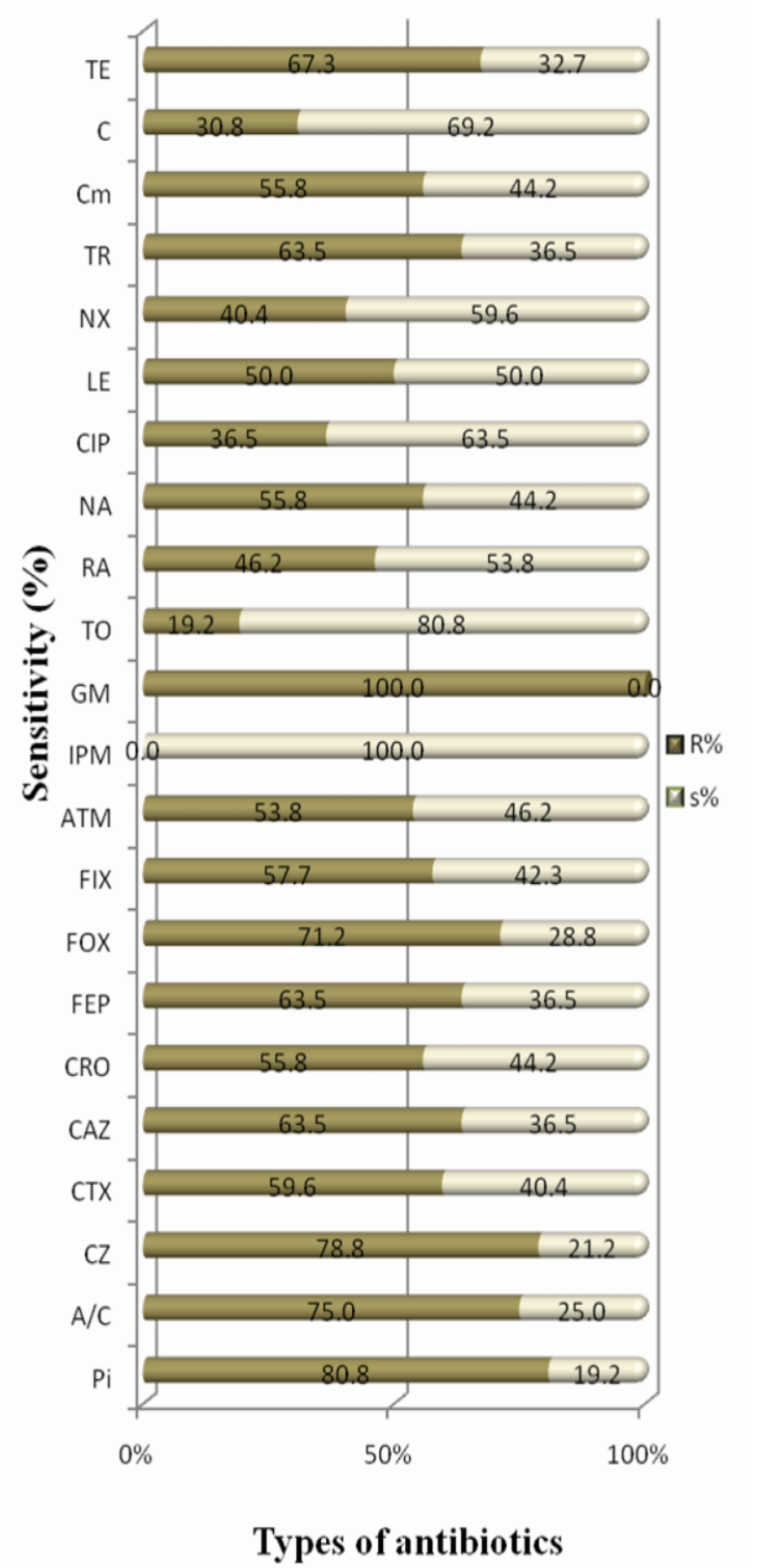

Figure (4-4): Resistivity patterns of 52 *E. coli* against different antibiotics.

and ciprofloxacin, respectively. On the other hand, 100% of the *E. coli* isolates were found to be sensitive to imipenem (Figure 4-4). This result was agreed with previous studies that show similar of imipenem against all *E. coli* isolates (Al-Lawati *et al*., 2000; Srifuengfung *et al*., 2005; Tonkie *et al*., 2005; Messai *et al*., 2006; Tantracheewathorn *et al*., 2007; Jayapradha *et al*., 2007). The studies in Addis Ababa, Asrat and Wolde Amanuel (2001) showed that *E. coli* resistance was 75% to trimethoprim sulfamethoxazole, 64% to augmentin, 64% to chloramphenicol, 36% to gentamicin. Isibor *et al*. (2008), in Nigeria, found low resistant to gentamicin (57%) and higher to amoxicillin/clavulanic acid (100%). Previous studies in USA (Bolon *et al.*, 2004) reported lower resistant to ciprofloxacin (11%) and norfloxacin (13%). These differentiations between studies may be reflect antibiotics may not have been misused or the bacteria did not exposed to these antibiotics in those regions.

The ability of *Klebsiella* spp. isolates to grow in the different of antibiotics was tested (Figure 4-5). All these isolates were resistance to gentamicin (100%), followed by piperacillin (93.4%). Resistance to cefazolin, amoxicillin/clavulanic acid, cefoxitin, and cefixime were 82.0%, 78.7%, 78.7%, and 75.4%, respectively. Moreover, 83.6%, 80.3%, and 73.8% of *Klebsiella* spp. isolates showed susceptibility to norfloxacin, ciprofloxacin, and nalidixic acid, respectively. Among the antimicrobials tested, the floroquinolones seem to be effective in treating infections caused by *Klebsiella* spp., the resistance rates were (34.4%) to levofloxacin, (19.7%) to ciprofloxacin, and (16.4%) to norfloxacin, in spite of the fact that resistance to

Tetracycline (TE)

Chloramphenicol (C)

Co-trimazine (Cm)

Trimethoprim (TR)

Norfloxacin (NX)

Levofloxacin (LE)

Ciprofloxacin (CIP)

Nalidixic acid (NA)

Rifampicin (RA)

Tobramycin (TO)

Gentamicin (GM)

Imipenem (IPM)

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Ceftriaxone (CRO)

Ceftazidime (CAZ)

Cefotaxime (CTX)

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Amoxicillin/clavulanate(A/C)

Piperacillin (Pi)

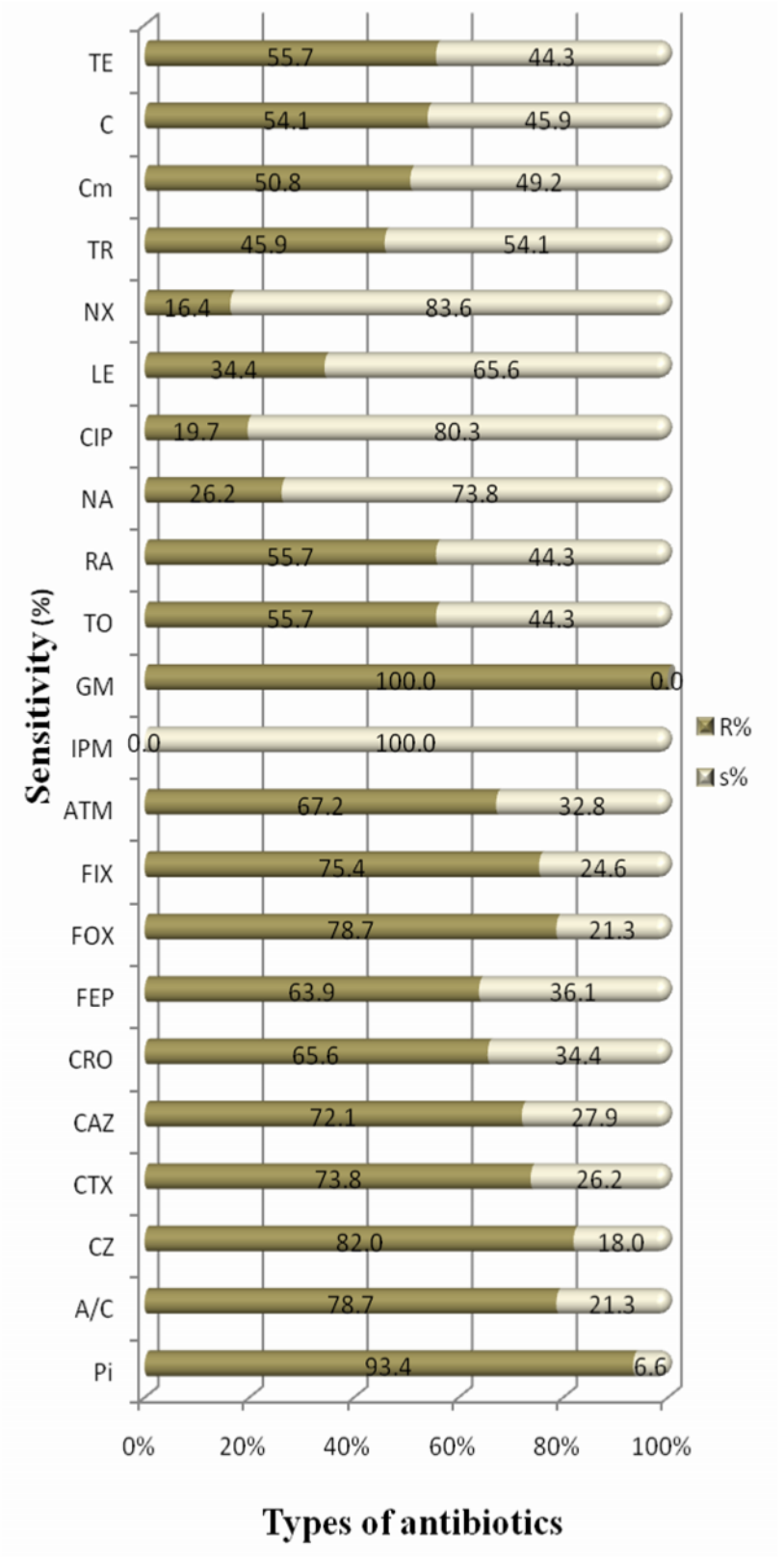

Figure (4-5): Resistivity patterns of 61 *Klebsiella* spp. against different antibiotics.

floroquinolones is increasing in Europe, Asia and America and its use is significantly associated with the isolation of resistant *Enterobacteriacaeae* (Sheng *et al.*, 2002; Bolon *et al.*, 2004). On the other hand, all *Klebsiella* spp. isolates were found to be susceptible imipenem (100%). Other previous studies have shown similar result of imipenem against all *Klebsiella* spp. isolates in different countries, in Sultanate of Oman (Al-Lawati *et al*., 2000), in Russian from ICUs (Reshedko *et al*., 2004), and in Jordan (Bataineh and Alrashed, 2007).

Among the 21 isolates of *Proteus* spp. that were tested for antimicrobial susceptibility, all isolates were resistance to gentamicin (100%). Resistance to amoxicillin/clavulanic acid, cefoxitin, cefazolin, and piperacillin, was shown in 90.5% of these isolates, while 71.4% to each of cefixime, cefotaxime, levofloxacin. On the other hand, all *Proteus* spp. isolates were found to be sensitive to imipenem. Moreover 66.7% of *Proteus* spp. isolates showed susceptibility to each of chloramphenicol and ciprofloxacin (Figure 4-6).

Other previous studies in different places, in Cameroon (Gangoue-Pieboji *et al*., 2006) and in Greece (Reslinski *et al*., 2005) have shown similar results to imipenem against all *Proteus mirabilis* isolates. This sensitivity to imipenem reflect good permeability of this antibiotic to across the cell wall of Gram-negative bacteria (Labombardi, 2007), or may mean that these isolates have not any carbapenemase enzymes.

However, other studies revealed high sensitivity of these isolates to different antibiotics, Gangoue-Pieboji *et al*. (2006) found the sensitivity of these isolates to antibiotics as follows 100% to both aztreonam and ceftazidime, 98% to cefotaxime, 97% to cefoxitin, 69% to amoxicillin/

Tetracycline (TE)

Chloramphenicol (C)

Co-trimazine (Cm)

Trimethoprim (TR)

Norfloxacin (NX)

Levofloxacin (LE)

Ciprofloxacin (CIP)

Nalidixic acid (NA)

Rifampicin (RA)

Tobramycin (TO)

Gentamicin (GM)

Imipenem (IPM)

Aztreonam (ATM)

Cefixime (FIX)

Cefoxitin (FOX)

Cefepime (FEP)

Ceftriaxone (CRO)

Ceftazidime (CAZ)

Cefotaxime (CTX)

Cefazolin (CZ)

Amoxicillin/clavulanate(A/C)

Piperacillin (Pi)

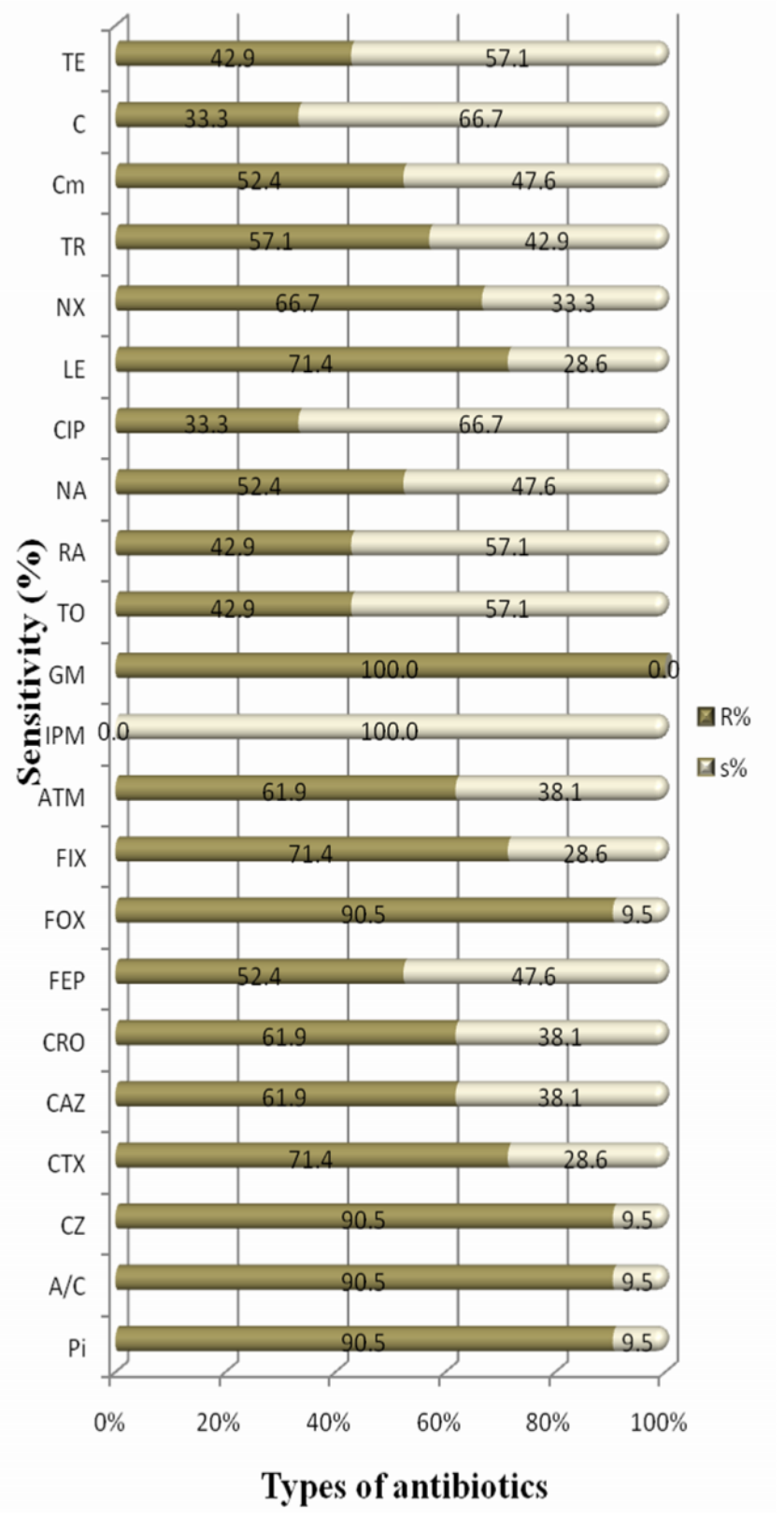

Figure (4-6): Resistivity patterns of 21 *Proteus* spp. against different antibiotics.

clavulanic acid, 68% to gentamicin, 48% to cefazolin, and 40% to amoxicillin. Khushal (2004) reported that 53% of *P. mirabilis* were sensitive to cefixime, 65% to ceftazidime, 67% to ceftriaxone, and 100% to cefotaxime. Reslinski *et al*. (2005) found that *P. mirabilis* isolates were highly resistant to cephalosporins 100 %, co-trimazine 98.9%, quinolones (e.g. nalidixic acid) 77.7%, and tetracycline 63.8%.

Clinical isolates are considered multi-drug resistant (MDR) if an isolate is resistance to representatives of three or more main classes of the following antimicrobial agents; penicillins, cephalosporins, monobactams, carbapenems, aminoglycosides, quinolones, sulfamides, and tetracyclins (Obritsch *et al.*, 2005). However, in the present study, it has been found that 174 (85.7%) of the isolates were characterized as a multi-drug resistant (Table 4-9). Bacteria resistance to antibiotics are widespread nowadays and constitutes serious clinical threats (Mathur *et al.*, 2002). Infections due to multidrug-resistant strains of *E. coli*, *K. pneumoniae* and other Gram-negative bacteria have been causing significant diagnostic and therapeutic problems (Kader *et al*. 2006).

Multi-drug resistant rate varies from country to country, in Iraq Al-Mohana (2004) reported that 56.8% of clinical isolates of *E. coli* were resistant to more

than five antimicrobial agents in Najaf. Al-Asady (2009) found that all 15 (100%) of the β-lactam resistant *E. coli* and *Klebsiella* spp. isolates obtained from children with bacteremia in Hilla city were multi-drug resistance. However, over all Gram-negative isolates from Latin American countries showed the lowest susceptibility rates to all antimicrobial agents followed by Asian and European isolates (Gals *et al.*, 2001).

Table (4-9): Occurrence of MDR in GNB isolates

Type of isolates	Total of isolates	No. % of MDR	
		No.	%
Ps. aeruginosa	59	58	98.3
E. coli	52	45	86.5
K. pneumoniae* subsp. *pneumoniae	19	19	100
K. pneumoniae* subsp. *ozaenae	15	13	86.7
K. pneumoniae* subsp. *rhinoscleromatis	14	12	85.7
K. pneumoniae* subsp. *aerogenes	7	3	42.9
K. oxytoca	6	5	83.3
P. mirabilis	16	15	93.8
P. vulgaris	5	4	80.0
Total	**203**	**174**	**85.7%**

In this study, among 59 β-lactam resistant *Ps. aeruginosa* isolates, 58 (98.3%) were identified as multi-drug resistance (Table 4-9). *Ps. aeruginosa* infections are difficult to treat because the responsible strains may be resistance to multiple antibiotics. Antibiotics resistance may emerge during or after

treatment of *Ps. aeruginosa* infections with antibiotics (Fonseca *et al*., 2005). However, the susceptibility pattern of isolates to some commonly used antibiotics as reported in this study is similar to that found in other literatures. Hassanien (2004) in Egypt, found multiple drug resistance against most of the examined poultry pathogens *Ps. aeruginosa, E. coli, K. pneumoniae* and *S. aureus*.

4-5 Frequency of Extended Spectrum β-Lactamases Production:

Extended Spectrum β-Lactamases (ESBLs) are defined as acquired, transferable β-lactamases that can significantly hydrolyse third generation cephalosporins (e.g. cefotaxime, ceftazidime, and ceftriaxone) and monobactam (aztreonam), but inactive against cephamycins, carbapenems (Paterson *et al.*, 2004; Chaudhary and Aggarwal, 2004), and β-lactamase-inhibitors such as clavulanic acid and tazobactam (Livermore, 2008).

The ESBL detection methods can be roughly divided into two groups: phenotypic methods, which detect the ability of the ESBL enzymes to hydrolyse different cephalosporins, and genotypic (molecular techniques) methods, which detect the genes responsible for the production of the ESBL. The phenotypic methods are commonly used by clinical diagnostic laboratories since these tests are easy to perform and they are also cost effective. In addition, the incorporation to automated susceptibility systems, have made them easily accessible (Wiegand *et al.*, 2007).

The present study was conducted on the presence of ESBL-production in 193 β-lactam resistant *Ps. aeruginosa, E. coli, Klebsiella* spp. and *Proteus* spp. clinical isolates recovered from patients with wound and lower respiratory tract infections in Najaf hospitals. However, Gram-negative pathogens, such as

Enterobacteriaceae and *Ps. aeruginosa* have acquired an important role in hospital infections, which is of particular concern because of the associated broad spectrum of antibiotic resistance (Pérez-Llarena and Bou, 2009). Early detection of ESBLs is necessary in infection control. But silent genes occasionally be activated or modestly functional genes may be enhanced as a result of selective pressure of antibiotic therapy (Jain *et al*., 2003). There is not one single detection method that covers all different β-lactamase resistance mechanisms (Drieux *et al.*, 2008), the sensitivity and specificity of a susceptibility test to detect ESBLs vary with the cephalosporin tested (Navon-Venezia *et al.*, 2003; Tofteland *et al.*, 2007).

4-5-1 Phenotypic Methods:

4-5-1-1 Initial Screen Test:

The initial screening and confirmation methods only indicate the possible presence of ESBLs (Livermore and Brown 2005). In this study, isolates of Gram-negative bacteria were screened for ESBLs by the National Committee for Clinical Laboratory Standards (CLSI, 2007), initial screen disc test using third generation cephalosporins and monobactam discs. When the inhibition zones of the antibiotics used for screening ESBL was ≤ 27 for cefotaxime (30µg), ≤ 22 for ceftazidime (30µg), ≤ 25 for ceftriaxon (30µg), and ≤ 27 for aztreonam (30µg) respectively, the strain was suspected as a potential ESBL producer (CLSI, 2007).

The results of this study show that the best indicator cephalosporin for detection of ESBLs in all isolates is cefotaxime. This cephalosporin demonstrated the highest rate (97.9%) of ESBL detection among isolates in the

susceptibility test. On the other hand, ceftriaxone demonstrated the lowest percentage (76.7%) of isolates in the susceptibility test. However, the initial screening for reduce susceptibility to third generation cephalosporins and aztreonam in the present study showed that 189 (97.9%) of GNB isolates tested were revealed a potential ESBL-producers, initially (Table 4-10).

Table (4-10): Extended-spectrum β-lactamase (ESBL) producing GNB isolated from the patients by phenotypic initial screen test

Type of isolate	No. of isolates	No. (%) of the potential ESBL producer
Ps. aeruginosa	59	58 (98.3%)
E. coli	52	51 (98.1%)
K. pneumoniae subsp. *pneumoniae*	19	19 (100%)
K. pneumoniae subsp. *ozaenae*	15	15 (100%)
K. pneumoniae subsp. *rhinoscleromatis*	14	13 (92.9%)
K. pneumoniae subsp. *aerogenes*	7	6 (85.7%)
K. oxytoca	6	6 (100%)
Proteus mirabilis	16	16 (100%)
Proteus vulgaris	5	5 (100%)
Total (%)	**193**	**189** (97.9%)

Conclusions and Recommendations

From different places, other studies are agreement with present study, in New Zealand reported that all *E. coli* and *Klebsiella* spp. isolates (n = 87) that were screen positive (Blackmore, 2006), in India, all *K. pneumoniae* were suspicious of ESBL-producers when used cefotaxime and ceftriaxone (Chiangjong, 2006), and in Germany reported that 85% of *E. coli* and 94% of *Klebsiella* spp. were suspected as ESBL-producers (Svärd, 2007). The persistent exposure of bacteria to a multitude of β-lactams has induced dynamic changes in terms of increasing production of β-lactamases and mutations in their restricted spectrum enzymes to become ESBLs (Pongpech *et al*., 2008).

In other studies (by this method) were contrary, that showed lower percentage of ESBL-producers. In Hilla city, only 6 (15.7%) of *Klebsiella* spp. isolates were potential ESBL-producers (Al-Charrakh, 2005). However, different frequency of ESBL-producers were observed, in Thailand (38.3%) (Pongpech *et al*., 2008) and in India (55%) (Goyal *et al*., 2009).

In the present study, all potential ESBL-producing isolates were resistant to most of the β-lactams and non β-lactams antibiotics. However genes that encode ESBLs are often located on plasmids together with genes encoding resistance to trimethoprim, aminoglycosides, sulphonamides, tetracyclines, fluoroqinolones, and chloroamphenicol (Potz *et al.*, 2006; Paterson, 2006), and therefore ESBLs are often MDR producers. The prevalence of MDR and resistance to fluoroqinolones e.g. ciprofloxacin is increasing among ESBL producers (Kang *et al.*, 2004; Livermore *et al.*, 2008); Nevertheless, present study revealed that all potential ESBL producers were susceptible to the carbapenems (e.g. imipenem) tested. Jamal *et al*. (2009), they indicated that carbapenems are the reliable drugs of choice for serious or life-threatening

infections caused by ESBLs-producing bacteria. Imipenem and meropenem were very active against ESBL and non-ESBL producers. In this study, the high resistance rate amongst Gram-negative bacilli to third-generation cephalosporins is a likely consequence of heavy empirical usage of this group of antibiotics, this high prevalence of ESBL-production among Gram-negative bacilli were commonly observed in *K. pneumoniae* and *E. coli* in Asia (Kader and Veena Kumar, 2005; Rossi *et al*., 2006).

4-5-1-2 Phenotypic Confirmatory Test

According to the guidelines provided by the Clinical and Laboratory Standards Instituted (CLSI, 2006), ESBL screen was followed by ESBL confirmatory tests using double disc synergy (DDS) test (which also called Jarlier method). This test is placed cefotaxime, ceftazidime, ceftriaxone, and aztreonam antibiotic discs (20 µg for each one) on Muller Hinton agar at a same distances (30 mm from center to center) from the amoxicillin/clavulanic acid disc (which placed in the center of the plate). The clavulanic acid is separated and distributed around disc, this component can permeate the bacterial cell walls and can inactivate extracellular enzymes (Livermore and Brown, 2001; Bedenic *et al*., 2001); the synergy effect seen depended on the diffusion across the agar. A clear enhancement of the zone of inhibition was seen on sides of the amoxicillin/clavulanic acid disc toward other discs, these clear enhancement indicated some synergistic effect between the clavulanic acid and other an oxyimino-cephalosporin (third generation cephalosporins) and monobactam antibiotics which mean that these bacteria are ESBL producers, while the strains without synergistic effect considered non-ESBL producers (Al-Jasser, 2006).

Conclusions and Recommendations

In the present study, the result showed that only 10 (5.2%) of the 193 isolates were confirmed as ESBL producers. These results are summarized in Table (4-11). The majority of the ESBL-producing GNB were shown to be from wound infections (9, 90%), while, only one (10%) were from lower

Table (4-11): Frequency of ESBL producing-GNB isolates by double disc synergy method

Type of isolate	No. of isolates	No. (%) of the ESBL producer by Confirmatory test
Ps. aeruginosa	59	2 (3.4%)
E. coli	52	5 (9.6%)
K. pneumoniae subsp. *pneumoniae*	19	0.0
K. pneumoniae subsp. *ozaenae*	15	0.0
K. pneumoniae subsp. *rhinoscleromatis*	14	3 (21.4%)
K. pneumoniae subsp. *aerogenes*	7	0.0
K. oxytoca	6	0.0
P. mirabilis	16	0.0
P. vulgaris	5	0.0
Total (%)	**193**	**10 (5.2%)**

respiratory tract infections. Occurrence of ESBL in isolates was as follows: 5 (9.6%) were *E. coli*, 3 (21.4%) were *K. pneumoniae* subsp. *rhinoscleromatis*, and 2 (3.4%) were *Ps. aeruginosa*.

Double disc synergy method is widely used because it is the more commonly and reliable method (Bedenic *et al*., 2001), as well as easily adopted by laboratories for detection of ESBLs among clinical isolates. DDS tests are sensitive and specific in *Enterobacteriaceae* (Livermore *et al*., 2001; Florijn *et al*., 2002), but this test may not be as useful for detection of ESBLs in many cases; if the optimum disc placement is not exactly, the inability of clavulanic acid to inhibit all ESBLs, the loss of clavulanic acid disc potency during storage, and the inability to detect ESBLs isolates which have ability to produce chromosomal and plasmid-mediated cephalosporinases (Hemalatha *et al*., 2007; Fam and El-Damarawy, 2008).

In this study, the proportion of ESBL producers is considered low when compared with the results of initial screen disc test, which all these isolates were cefoxitin resistant, and any isolate was cefoxitin resistant indicates that it is possibly AmpC β-lactamase producers which can mask ESBL production in the standard CLSI ESBL confirmatory tests. False results are supposed to occur if the AmpC activity is larger than activity of ESBL which may lead to failure treatment (Yan *et al*., 2002). Therefore, it can be said that these isolates may have ESBL enzymes, but they can't be detectable by third generation cephalosporins with amoxicillin/clavulanic acid may be due to the existence AmpC enzymes which act as a mask against the production of ESBL enzymes in confirmatory tests. Unlike ESBLs, AmpC β-lactamases do not confer

resistance to fourth-generation cephalosporins. Therefore, the use of fourth-generation cephalosporins, such as cefepime and cefpirome, should facilitate the detection of ESBLs in organisms that also produce AmpC β-lactamases (Al-Jasser, 2006; Svärd, 2007). On the other hand, ESBL producing isolates don't always show *in vitr*o, because the cases may show that especially simultaneous presence of metallo-enzymes with carbapenem hydrolyzing activity (Mavroidi *et al*., 2000; Docquier *et al*., 2001; Paterson *et al*., 2001), extended spectrum oxacillinases (e.g. OXA-10) (Girlich *et al*., 2002), effective of GES-2 on clavulanic acid (Poirel *et al*., 2001), or may combine mechanisms of resistance like efflux pumping and impermeability (Weldhagen *et al*., 2003). Therefore, Navon-Venezia *et al*. (2005); Drieux *et al.* (2008); and Kahlmeter (2008) mentioned that ESBL enzymes is difficult to detect phenotypically with the existing AmpC β-lactamase genes. By the way, infections caused by ESBL- and AmpC β-lactamase-producing Gram-negative bacteria complicate therapy and limit treatment options (Färber *et al*., 2008); ESBL producing organisms mainly *Enterobacteriaceae* have been an emergent problem in hospitals and other health care centers as a cause of nosocomial outbreaks worldwide (d' Azevedo *et al.*, 2004). Different prevalence reports have been observed in different geographical areas and are rapidly changing over times (Babypadmini and Appalaraju, 2004). The most effective and reliable is carbapenem antibiotics e.g. meropenem and imipenem (Samaha-Kfoury and Araj, 2003) which has highly active *in vitro* against *Enterobacteriaceae* including organisms that produce ESBLs (Paterson *et al*., 2005).

This study show lower proportion of (5.2%) ESBL producing isolates than other studies in different places, in Hilla city, 46.7% of the β-lactam resistant isolates were ESBL producers (Al-Asady, 2009); the percentage expression ESBL phenotype was higher in Gram-negative isolates from Norway (63.2%)

(Tofteland *et al*., 2007), the Thailand (31%) (Pongpech *et al*., 2008), and the spain (72%) (Escudero *et al*., 2009). On the other hand, other studies revealed lower proportion of ESBL producers than this study, in South India (2.9%) (Menon *et al*., 2004) and in Korea (4.8%) (Shah *et al*., 2004).

Although ESBLs are theoretically inhibited by β-lactamase inhibitors (e.g. clavulanic acid), the present study found that the 90% of ESBLs producing bacteria were resistant to β-lactam/β-lactamase inhibitor combinations, this may be because of production of AmpC and carbapenemase enzymes.

In the present study, of the 52 *E. coli* isolates, only five (9.6%) were ESBL producers by using double disc synergy test (Table 4-11) and Figure (4-7), all these isolates were obtain from patient with wound infections. A similar isolation rate were observed in Korea (Ryoo *et al*., 2005), in Latin America (Villegas *et al*., 2004), and in South India (Jayapradha *et al*., 2007) were 9.3%, 8.5%, and 8.3%, respectively.

On the other hand, the occurrence of ESBL producing *E. coli* in this study is considered lower than other studies in different places, in Najaf city, that ESBL producing *E. coli* was 15.8 (Hadi, 2008); in Hilla city, Al-Asady (2009) reported that 13.3% of *Enterobacteriaceae* isolates was ESBL producers; in Kuwait, ESBL producing *E. coli* was 62% (Jamal *et al*., 2009); in Iran, only 16.8% was ESBL producers (Mansouri and Ramazanzadeh, 2009).

As shown in Table (4-11) and Figure (4-8), only 3 (4.9%) out of 61 *Klebsiella* spp. isolates were confirmed as ESBL producers. All these ESBL-producing isolates were *K. pneumoniae* subsp. *rhinoscleromatis*. Two of these isolates were from wound infections, while only one was from lower respiratory tract infection. This result is agreement with the research of Patrick

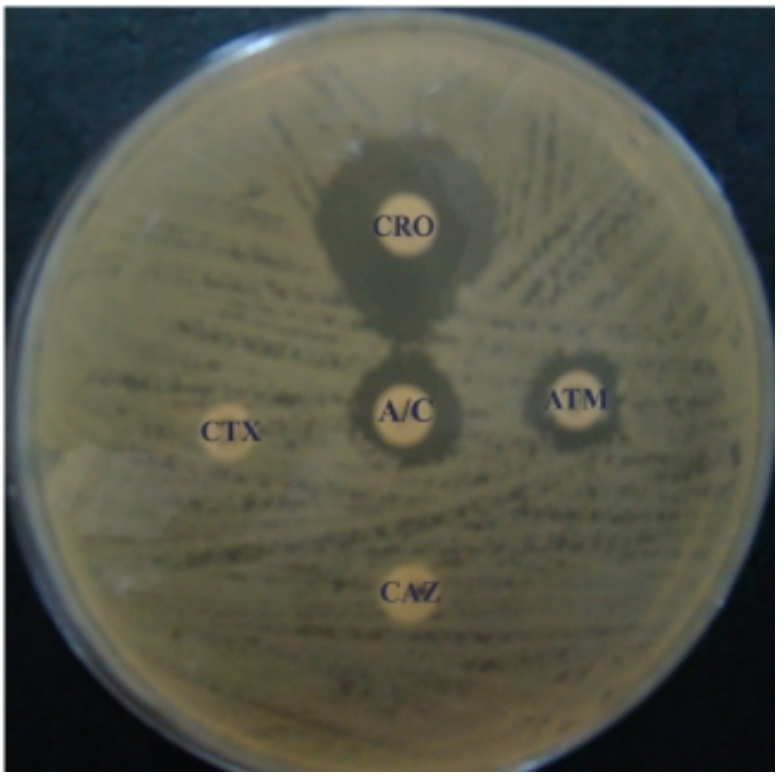

Figure (4-7): Double disc synergy test to detect ESBL producing in *E. coli* NE139 isolate.

A/C, Augmentin disc (20 µg of amoxicillin plus 10 µg of clavulanic acid) CRO, ceftriaxone 30 µg; CTX, cefotaxime 30 µg, CAZ, ceazidime 30 µg and ATM, aztreonam 30 µg.

et al. (2009) in Netherland which revealed that 5.2% of *K. pneumoniae* were ESBL producers. The overall rate of ESBL-producing *Klebsiella* spp. in this study was lower than that reported in other investigations. Al-Charrakh (2005) reported that 10.5% of all *Klebsiella* spp. were ESBL producers in Hilla city; Hadi (2008) found that 22.7% of *K. pneumoniae* collected from patient with significant bacteriuria in Najaf were ESBL producing isolates.

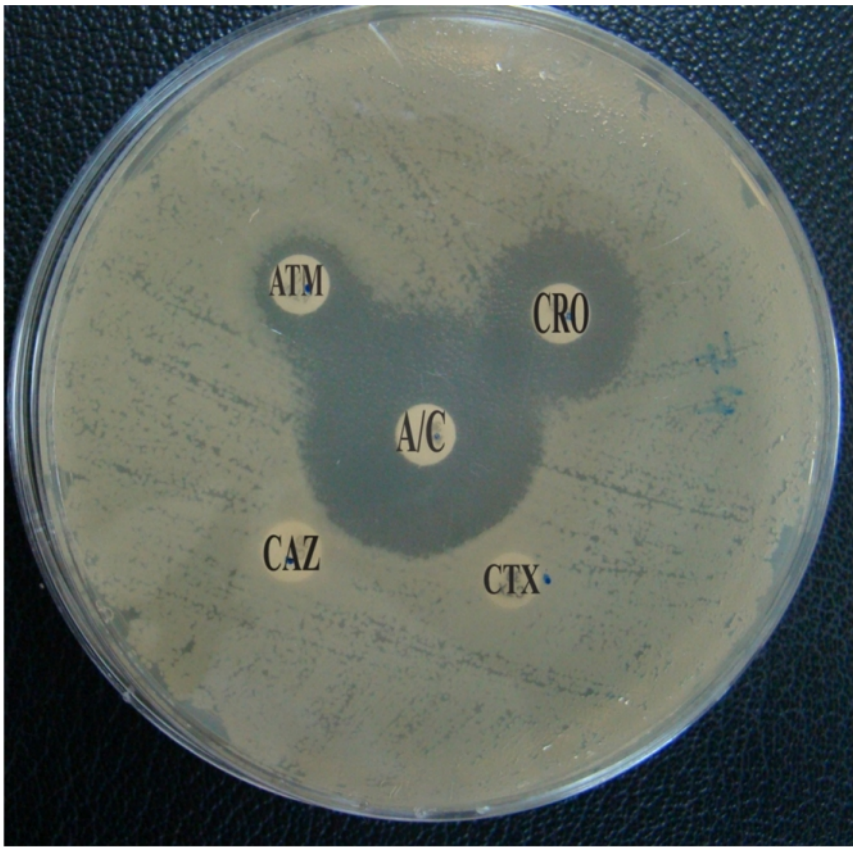

Figure (4-8): Double disc synergy test to detect ESBL producing in *K. pneumoniae* subsp. *rhinoscleromatis* NK74 isolate.

A/C, Augmentin disc (20 µg of amoxicillin plus 10 µg of clavulanic acid) is placed in the center that surrounded by 3 discs of third generation cephalosporins (CRO, ceftriaxone 30 µg; CTX, cefotaxime 30 µg; and CAZ, ceftazidime 30 µg) and one disc of monobactam (ATM, aztreonam 30 µg).

However, in India, Goyal *et al*. (2009) found that 66.7% of *Klebsiella* spp., isolates were identified as ESBL producers; and in Kuwait, ESBL production was detected in 82.1% of the *K. pneumoniae* isolates (Jamal *et al*., 2009).

Conclusions and Recommendations

In this investigation, out of 59 *Ps. aeruginosa* isolates, only 2 (3.4%) were positive for ESBL test (Table 4-11). The source of these isolates were from the wound infections. *Ps. aeruginosa* strains producing plasmid-mediated ESBL have been reported increasingly (Marchandin *et al.*, 2000). Most of them are non-SHV and non-TEM type of ESBLs such as; PER-1 and OXA type (Afzal-Shah *et al.*, 2001; Luzzaro *et al.*, 2001).

However, all of *Proteus* spp. isolates in the present study were non-ESBL producers. In previous studies of hospital outbreak reports have been associated with use and overuse of third generation cephalosporins as the major cause of ESBL producing organisms emergence worldwide (Saurina *et al.*, 2000; Bisson *et al.*, 2002). In addition, controlling the use of cephalosporin usage appeared to be useful in limiting some of the outbreaks.

4-5-2 Molecular Detection of ESBL Genes:

Molecular methods, particularly PCR, are widely used for confirmation and determination of ESBL genes. however, only genes in the families of TEM, SHV, OXA, and CTX-M were selected in the present study. The studies on the distribution of bla_{TEM}, bla_{SHV}, bla_{OXA}, and $bla_{CTX\text{-}M}$ in ESBL-producing GNB, have been performed worldwide, but only few data have been published in Iraq (Hadi, 2008). Nevertheless, the goal of present study was to evaluate to occurrence of bla_{TEM}, bla_{SHV}, bla_{OXA}, and $bla_{CTX\text{-}M}$ genes among 62 potential ESBL-producing clinical isolates recovered from patients in Najaf. PCR is considered the best efficiency method for ESBL detection because it is a faster than phenotypic detection method (Chiangjong, 2006), and also detect the presence of poorly or non-expressed (silent) genes difficult to determine by

phenotype; PCR may also be used to directly test patient samples as an early predictor of infection (Diekema *et al.*, 2004).

Of the 189 potential ESBL producing GNB isolates (and because of the expensive cost of the genotypic method), only 62 (32.8%) isolates were chosen to be screened by PCR for the presence of genes that encode TEM, SHV, OXA, and CTX-M. However, 51 (82.3 %) of 62 potential ESBL producing GNB isolates were carried at least one of the above mentioned ESBL genes; nevertheless, 34 (66.7%) of these isolates were recovered from patients with wound infections, while, only 17 (33.3%) were obtained from patients suffering from lower respiratory tract infections (Figure 4-9). Among these isolates, 17 (27.4%) were *Ps. aeruginosa*, 15 (24.2%) were *E. coli*, 15 (24.2%) were *Klebsiella* spp., and 4 (6.5%) were *Proteus* spp. **(**Figure 4-9).

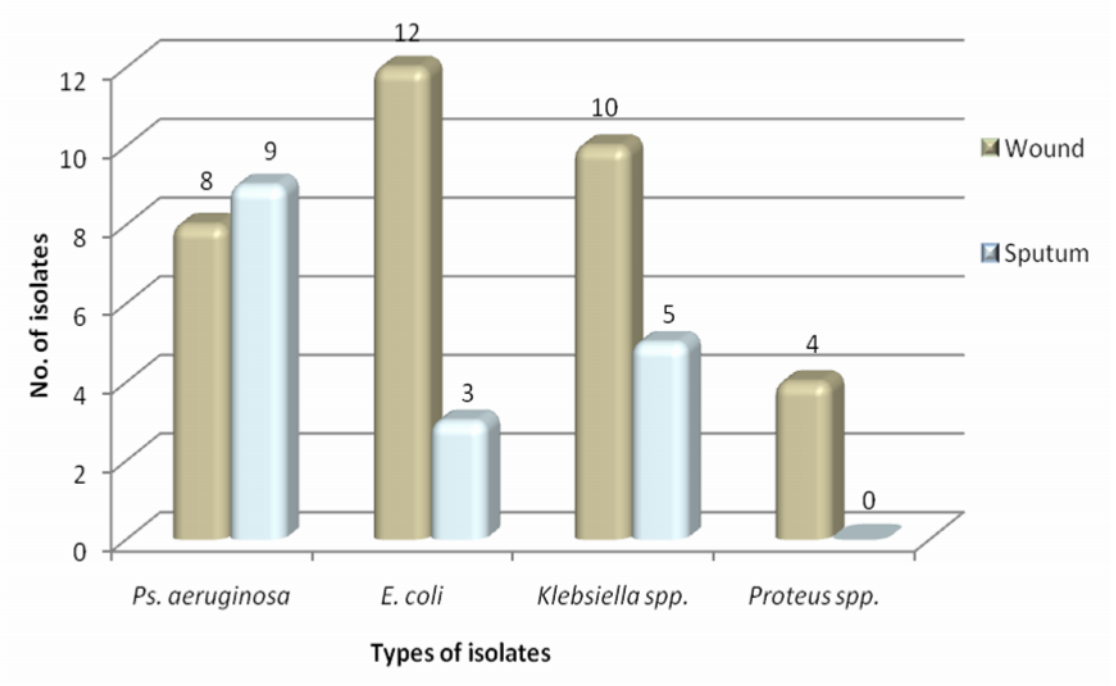

Figure (4-9): Distribution of ESBL genes among GNB isolates according to the source of samples.

Conclusions and Recommendations

The results revealed that 27 bacterial isolates were contained only one type of ESBL genes: nine *bla*$_{CTX-M}$ genes, eight *bla*$_{SHV}$ genes, eight *bla*$_{OXA}$ genes, and two *bla*$_{TEM}$ genes. While, 21 isolates had the combination of two genes: 13 *bla*$_{TEM}$ genes (five in combination with *bla*$_{SHV}$ genes; two in combination with *bla*$_{OXA}$ genes; six in combination with *bla*$_{CTX-M}$ genes), five *bla*$_{SHV}$ genes (four in combination with *bla*$_{CTX-M}$ genes and one in combination with *bla*$_{OXA}$ gene), and three *bla*$_{CTX-M}$ genes in combination with *bla*$_{OXA}$ genes, and two (3.2%) isolates had three types of ESBL genes, *Ps. aeruginosa* (combination of *bla*$_{SHV}$, *bla*$_{OXA}$, and *bla*$_{CTX-M}$ genes) and *E. coli* (combination of *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{OXA}$ genes). As well as one (1.6%) isolate (*K. pneumoniae* subsp. *rhinoscleromatis*) carried the combination of four genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, and *bla*$_{CTX-M}$ genes) **(**Table 4-12). The prevalence of bacteria which produced more than one type of ESBL enzymes is considered more dangerous for human hygiene (Erlandsson, 2007); therefore, different studies are needed to find more effective strategies to improve antibiotic use and hospital hygiene in order to minimize the emergence and spread of resistant organisms in Najaf hospitals.

The present study revealed that CTX-M β-lactamase was the most prevalent (38.7%) among the ESBL producing isolates; followed by SHV (33.9%); while, TEM and OXA β-lactamases were the less (27.4% for each). Overall, this result is in agreement with many Asia`s studies like Yan *et al*. (2000) in Taiwan and Goyal *et al*. (2009) in India, who reported that CTX-M β-lactamases were the commonest enzymes than TEM and SHV in clinical GNB isolates. The CTX-M and SHV β-lactamases were mostly found among the *Ps. aeruginosa* isolates (10, 17.2% and 7, 12.1% respectively), while TEM and OXA β-lactamases were the most prevalence among *Klebsiella* spp.

Table (4-12): Extended spectrum β-lactamase (ESBL) genotypes in ESBL-producing Gram-negative isolates

Positive by PCR for ESBL genes	Number amplified				Total
	Ps. aeruginosa	***E. coli***	***Klebsiella* spp.**	***Proteus* spp.**	
A. Single ESBL gene:					
TEM	0	1	1	0	**2**
SHV	3	2	2	1	**8**
CTX-M	5	2	1	1	**9**
OXA	2	2	4	0	**8**
B. Two or more ESBL genes:					
TEM, SHV	1	1	3	0	**5**
TEM, CTX-M	2	2	2	0	**6**
TEM, OXA	1	0	1	0	**2**
SHV, CTX-M	2	2	0	0	**4**
SHV, OXA	0	0	0	1	**1**
CTX-M, OXA	0	2	0	1	**3**
TEM, SHV, OXA	0	1	0	0	**1**
SHV, CTX-M, OXA	1	0	0	0	**1**
TEM, SHV, CTX-M, OXA	0	0	1	0	**1**
Total	**17**	**15**	**15**	**4**	**51**

isolates (8, 13.6% and 6, 10.2% respectively) as showed in Table (4-13). Many of GNB contain large or mega plasmid when these isolates carry β-lactams resistance and non-β-lactams resistance genes that confers ability to resistance

different types of antibiotics; additionally, the activity of transferable and conjugatable can facilitate plasmids and integrons to distribution resistant genes among different bacteria (Jacoby and Munoz-Price, 2005).

All the TEM-, SHV-, CTX-M-, OXA-positive isolates were resistant to cefoxitin and piperacillin. While, 94.1%-95.8% of these isolates were, resistant to the oxyimino-cephalosporins (cefazolin, cefixime, cefotaxime, and ceftazidime); Nevertheless, the present study revealed that all these isolates were susceptible to imipenem which is in agreement with other studies, for example, Paterson (2006) and Livermore *et al.* (2008) who showed that the vast majority of *Enterobacteriaceae*, including ESBL producers, remain susceptible to carbapenems, because they are highly stable to β-lactamase hydrolysis; therefore, carbapenems are the reliable drugs of choice for serious or life-threatening infections caused by ESBLs-producing bacteria. However, Jamal *et al*. (2009) found that imipenem and meropenem were very active against ESBL producers.

On the other hand, the present study revealed that, out of the 62 GNB, a total of 51 isolates were identified as ESBL positive by PCR method, when compared to the phenotype data confirmed by double disc synergy method (only 10 isolates gave positive results).

These results confirmed the possibility of existing of AmpC β-lactamase enzyme with larger activity which can mask ESBL production, this idea was confirmed by Yan *et al*. (2002) and Pitout *et al*. (2003) who reported that the

Table (4-13): Distribution of the ESBL genes among 62 of GNB

Type of isolate	No. of potential ESBL isolates	No. (%) of the ESBL genes			
		***bla*$_{TEM}$**	***bla*$_{SHV}$**	***bla*$_{CTX-M}$**	***bla*$_{OXA}$**
Ps. aeruginosa	18	4 (22.2%)	7 (38.9%)	10 (55.6%)	4 (22.2%)
E. coli	20	5 (25%)	6 (30%)	8 (40%)	5 (25%)
K. pneumoniae* subsp. *pneumoniae	7	1 (14.3%)	0 (0.0%)	0 (0.0%)	5 (71.4%)
K. pneumoniae* subsp. *ozaenae	3	1 (33.3%)	1 (33.3%)	1 (33.3%)	0 (0.0%)
K. pneumoniae* subsp. *rhinoscleromatis	5	4 (80%)	4 (80%)	2 (40%)	1 (20%)
K. pneumoniae* subsp. *aerogenes	0	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
K. oxytoca	2	2 (100%)	1 (50%)	1 (50%)	0 (0.0%)
Proteus mirabilis	6	0 (0.0%)	2 (33.3%)	2 (33.3%)	2 (33.3%)
Proteus vulgaris	1	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Total (%)	**62**	**17 (27.4%)**	**21 (33.9%)**	**24 (38.7%)**	**17 (27.4%)**

AmpC-producing organisms can act as hidden reservoirs for ESBLs, therefore, this case will certainly necessitate the use of a molecular diagnostic method

and additionally enhancement of the clinical microbiology laboratories to so as be able to detect an ESBL production in these organisms on a routine basis.

TEM-type ESBLs are the first plasmid-mediated ß-lactamase that are often found in genera of *Enterobacteriaceae* such as *E. coli*, *K. pneumoniae*, and *Proteus mirabilis*; and also in non-*Enterobacteriaceae* like *Ps. aeruginosa* (Shah *et al.*, 2004). Currently, more than 170 bla_{TEM} gene variants that are resistant to inhibitors like clavulanic acid (Jacoby and Bush, 2009) that occur by single amino acid substitutions at positions 104, 164, 238, and 240 (Bradford, 2001). Reports originated from geographically diverse locations, suggest that local antibiotic usage and practices may play an active role in promoting the selection of point mutations in *bla* $_{TEM}$-type genes (Vourli *et al*., 2004; Wachino *et al*., 2004).

In this study, a total of 17 (27.4%) GNB which carried *bla* $_{TEM}$ genes. Of these isolates, eight (12.9%) were *Klebsiella* spp. (four isolates were *K. pneumoniae* subsp. *rhinoscleromatis*, one isolate was *K. pneumoniae* subsp. *pneumoniae*, one isolate was *K. pneumoniae* subsp. *ozaenae*, and two isolates were *K. oxytoca*), five (8.1%) were *E. coli*, and four (6.5%) were *Ps. aeruginosa* isolates (Table 4-13 and Figure 4-10).

In previous local studies, Hadi (2008) reported that 41.2% among *E. coli* and *K. pneumoniae* isolated from patients with significant bacteriuria had *bla* $_{TEM}$ genes, in Hilla Al-Asady, (2009) showed that 57.1% of *E. coli K. pneumoniae* isolates carried genes of *bla* $_{TEM}$. The result of this study is considered higher than any other study, the study reported by Shahcheraghi *et al.* (2009) (from Iran) found that the frequency of *bla* $_{TEM}$ genes among the ESBL Gram-negative isolates were 9.0%. While the present result was lower than other studies in other parts of the world, in Germany, Svärd (2007) reported that 70% of *E. coli* and *Klebsiella* spp. had *bla* $_{TEM}$ genes, in Thailand,

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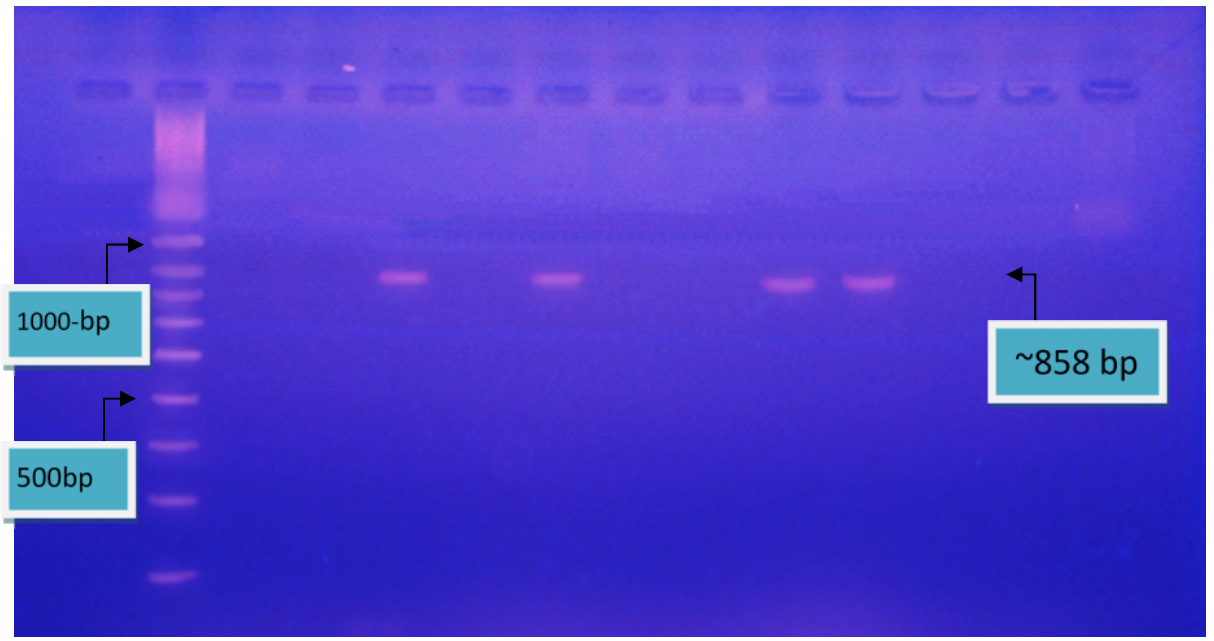

Figure (4-10): Ethidium bromide stained agarose gel showing PCR amplification products with TEM primers.

Lane 1: negative control was reagent blank with no DNA.

Lane 2: 100 bp standard size reference marker (500 & 1000 bp segment indicated as a pointed band).

Lanes: 3, NE183; **4**, NP179; **5**, NE121; **6**, NP106; **7**, NE99; **8**, NP82; **9**, NP72; **10**, NK74; **11**, NK69; **12**, NE61; **13**, NK60; **14**, NP57.

Pongpech *et al*. (2008) found that *bla* $_{TEM}$ genes were 78% of the confirmed ESBL producers, and in India, Goyal *et al*. (2009) revealed this gene was 54.9% among *E. coli* and *K. pneumoniae* isolates.

SHV enzymes are the ß-lactamases that were also mainly found in GNB (Huang *et al*., 2004), these enzymes possess variants because of substituted serine instead of glycine at position 238 and lysine instead of glutamate at position 240 (Poole, 2004). There are more than 125 SHV varieties described worldwide (Jacoby and Bush, 2009). The SHV ß-lactamases were the predominant ESBL types in Europe and United States. The SHV-2, SHV-5 and SHV-12 variants are the most common ones (Paterson *et al*., 2003).

By using of PCR amplification method, a *bla* $_{SHV}$ ESBL gene was detected in 21 (33.9%) of 62 of GNB isolates, of these, seven (11.3%) were *Ps. aeruginosa*, six (9.7%) were *Klebsiella* spp. (four isolates were *K. pneumoniae* subsp. *rhinoscleromatis*, one isolate was *K. pneumoniae* subsp. *ozaenae*, and one isolate was *K. oxytoca*, six (9.7%) were *E. coli*, and only two (3.2%) were *Proteus mirabilis* (Table 4-13, Figure 4-11). This study showed a lower result of *bla* $_{SHV}$ producing Gram-negative bacilli than the research of Al-Asady (2009) in Hilla who reported that 78.6% of ß-lactamase producing *E. coli* and *K. pneumoniae* isolates produced SHV enzymes, while Hadi (2008) in Najaf revealed that 29.4% of confirmed of ESBL-producing *E. coli* and *K. pneumoniae* isolates had *bla* $_{SHV}$ genes. However, in other parts of the world, investigators got lower results than in this study, Svärd (2007) showed that 15% of clinical isolates of *E. coli* are suspected to be ESBL producers and carried SHV enzymes in Germany, Pongpech *et al*. (2008) in Thailand, reported that The frequency of *bla* $_{SHV}$ genes were 8% of the confirmed ESBL producing *E. coli* isolates. Shahcheraghi *et al.* (2009) in Iran showed that

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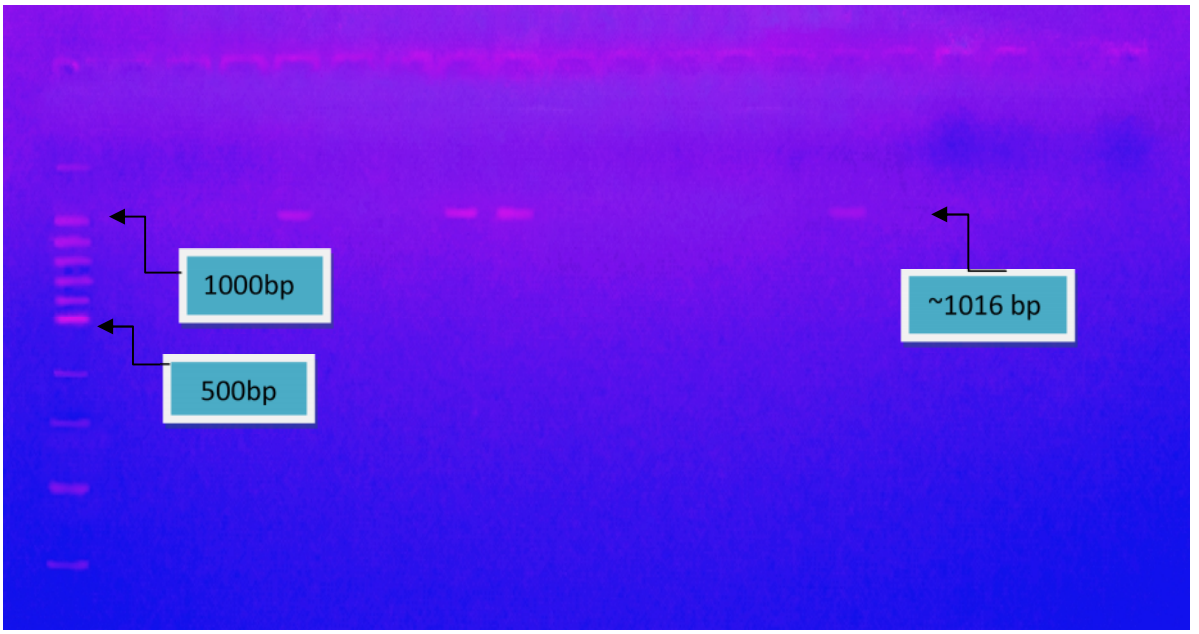

Figure (4.11): Ethidium bromide stained agarose gel showing PCR amplification products with SHV primers.

Lane 1: 100 bp standard size reference marker (500 & 1000 bp segment indicated as a pointed band).

Lanes: 2, NK2; **3**, NP8; **4**, NE12; **5**, NE15; **6**, NK16; **7**, NP20; **8**, NE26; **9**, NP48; **10**, NK60; **11**, NE61; **12**, NP63; **13**, NK64; **14**, NK69; **15**, NK74; **16**, NK91; **17**, NE99; **18**, NP106; **19**, NK118.

Lane 20: negative control was reagent blank with no DNA.

frequency of *bla* $_{SHV}$ genes were 22% among the ESBL Gram-negative isolates.

On the other hand, the present study is considered to be lower than other studies in ratio of *bla* $_{SHV}$ genes in other places, for example the research of Chiangjong (2006) showed that all of ESBL producing *K. pnumoniae* isolates had SHV enzymes.

The dissemination of CTX-M and OXA genes among bacteria are considered a novel study in this region. CTX-M-encoding genes are a more recent family of plasmid-mediated ESBLs; some of them are part of transposons or constitute gene cassettes in integrons (Livermore *et al*., 2007). The bla_{CTX-M} gene variants show less than 40% identity to bla_{SHV} and bla_{TEM}, and are hence not closely related. The CTX-M variants are mostly found in *Enterobacteriaceae*, most prevalently in *E. coli*, *K. pneumoniae*, and *Proteus miriabilis*. It is not clear why the spread of CTX-M has been more extensive than the spread of TEM and SHV, may be beside of hydrolysis ceftazidime antibiotic, these enzymes are initially identified by preferential hydrolysis of cefotaxime (Bonnet, 2004). Recently, over 80 different variants of CTX-M have been found (Jacoby and Bush, 2009). CTX-M enzymes can be classified by amino acid sequence similarities to five major groups of acquired CTX-M enzymes which included plasmid-mediated enzymes: The CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 groups (Bonnet, 2004).

During the study period, the bla_{CTX-M} genes were detected in 24 (38.7%) of the 62 potential ESBL-producing GNB isolates, these enzymes were the most common among the *Ps. aeruginosa* (10, 16.1%), followed by *E. coli* (8, 12.9%), *Klebsiella* spp. were (4, 6.5%) (2 isolates were *K. pneumoniae* subsp.

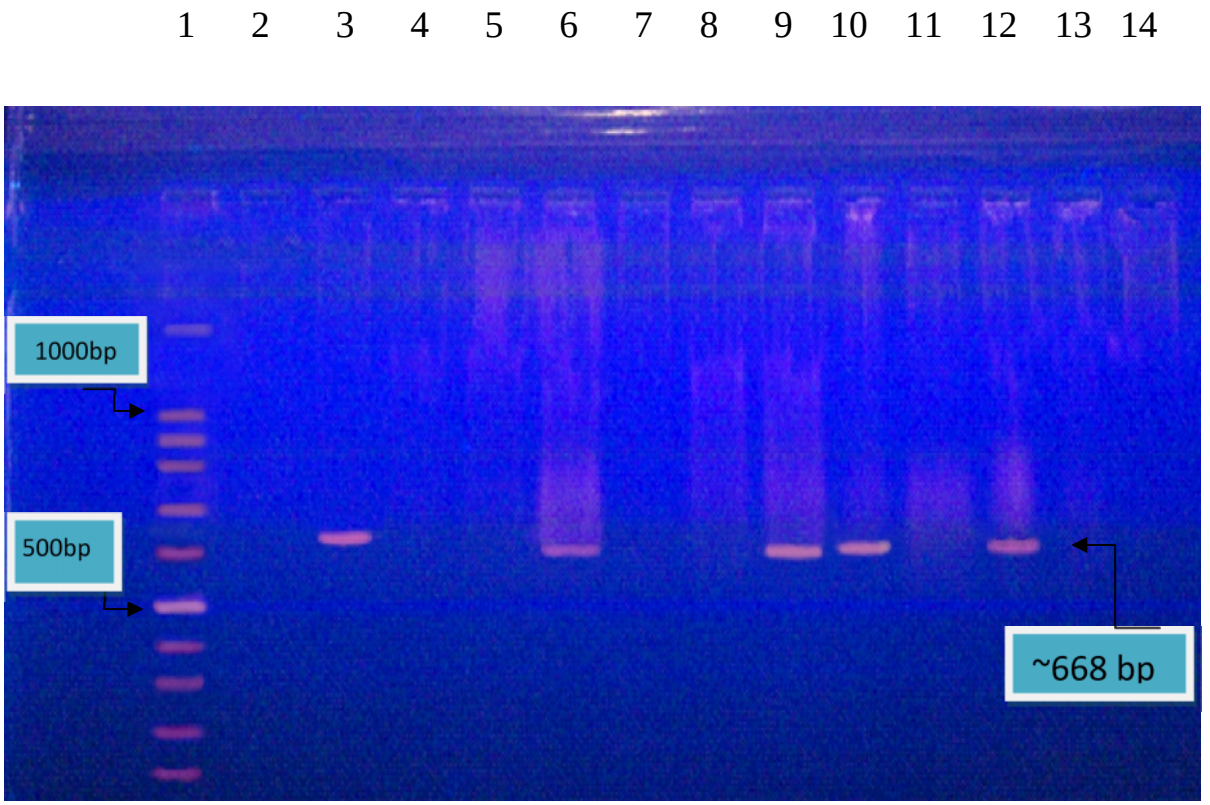

Figure (4.12): Ethidium bromide stained agarose gel showing PCR amplification products with CTX-M primers.

Lane 1: 100 bp standard size reference marker (500 & 1000 bp segment indicated as a pointed band).

Lanes: 2, NK4; **3**, NP8; **4**, NE15; **5**, NK16; **6**, NE18; **7**, NP20; **8**, NK24; **9**, NP32; **10**, NP40; **11**, NP48; **12**, NK64; **13**, NP82.

Lane 14: negative control was reagent blank with no DNA.

rhinoscleromatis, 1 isolate was *K. pneumoniae* subsp. *ozaenae*, and 1 isolate was *K. oxytoca*), and *Proteus mirabilis* (2, 3.2%) (Table 4-13, Figure 4-12).

Overall, the spreading of the bla_{CTX-M} genes in the GNB clinical isolates collected from Najaf and included in this study was low. Nevertheless, high prevalence has been detected in different parts of the world such as Norway (90%) (Tofteland *et al.*, 2007), Slovenia (59%) (Istinič, 2008), India (85.4%) (Goyal *et al.*, 2009). Low prevalence of bla_{CTX-M} genes has also been reported from India, the prevalence of bla_{CTX-M} genes was 15.8% among ESBL positive *Enterobacteriaceae* isolates (Jemima and Verghese, 2008).

The most of OXA derivative genes are plasmid and integron located (Poirel *et al.*, 2001). While some OXA-type ß-lactamases are encoded by chromosomal genes that appear to be resident in some microbial genomes such as those in *Ps. aeruginosa* (Giuliani *et al.*, 2005). The presence of co-resistance gene cassettes on integrons make these genetic elements useful to bacteria by facilitating widespread dissemination through patients from a wide variety of clinical disciplines (Poirel *et al.*, 2002). OXA-type enzymes hydrolyse cloxacillin and oxacillin are faster than benzylpenicillin and are generally not inhibited by clavulanic acid (except OXA-18 and OXA-45) (Toleman *et al.*, 2003). Currently, 139 OXA-type β-lactamases have been detected (Jacoby and Bush, 2009).

Of the 62 potential ESBL-producing GNB isolates tested, the distribution of bla_{OXA} genes is summarized in Table (4-13) and Figure (4-13). However, these genes were detected in 17 (27.4%) isolates, including six (9.7%) *Klebsiella* spp. (five isolates were *K. pneumoniae* subsp. *pneumoniae* and one *K. pneumoniae* subsp. *rhinoscleromatis*; while none of the *K. pneumoniae*

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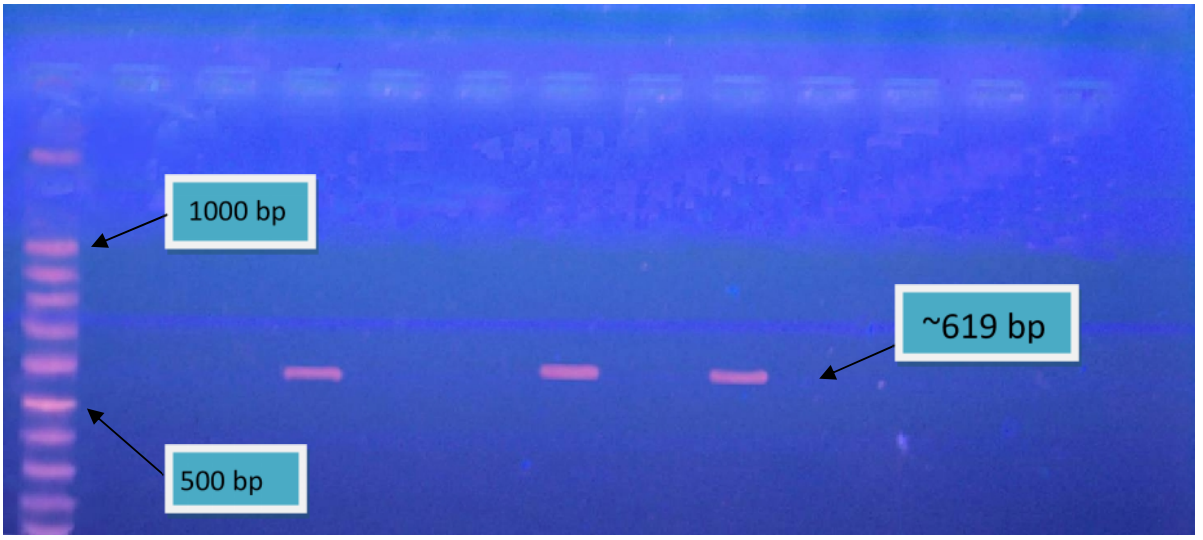

Figure (4.13): Ethidium bromide stained agarose gel showing PCR amplification products with OXA primers.

Lane 1: 100 bp standard size reference marker (500 & 1000 bp segment indicated as a pointed band).

Lanes: 2, NP3; **3**, NK4; **4**, NE12; **5**, NE15; **6**, NE18; **7**, NP20; **8**, NE25; **9**, NE26; **10**, NP32; **11**, NK39; **12**, NE45.

Lane 13: negative control was reagent blank with no DNA.

subsp. *ozaenae*, *K. pneumoniae* subsp. *aerogenes*, and *K. oxytoca* isolates were carried *bla* $_{OXA}$ genes), five (8.1%) *E. coli*, four (6.5%) *Ps. aeruginosa*, and only two (3.2%) *Proteus mirabilis*. In contrast, lower prevalence has been reported from Spain (3.8%) (Tenover *et al.*, 2003), Germany (15%) (Svärd, 2007), Malaysia (5%) (Lim *et al.*, 2009). On the other hand, high prevalence was detected in Slovenia (41%) (Istinič, 2008) and in USA (40%) (Jones *et al.*, 2009).

CONCLUSIONS

and

RECOMMENDATIONS

Conclusions:

The main findings of this thesis are:

1) The majority of GNB isolates are multi-drug resistant and pose a challenge to antibiotic therapy.
2) PCR technique is an accurate genotypic method used for detection of ESBL genes types.
3) The vast majority of GNB clinical isolates have TEM, SHV, CTX-M, and OXA β-lactamases which confirmed by PCR technique.
4) *bla* CTX-M was the most frequently β-lactamase gene detected in the isolates analysed.
5) Imipenem holds promise as an alternative choice of therapy for infections caused by ESBL-producing isolates and multi-drug resistant GNB.

Recommendations:

The following recommendations are put forward based on the study conducted:

1) There is a need of continuous surveillance of the prevalence and antibiotic susceptibility pattern of bacterial isolates in hospitals and in the community which should be the basis for empiric therapy.
2) Phenotypic and genotypic methods to identify the presence of an ESBL should be carried out in all hospitals laboratories routinely in Najaf.
3) Molecular methods, particularly PCR method, should be employed in future work. That can discriminate and detect all variants of ESBLs.
4) Promote patient education, which is also crucial in ensuring that the public understands and participates in efforts to control the spread of

antibiotic resistant bacteria, that be guidelines for improving antibiotic use in the community and within institutional health care settings.

5) Monitoring ESBL-producing bacteria in human and animal isolates to investigate their evolution and to analyse the factors that contribute to their selection and spread.

REFERENCES

References:

Afzal-Shah, M.; Woodford N. and Livermore D.M. (2001). Characterization of OXA-25, OXA-26, and OXA-27, molecular class D β-lactamases associated with carbapenem resistance in clinical isolates of *Acinetobacter baumannii*. Antimicrob Agents Chemother., **45**: 583-588.

Al-Asady, F.M.H. (2009). Bacteriological study on extended-spectrum beta-lactamases produced by *Enterobacteriaceae* isolated from children with bacteremia in Hilla city. MSc. Thesis. college of Medicine; Babylon University.

Al-Charrakh, A.H. (2005). Bcteriological and genetic study on extended-specturm β-lactamases and bacteriocins of *Klebsiella* isolated from Hilla city. Ph.D. Thesis. College of Science. Baghdad University.

Al-Jasser, A.M. (2006). Extended-spectrum beta-lactamases (ESBLs): a global problem. Kuwait Med J., **38** (3):171-185.

Al-Lawati, A.M.; Crouch, N.D.; Elhag, K.M. (2000). Antibiotic consumption and development of resistance among gram-negative bacilli in intensive care units in Oman. Annals Saudi Med., **20**: 3-4.

Al-Mohana, A.M. (2004). Prevalence and characterization of verotoxin producing *Escherichia coli* isolated from patients with diarrhea Baghdad and Najaf. Ph.D. Thesis. Al-Mustansiryia University.

Al-Zahrani, A.J. and Akhtar, N. (2005). Susceptibility patterns of extended-spectrum beta-lactamases (ESBL)-producing *Eschericha coli* and *Klebsiella pneumoniae* isolated in teaching hospital. Pakistan J Med Res., **44** (2): 64-67.

Ambler, R.P.; Coulson, A.F.; Frère, J.M.; Ghuysen, J.M.; Joris, B.; Forsman, M.; Levesque, R.C.; Tiraby G. and Waley S.G. (1991). A standard numbering scheme for the class A beta-lactamases. Biochem J., **276**: 269-270.

Amersfoort, E.S.V.; Berkel, T.J.C.V. and Kuiper, J. (2003). Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. Clin Microbiol Rev., **16**: 379-414.

Amin, R.; Hoque, A.M.W.; Khan, R.F.; and Rahman, M. (2009). Considering respiratory tract infections and antimicrobial sensitivity: an exploratory analysis; Malaysian J Microb., **5** (2): 1-4.

Amyes S.G. (2003). Resistance to beta-lactams-the permutations. J Chemother., **15** (6): 525-535.

Ananthan, S. and Subha, A. (2005). Cefoxitin resistance mediated by loss of porin in clinical strains of *Klebsiella pneumoniae* and *Eschericha coli*. Indian J Med Microbiol., **23**: 20-23.

Anupurba, Sh.; Bhattacharjee, A.; Garg, A.; Sen, M. R. (2006). Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolated from wound infections. Indian J Dermatol., **51** (4): 286-8.

Asrat, D. and Wolde Amanuel Y. (2001). Prevalence and antibiotic susceptibility pattern of bacterial isolates from blood culture in Tikur Anbassa hospital, Addis Ababa Ethiopia. Ethiop Med J., **39**: 97-104.

Babic, M.; Hujer, A.M. and Bonomo, R.A. (2006). What's new in antibiotic resistance? Focus on beta-lactamases. Drug Resistance Updates, **9**: 142-156.

Babypadmini, S. and Appalaraju, B. (2004). Extended-spectrum β -lactamases in urinary isolates of *Escherichia coli* and *Klebsiella pneumoniae* - prevalence and susceptibility pattern in a tertiary care hospital. Indian J Med Microbiol., **22** :172-4.

Bataineh, H.A. and Alrashed, K. M. (2007). Resistant Gram-negative bacilli and antibiotic consumption in Zarqa, Jordan. Pak J Med Sci., **23** (1): 59-63.

Bauer, A.W.; Kirby, W.M.M.; Sherris, J.C.; Jurek, M. (1966). Antibiotic susceptibility testing by a standardized single disc method. Am J Clin Pathol; **45**: 493-496.

Bedenic, B.; Beader, N.; Zagar, Z. (2001). Effect of inoculum size on the antibacterial activity of cefpirome and cefepime against *Klebsiella pneumoniae* strains producing SHV extended-spectrum beta-lactamases. Clin Microbiol Infect., **7**: 626-635.

Bergey's Manual for Systematic Bacteriology, (2001). Vol. 2. Ed. Krieg, N.R. and Holt, J.G.; Williams and Wilkins Publishers, Baltimore.

Bisson, G.; Fishman, N.O.; Patel, J.B. (2002). Extended-spectrum b-lactamase-producing *Escherichia coli* and *Klebsiella* species: Infect Control Hosp Epidemiol., **23**: 254–260.

Black, J.G. (2002). Antimicrobial therapy: the resistance of microorganisms In Microbiology principles and explorations. Ed Roesch B, Swain E. J Wiley & Sons, New York. 5 th edition: 342-343.

Blackmore, T. (2006). Prevalence of extended-spectrum β-lactamases among urinary *Escherichia coli* and *Klebsiella* in New Zealand in 2006; Communicable Disease Group, ESR. Porirua; New Zealand.

Bolon, M.K.; Wright, S.B.; Gold, H.S. and Carmeli, Y. (2004). The magnitude of the association between floroquinolones use and quinolone-resistant *Escherichia coli* and *Klebsiella pneumoniae* may be lower than previously reported. Antimicrob Agent Chemother., **48**:1934-1940.

Bonfiglio, G.; Russo, G.; Nicoletti, G. (2002). Recent developments in carbapenems. Exp Opin Invest Drugs, **11**: 529–544.

Bonnet, R. (2004). Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother., **48**: 1–14.

Bonomo, R.A. and Rice, L.R. (1999). Inhibitor resistant class A beta-lactamases, Front Biosci., **4**: 34-41.

Bowler, P.G.; Duerden, B.I.; Armstrong, D.G. (2001). Wound microbiology and associated approaches to wound management. Clin Microbiol Res., **14**: 244-269.

Bradford, P.A. (2001). "Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat." Clin Microbiol Rev., **14**: 933-951.

Bush, K.; Jacoby, G. and Medeiros A. (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother., **39**: 1211-1233.

Bush, K. (1996). Other β-lactams. Antibiotic and Chemotheraphy F. O´Grady, H. P. Lambert, R. G. Finch and D. Greenwood. New York, Churchill Livingstone: 306-327.

Bush, K. (2001). New beta-lactamases in Gram-negative bacteria: diversity and impact on the selection of antimicrobial therapy. Clin Infect Dis., **32:**1085-1089.

Buynak, J.D. (2006). Understanding the longevity of the beta-lactam antibiotics and of antibiotic/beta-lactamase inhibitor combinations. Biochem. Pharmacol., **71:** 930-940.

Casin, I.; Hanau-Berçot, B.; Podglajen, I.; Vahaboglu, H. and Collatz, E. (2003). *Salmonella enterica* serovar Typhimurium *bla*$_{PER}$-1-carrying plasmid pSTI1 encodes an extended-spectrum aminoglycoside 6'-*N*-acetyltransferase of type Ib. Antimicrob Agents Chemother., **47**: 697-703.

Chambers, H.F. (2001). Antibiotics in basic & Clinical Pharmacology, Katzung BG. Editor, 8th edition, New York, McGraw-Hill.

Chaudhary, U. and Aggarwal, R. (2004). Extended-spectrum β-lactamases (ESBL) An emerging threat to clinical therapeutics. Indian J Med Microb., **22** :75-80.

Chayakulkeeree, M.; Junsriwong, P.; Keerasuntonpong, A.; Tribuddharat, C. and Thamlikitku, V. (2005). Epidemiology of extended-spectrum beta-lactamase producing gram-negative bacilli at Siriraj hospital, Thailand, 2003. Southeast Asian J Trop Med Public Health; **36** (6): 1503-1509.

Chiangjong, W. (2006). Study of extended-spectrum beta-lactamase (ESBL)-producing *Klebsiella pneumoniae*: phenotypic and genotypic characteristics.

Clinical and LaboratoIy Standards Institute (CLSI), (2006). Performance Standards for Antimicrobial disk Susceptibility Tests. Approved Standard. 9th Edn, Clinical and LaboratoIy Standards Institute, Wayne, Pennsylvania.

Clinical and Laboratory Standards Institute (2007). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard. M7-A7, Wayne, Pa.

Clinical and Laboratory Standards Institute (2009). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard. M8-A8, Wayne, Pa.

Collee, J. G.; Fraser, A. G.; Marmiom, B. P.; and Simmon, A. (1996). Mackie and McCarteny Practical Medical Microbiology. 4th ed. Churchill Livingstone Inc., USA.

Correa, A.G. (2001). Update on cephalosporins in pediatrics. Infect Dis., **4**: 325-335.

Coudron, P.E.; Moland, E.S.; and Sanders, C.C. (1997). Occurrence and detection of extended-spectrum β-lactamases in members of the family Enterobacteriaceae at a Veterans Medical Center: Seek and you may find. J. Clin. Microbiol. **35**: 2593-2597.

Courvalin, P. (2006). Antibiotic resistance: the pros and cons of probiotics. Digestive and Liver Dis., **38** (2): 261-265.

Cowan, S.T. (1985). Cowan and Steels Manual for Identification of Medical Bacteria. 2nd ed., Cambridge Univ. Press, U.K.

d'Azevedo, P.A.; Goncalves, A.L.S.; Musskopf, M.I.; Ramos, C.G.; and Dias, C.A.G. (2004). Laboratory tests in the detection of extended spectrum beta-lactamase production: National committee for clinical laboratory standards

(NCCLS) screening test, the E-test, the double disk confirmatory test, and cefoxitin susceptibility testing. Braz Infect Dis., **8**: 372-377.

Dancer, S. J. (2004). What's under your bed?! ESBLs: Infection Control in the hospital environment; Health Protection Scotland, Supplement to Scieh Weekly Report, **38** (50): 6.

Datta, P.; Thakur, A.; Mishra, B. and Gupta, V. (2004). Prevalence of Clinical Strains Resistant to Various β-Lactams in a Tertiary Care Hospital in India, Jpn. J. Infect. Dis., **57**: 146-149.

Deplano, A.; Denis, O.; Poirel, L.; Hocquet, D.; Nonhoff, C.; Byl, B.; Nordmann, P.; Vincent, J.L.; Struelens, M.J. (2005). Molecular characterization of an epidemic clone of panantibiotic-resistant *Pseudomonas aeruginosa*. J Clin Microbiol., **43**: 1198-1204.

Diekema, D.J.; Boots-Miller, B.J.; Vaughn, T.E.; Woolson, R.F.; Yankey, J.W.; Ernst, E.J.; Flach, S.D.; Ward, M.M.; Franciscus, C.L.; Pfaller, M.A.; Doebbeling, B.N. (2004). Antimicrobial resistance trends and outbreak frequency in United States hospitals. Clin Infect Dis., **38** (1): 78-85.

Ding, J-G.; Sun, Q-F.; Li, K-Ch.; Zheng, M-H.; Miao, X-H.; Ni, W.; Hong, L.; Yang, J-X.; Ruan, Z-W.; Zhou, R-W.; Zhou, H-J. and He, W-F. (2009). Retrospective analysis of nosocomial infections in the intensive care unit of a tertiary hospital in China during 2003 and 2007; BMC Infect Dis., **9** (115): 1-6.

DiPersio, J.R.; Deshpande, L.M.; Biedenbach, D.J.; Toleman, M.A.; Walsh, T.R.; Jones, R.N. (2005). Evolution and dissemination of extended-spectrum β-lactamase producing *Klebsiella pneumoniae*: epidemiology and molecular report from the SENTRY antimicrobial surveillance program (1997-2003). Diagn Microbial Infect Dis., **51**:1-7.

Dobrindt, U.; Hochhut, B.; Hentschel, U. and Hacker, J. (2004). Genomic islands in pathogenic and environmental microorganisms. Nature Rev Microb., **2**: 414-424.

Docquier J.D.; Luzzaro, F.; Amicosante G. (2001). Multidrug-resistant *Pseudomonas aeruginosa* producing PER-1 extended-spectrum-serine-β-Lactamase and VIM-2 metallo-β-Lactamase. Emerg Infect Dis., **7**: 910-1.

Doucet, N. (2006). Mutagenèse semi-aléatoire et analyse dynamique de la β-lactamase TEM-1 de *Escherichia coli*. Ph.D. Thesis. Département de biochimie, Faculté de medicine, Université de Montréal.

Drieux, L.; Brossier, F.; Sougakoff, W. and Jarlier, V. (2008). Phenotypic detection of extended-spectrum β-lactamase production in Enterobacteriaceae: review and bench guide. Clin Microbiol Infect., **14**: 90-103.

Ekalaksananan, T.; Pientong, Ch.; Kongyingyoes, B.; Pairojkul, S.; Teeratakulpisarn, J. and Heng, S. (2001). Etiology of acute lower respiratory tract infection in children at Srinagarind hospital, Khon Kaen, Thailand. Southeast Asian J Trop Med Public Health, **32** (3)

El-Enbaawy, M.I. and Yousif, A.A. (2006). Beta-lactamase gene in multi-drug-resistant clinical bacterial isolates from Egyptian food animal species. Arab J. Biotech., **9** (1): 71-82.

Erlandsson, M. (2007). Surveillance of antibiotic consumption and antibiotic resistance in Swedish intensive care units. Linköping University Medical Dissertations No. 1019 Sweden.

Escudero E.; Vinuē, L.; Teshager, T.; Torres, C. and Moreno, M.A. (2009). Resistance mechanism and farm-level distribution of fecal *Escherichia coli* isolates Resistant to extended-spectrum cephalosporins in pigs in Spain. Res Vet Sci., **88** (1): 83-87.

Espy, M.J. (2006). Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev; **19**:165.

Essack, S.Y. (2001). The Development of β-Lactam Antibiotics in Response to the Evolution of β-Lactamases. Pharm Res., **18**: 1391-1399.

Fajardo, A.; Martinez-Martin, N.; Mercadillo, M.; Galan, J.C.; Ghysels, B.; Matthijs, S.; Cornelis, P.; Wiehlmann, L.; Tummler, B.; Baquero, F. and Martinez, J.L. (2008). The neglected intrinsic resistome of bacterial pathogens. PLoS ONE, **3:** e1619.

Fam, N.S. and El-Damarawy, M.M. (2008). CTX-M-15 extended-spectrum beta-lactamases detected from intensive care unit of an Egyptian medical research institute, Res J Med and Medical Sciences, **3** (1): 84-91.

Färber, J.; Moder, K.A.; Layer, F.; Tammer, I.; König, W.; König, B. (2008). Extended-spectrum Beta-lactamase detection with different panels for automated susceptibility testing and with a chromogenic medium. J Clin Microbiol., **46** (11): 3721-3727.

Ferguson, D. (2007). A study of clinical strains of *Pseudomonas aeruginosa* and the investigation of antibiotic resistance mechanisms in the multidrug resistant strain PA13 Ph.D. Thesis. School of Biotechnology and National Institute for Cellular Biotechnology, Dublin City University.

Flaih, R.A. (2005). The clinical important of bacterial beta-lactamase. MSc. thesis. College of Science. Babylon University.

Florijn, A.; Nijssen, S.; Smitz, F.; Verhoef, J.; Fluit, A. (2002). Comparison of E-tests and double disk diffusion tests for the detection of extended spectrum beta-lactamases (ESBLs) Europ J Clin Microb Infect Dis., **21**: 241-243

Fonseca, E.L.; Vieira, V.V.; Cipriano, R.; Vicento, A.C. (2005). Class1 integrons in *Pseudomonas aeruginosa* isolates from clinical settings in Amazon region, Brazil. FEMS Immunol Med Microbiol., **44**: 303-309.

Forssten, S. (2009). Genetic Basis and diagnostics of extended-spectrum β-lactamases among *Enterobacteriaceae* in Finland. Thesis; Annales Universitatis Turkuensis, University of Turku, Finland.

Franiczek, R.; Dolna, I.; Krzyżanowska, B.; Szufnarowski, K.; Kowalska-Krochmal, B. (2007). Conjugative Transfer of Multiresistance Plasmids from ESBL-positive *Escherichia coli* and *Klebsiella* spp. Clinical Isolates to *Escherichia coli* Strain K12 C600; Adv Clin Exp Med., **16** (2): 239–247.

French, G.; and Ransjö, U. (2006). Prevention of lower respiratory tract infection. IFIC basic concepts of infection control.

Gad, F.G.; El-Domany, A.R. and Ashour, M.H. (2008). Antimicrobial susceptibility profile of *Pseudomonas aeruginosa* isolates in Egypt, J Urol., **180** (1): 176-181.

Galimand, M.; Sabtcheva, S.; Courvalin, P. and Lambert, T. (2005). Worldwide disseminated *arm*A aminoglycoside resistance methylase gene is borne by composite transposon Tn 1548. Antimicrob Agents Chemother., **49:** 2949-2953.

Gals, A.C.; Jones, R.N.; Forward, K.R.; Linares, J.; Sader, H.S.; and Verhoef, J. (2001). Emerging importance of multidrug-resistant *Acinetobacter* species and *Stenotrophomonas maltophilia* as pathogens in seriously ill patients: Geographic patterns, epidemiological features, and trends in the SENTRY antimicrobial surveillance program (1997-1999). Clin Infect Dis., **32**: 104-113.

Gangoue-Pieboji, J.; Koulla-Shiro, S.; Ngassam, P. (2006). Antimicrobial activity against Gram-negative bacilli from Yaounde central hospital, Cameroon, Afr Health Sci., **6**: 232-235.

Garau, G.; Bebrone, C.; Anne, C.; Galleni, M.; Frere, J.-M. and Dideberg, O. (2005). A Metallo-β-lactamase Enzyme in Action: Crystal Structures of the

Monozinc Carbapenemase CphA and its Complex with Biapenem. J Mol Biol., **345**: 785-795.

Girlich, D.; Naas, T.; Leelaporn, A.; Poirel, L.; Fennewald, M.; Nordmann, P. (2002). Nosocomial spread of the integron located VEB-1-like cassette encoding an extended-spectrum beta-lactamases in *Pseudomonas aeruginosa* in Thailand. Clin Infect Dis., **34**: 603-11

Giuliani, F.; Docquier, J.D.; Riccio, M.L.; Pagani, L. and Rossolini, G.M. (2005). OXA-46, a new class D β-lactamase of narrow substrate specificity encoded by a *bla*VIM-1-containing integron from a *Pseudomonas aeruginosa* clinical isolate. Antimicrobial Agents Chemother., **49**: 1973-1980.

Goyal, A.; Prasad K. N.; Prasad, A,; Gupta, S.; Ghoshal, U.; Ayyagari, A. (2009). Extended-spectrum beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* & associated risk factors. Indian J. Med. Res., **129** (6): 695-700.

Grohmann, E.; Muth, G. and Espinosa, M. (2003). Conjugative plasmid transfer in Gram-positive bacteria. Microb and Molecular Biology Reviews., **67** (2): 277-301.

Hadi, Z.J. (2008). Detection of extended-spectrum beta-lactamases of *Escherichia coli* and *Klebsiella* spp. isolated from patients with bacteriuria in Najaf. MSc. thesis. College of Medicine. Kufa University.

Hall, B.G. and Barlow, M. (2004). Evolution of the serine β-lactamases: past, present and future. Drug Resistance Update, **7**: 111-23.

Hall, B.G. and Barlow, M. (2005). Revised Ambler classification of β-lactamases. J Antimicrob Chemother., **55**: 1050-1051.

Hanson, N.D. (2003). AmpC b-lactamases: what do we need to know for the future?; J Antimicrob Chemother., **52**: 2-4.

Harbottle, H.; Thakur, S.; Zhao, S.; White, D.G. **(**2006). Genetics of Antimicrobial Resistance. Animal Biotechnol., **17**: 111-124.

Hassanien, H.S.M. (2004). Sensitivity and resistance patterns of some fluoroquinolones. M.V.Sc. (Microbiology), Fac. Vet. Med. Cairo University, Giza, Egypt.

Hemalatha, V.; Padma, M.; Sekar, U.; Vinodh, T. M. and Arunkumar, A.S. (2007). Detection of Amp C beta lactamases production in *Escherichia coli* & *Klebsiella* by an inhibitor based method. Indian J Med Res., **126**: 220-223.

Holt, J.G.; Krieg, N.R.; Sneath, H.A.; Stanley, J.T.; and Williams, S.T. (1994). Bergey`s manual of determinative bacteriology. 9th ed., Baltimore; Wiliams and Wilkins, USA.

Huang, ZM.; Mao, PH.; Chen, Y.; Wu, L.; Wu, J. (2004). Study on molecular epidemiology of SHV type beta-lactamaseencoding genes of multiple-drug - resistant *Acinetobacter baumannii*. Zhonghua Liu Xing Bing Xue Za Zhi., **25**: 425-427.

Isibor, J. O.; Oseni, A.; Eyaufe, A.; Osagie, R. and Turay, A. (2008). Incidence of aerobic bacteria and *Candida albicans* in post-operative wound infections African Journal of Microbiology Research., **2**: 288-291.

Istinič, I. (2008). Plasmid mediated quinolone and extended-spectrum beta-lactam resistance of selected *Escherichia coli* isolates. Graduation thesis. Biotechnique college. Ljubljana University.

Jacoby GA. and Munoz-Price LS. (2005). The New β-Lactamases; The New England J of Med., **352**: 380-91.

Jacoby, G.A. and Bush, K. (2009). "Amino Acid Sequences for TEM, SHV and OXA extended-spectrum and inhibitor resistant ß-lactamases.", from http://www.lahey.org/Studies/.

Jain, A.; Roy, I.; Gupta, M. K.; Kumar, M.; Agarwal, S. K. (2003). Prevalence of Extended spectrum-β-lactamase producing gram negative bacteria in septicaemic neonates in a tertiary care hospital. J Med Microbiol., **52** :421-5.

Jamal W.Y.; Al Hashem G.; Khodakhast F.; Rotimi V.O. (2009). Comparative in vitro activity of tigecycline and nine other antibiotics against gram-negative bacterial isolates, including ESBL-producing strains. J Chemother., **21**(3):261-266.

Jarlier, V.; Nicolas, M. H.; Fournier, G.; and Philippon, A. (1988). Extended spectrum beta-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev Infect Dis., **10**: 867-878.

Javiya V.A.; Ghatak, S.B.; Patel K.R.; Patel J.A. (2008). Antibiotic susceptibility patterns of *Pseudomonas aeruginosa* at a tertiary care hospital in Gujarat, India, Indian J Pharmacol., **40** (5): 230-234

Jayapradha, R.; Murugesh, S.; Mahesh, N.; and Brahatheeswaran, D. (2007). Prevalence of ESBL Producing Strains in Tuberculosis Patients. Res J Microb., **2** (5): 491-495.

Jeannot, K.; Sobel, M. L.; El Garch, F.; Poole, K. and Plesiat, P. **(**2005). Induction of the MexXY efflux pump in *Pseudomonas aeruginosa* is dependent on drug-ribosome interaction. J Bacteriol., **187**:5341-5346.

Jemima, S.A. and Verghese, S. (2008). Multiplex PCR for *bla* $_{\text{CTX-M}}$ and *bla* $_{\text{SHV}}$ in the extended-spectrum beta-lactamase (ESBL) producing Gram-negative isolates. Indian J Med Res., **128** (3): 313-317.

Jeong J-Y.; Yoon, H.J.; Kim, E.S; Lee, Y.; Choi, S-H.; Kim, N.J.; Woo, J.H. and Kim, Y.S. (2005). Detection of *qnr* in Clinical Isolates of *Escherichia coli* from Korea. Antimicrob Agents Chemother., **49**: 2522-2524.

Jones, R.N.; Ross, J.E.; Rhomberg, P.R. and Quality control working group. (2009). MIC quality control guidelines and disc diffusion test optimization for CEM-101, a novel fiuoroketolide. J Clin Microbiol., 10: 92-109.

Julio, R.A.; Thilo, K.; Hiroshi, N. and Patrick, P. (1999). Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob agents chemother., **43** (11): 2624-2628.

Kader, AA.; Kumar, A. (2005). Prevalence and antimicrobial susceptibility of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in a general hospital. Ann Saudi Med; **25**: 239-242.

Kader A.A.; Kumar, A.; Krishna, A.; Zaman, M.N. (2006). An accelerated method for the detection of extended-spectrum β-lactamases in urinary isolates of *Escherichia coli* and *Klebsiella pneumoniae*. Saudi J Kidney Dis Transplantation, **17** (4): 535-539.

Kahlmeter, G. (2008). Breakpoints for intravenously used cephalosporins in *Enterobacteriaceae* -EUCAST and CLSI breakpoints. Clin Microbiol Infect., **14**: 169-174.

Kang, C.I.; Kim, S.H.; Kim, D.M.; Park, W.B.; Lee, K.D.; Kim, H.B.; Oh, M.D.; Kim, E.C. and Choe, K.W. (2004). "Risk factors for ciprofloxacin resistance in bloodstream infections due to extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*." Microb Drug Res., **10**: 71-6.

Kang, H. Y.; Jeong, Y. S.; Oh, J. Y. (2005). Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea. J Antimicrob Chemother., **55** (5): 639-644.

Kaur, H.; Bhat, J.; Anvikar, A. R.; Rao, S.; Gadge, V. (2006). Bacterial profile of blood and burn wound infections in burn patients. Proceeding of national symposium on Tribal Health, 89-95.

Khushal, R. (2004). Prevalence, Characterization and development of Resistance pattern in indigenous clinical isolates against cephalosporins Ph.D. Thesis, Microbiology. Quaid-I-Azam University.

Koletar, S.L. (2000). Antimicrobial mechainism of action. In: Mahon, CR. Manuselis, G. Editor. Textbook of diagnosis microbiology. 2nd ed. Pennsylvania; W.B. Saunder, 54-56.

Komatsu, M.; Aihara, M.; Shimakawa, K.; Iwasaki, M.; Nagasaka, Y.; Fukuda, S. (2000). Evaluation of MicroScan ESBL confirmation panel for *Enterobacteriaceae*-producing, extended-spectrum beta-lactamases isolated in Japan. Diagn Microbiol Infect Dis., **46** : 125-30.

Kumari, S.; Tamura, K. Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics **5**: 150-163.

Labombardi, V.J. (2007). The emergence of the KPC carbapenemases. Clinical and laboratory issues, **18** (2): 29-34.

Li, X. Z. and Nikaido, H. (2004). Efflux-mediated drug resistance in bacteria. Drugs, **64:**159-204.

Licht, T.R. & Wilcks, A. (2006). Conjugative gene transfer in the gastrointestinal environment. Adv Appl Microbiol., **58**: 77-95.

Lim, K.T.; Yasin, R.; Yeo, Ch.; Puthucheary, S.; Thong, K.L. (2009). Characterization of multidrug resistant ESBL-producing *Escherichia coli* isolates from hospitals in Malaysia. J Biomed Biotechnol., Article ID 165637.

Livermore, D.M. and Williams, J.D. (1996). β-Lactams: mode of action and mechanisms of bacterial resistance antibiotics in laboratory medicine. V. Lorian. New York, Williams & Wilkins, Baltimore**,** USA.

Livermore, D.M. and Brown, D.F.J. (2001). Detection of β-lactamase-mediated resistance. Antimicrob Chemother., **48**: 59-64.

Livermore, D.M.; Winstanley, T.B.; Shannon, K.P. (2001). Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. J Antimicrob Chemother., **48** (1): 87-102

Livermore, D.M. and Brown, D.F.J. (2005). "Detection of β-lactamase-mediated resistance." Retrieved 31. 3 2007, 2007.

Livermore, D.M.; Hope, R.; Fagan, E.J.; Warner, M.; Woodford, N. and Potz, N. (2006). Activity of temocillin against prevalent ESBL- and AmpC producing *Enterobacteriaceae* from south-east England. J Antimicrob Chemother., **57**: 1012-1014.

Livermore, D.M.; Canton, R.; Gniadkowski, M.; Nordmann, P.; Rossolini, G. M.; Arlet, G.; Ayala, J.; Coque, T.M.; Kern-Zdanowicz, I.; Luzzaro, F.; Poirel, L. and Woodford, N. (2007). CTX-M: changing the face of ESBLs in Europe. J Antimicrob Chemother., **59**: 165–174.

Livermore, D.M. (2008). Defining an extended-spectrum β-lactamase. Clin Microbiol Infect., **14**: 3-10.

Livermore, D.M.; Hope, R.; Brick, G.; Lillie, M.; Reynolds, R. and B. W. P. o. R. S. on behalf of the UK and Ireland. (2008). "Non-susceptibility trends among *Enterobacteriaceae* from bacteraemias in the UK and Ireland, 2001-06." J Antimicrob Chemother., **62**: 1141-54.

Luzzaro, F.; Mantengoli, E. M.; Perilli, G.; Lombardi, V.; Orlandi, A.; Orsatti, G.; Amicosante, G.; Rossolini, M. and Toniolo, A. (2001). Dynamics of a nosocomial outbreak of multidrug-resistant *Pseudomonas aeruginosa* producing the PER-1 extended-spectrum β-lactamase. J. Clin. Microbiol., **39:**1865-1870.

Luzzaro, F.; Mezzatesta, M.; Mugnaioli, C.; Perilli, M.; Stefani, S.; Amicosante, G. (2006). Trends in production of extended-spectrum β-lactamase among *Enterobacteriaeceae* of medical interest report of the second Italian Nationwide survey. J Clin Microbiol., **4** (5): 1659-64.

MacFaddin, J.F. (2000). Biochemical tests for identification of medical bacteria (3rd ed.), Lippincott Williams and Wilkins, Philadelphia, USA.

Macheboeuf, P.; Contreras-Martel, C.; Job, V.; Dideberg, O.; Dessen, A. (2006). Penicillin binding proteins: key players in bacterial cell cycle and drug resistance processes. FEMS Microbiol Rev., **30** (5): 673-91.

Madigan, M. T. and Martinko, J. M. (2006). Biology of the Microorganims. Pearson Prentice Hall, Upper Saddle River

Majiduddin, F.K.; Materon, I.C. and Palzkill, T.G. (2002). Molecular analysis of beta-lactamase structure and function. Int J Med Microbiol., **292**: 127-137.

Manchanda, V. and Singh, N. P. (2003). Occurrence and detection of AmpC β-lactamases among Gram-negative clinical isolates using a modified three-dimensional test at Guru Tegh Bahadur Hospital, Delhi, India. J Antimicrob Chemother. **51**: 415-418.

Mansouri, M. and Ramazanzadeh, R. (2009). Spread of extended-spectrum beta-lactamase producing *Escherichia coli* clinical isolates in Sanandaj hospitals, J Biological Sci., **9** (4): 362-366.

Marchandin, H.; Jean-Pierre, H.; De Champs, C.; Sirot, D.; Darbas, H.; Perigault, P.F.; Carriere, C. (2000). Production of a TEM-24 plasmid-mediated extended-spectrum β-lactamases by a Clinical Isolate of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother., **44**: 213-216.

Marin, M. and Gudiol, F. (2003). Antibiotics beta-lactamase. Enferm. Infect Microbiol Clin. **21**: 42-55.

Martinez, J.L.; Fajardo, A.; Garmendia, L.; Hernandez, A.; Linares, J.F.; Martinez-Solano, L. and Sanchez, M.B. (2009). A global view of antibiotic resistance. FEMS Microbiol Rev., **33:** 44-65.

Masaadeh, H.A. and Jaran, A.S. (2009). Incident of *Pseudomonas aeruginosa* in post-operative. American J Infect Dis., **5** (1): 1-6

Masterton, R. (2003). A quick guide to antibiotic resistance-antibiotic resistance in noscomial infections. Available online at http://www.infectionacademy.org (18th August 2005).

Mathur, P.; Tatman, A.; Das, B.; Dhawan, B. (2002). Prevalence of extended beta lactamase producing gram negative bacteria in a tertiary care hospital. Indian J Med Res., **115** :153-7.

Mavroidi, A.; Tsakris, A.; Tzelepi, E.; Pournaras, S.; Loukova, V.; Tzouvelekis, L.S. (2000). Carbapenem-hydrolysing VIM-2 metallo-beta-lactamase in *Pseudomonas aeruginosa* from Greece. J Antimicrob Chemother., **46**(6):1041-1042.

Mazel, D. (2004). Integrons and the origin of antibiotic resistance gene cassettes. ASM News., **70**: 520-525.

McDermott, P.F.; Walker, R.D.; White, D.G. (2003). Antimicrobials: modes of action and mechanisms of resistance. International J of Toxicology, **22**: 135-143.

Mendelson, G.; Hait, V.; Ben-Israel, J.; Gronich, D.; Granot, E.; Raz, R. (2005). Prevalence and risk factors of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in an long-term care facility. Eur J Clin Microbiol Infect Dis., **24**: 17-22.

Menon, Th.; Shanmugasundaram, S.; Kumar, M.P.; Kumar, C.P. (2004). Group A Streptococcal infections of the pharynx in a rural population in south India. Indian J Med Res., **119**: 171-173.

Messai, Y.; Benhassine, T.; Naim, M.; Paul G. and Bakour R. (2006). Prevalence of β-lactams resistance among *Escherichia coli* clinical isolates from a hospital in Algiers Rev Esp Quimioterap, **19** (2): 144-151

Meyer, M.M. (2006). Exploring Protein Sequence Space Using Computationally Directed Recombination. Ph.D. thesis; California Institute of Technology; Pasadena, California, USA.

Miró, E.; Rivera, A. and Mesa, R. (2004). Els β-lactamics, p. 91-106. In J. Ruiz edition, Antimicrobians. Societat Catalana de Biologia, Barcelona.

Mullany S. (2002). Cellular and molecular life sciences Birkhäuser Basel, Spain.

Murray, P.R.; Rosenthal, K.S.; Kobayashi, G.S.; Pfaller, M.A. (2002). Medical Microbiology, fourth edition, Mosby, USA.

National Committee for Clinical Laboratory Standards. **(**2002). Performance standards for antimicrobial susceptibility testing. Twelfth informational supplement. NCCLS document M100-S12. National Committee for Laboratory Standards, Wayne, Pa.

National Committee for Clinical Laboratory Standards (NCCLS). (2003a) Performance standards for disk susceptibility testing; approved standard M 100-S13. NCCLS, Wayne, Pa.

National Committee for Clinical Laboratory Standards. (2003b). Performance standards for antimicrobial susceptibility tests, 8th ed. Approved standard M 2-A8. NCCLS, Wayne, Pa.

Navaneeth, B.V.; Sridaran, D.; Sahay, D.; Belwadi, M.R.S. (2002). A preliminary study on metallo β-lactamase producing *Pseudomonas aeruginosa* in hospitalized patients. Indian J Med Res., **116**: 264-267.

Navon-Venezia, S.; Hammer-Munz, O.; Schwartz, D.; Turner, D.; Kuzmenko, B. and Carmeli Y. (2003). Occurrence and phenotypic characteristics of extended-spectrum β-lactamases among members of the family *Enterobacteriaceae* at the Tel-Aviv Medical Center and evaluation of diagnostic tests. J Clin Microbiol., **41**: 155-158.

Navon-Venezia, S.; Leavitt, A. ; Ben-Ami, R. ; Aharoni, Y. ; Schwaber, M.J.; Schwartz, D. (2005). Evaluation of an accelerated protocol for detection of extended-spectrum β-lactamase-producing Gram-negative bacilli from positive blood cultures. J Clin Microbiol., **43**(1): 439-441.

Noel, G.J. (2007). Clinical profile of ceftobiprole, a novel beta-lactam antibiotic. Clin Microbiol Infect., **13** (2): 25-29.

Nordmann, P. and Poirel, L. (2002). Emerging carbapenemases in Gram-negative aerobes. Clin Microbiol Infect., **8**: 321–331.

Normark, H.B. and Normark, S. (2002). Evolution and spread of antibiotic resistance. J Intern Med., **252**: 91-106.

Obritsch, M.D.; Fish, D.N.; MacLaren, R. and Jung, R. (2005). Nosocomial infections due to multi-drug-resistant *Pseudomonas aeruginosa*: epidemiology and treatment options. Pharmacother., **25**: 1353-1364.

Okesola, A.O. and Ige, O.M. (2008). Trends in Bacterial Pathogens of Lower Respiratory Tract Infections. Indian J Chest Dis Allied Sci; **50**: 269-272.

Oliver, A.; Weigel, L.M.; Rasheed, J.K.; McGowan, J.E.; Raney, P. and Tenover, F.C. (2002). Mechanisms of decreased susceptibility to cefpodoxime in *Escherichia coli*. Antimicrob Agent Chemother., **46**: 3829-3836.

Ozyilmaz, E.; Akan, O.A.; Gulhan, M.; Ahmed, K.; Nagatake, T. (2005). Major bacteria of community-acquired respiratory tract infections in Turkey. Jpn J Infect. Dis., **58**: 50-52.

Packham, D.R. (2007). Pneumonia with Bacteraemia Due to *Escherichia coli* T. C. Sorrell Internal Med J., **11** (5): 669 – 672.

Page, M.G. and Heim J. (2009). Prospects for the next anti-*Pseudomonas* drug. Curr Opin Pharmacol., PMID

Patel, J.; Bhatt, J.; Javiya, V.; & Patel, K. (2009). Anti-microbial susceptibility patterns of *Enterobacteriaceae* isolated from A tertiary care unit in Gujarat. The Internet J Microb., **6** (1)

Paterson, D.L.; Ko, W.C.; Von Gottberg, A.; Casellas, J.M.; Mulazimoglu, L.; Klugman, K.P.; Bonomo, R. A.; Rice, L.B.; Mccormack, J.G. and Yu, V.L. (2001). Outcome of cephalosporin treatment for serious infections due to

apparently susceptible organisms producing extended-spectrum β-lactamases: implications for the clinical microbiology laboratory. J Clin Microbiol., **39:** 2206–2212.

Paterson, D.L.; Hujer, K.M.; Hujer, A.M.; Yeiser, B.; Bonomo, M.D.; Rice, L.B.; Bonomo, R.A. and the International *Klebsiella* Study Group. (2003). Extended-spectrum ß-lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV- and CTX-M-type ß-lactamases. Antimicrob Agents Chemother., **47**: 3554-3560.

Paterson, D.L.; Ko, W.C.; VonGottberg, A. (2004). Antibiotic therapy for *Klebsiella pneumoniae* bacteremia: implications of production of extended-spectrum beta-lactamases. Clin Infect Dis., **39**:31-37.

Paterson, D.L.; Rossi, F.; Baquero, F.; Hsueh, P.R.; Woods, G.L.; Satishchandran, V.; Snyder, T.A.; Harvey, C.M.; Teppler, H.; Dinubile, M.J.; Chow, J.W. (2005). In vitro susceptibilities of aerobic and facultative Gram-negative bacilli isolated from patients with intra-abdominal infections worldwide: the 2003 Study for Monitoring Antimicrobial Resistance Trends (SMART). J Antimicrob Chemother., **55**(6):965-73.

Paterson, D.L. (2006). Resistance in Gram-Negative Bacteria: *Enterobacteriaceae*. American J Med., **119**: S20-S28.

Patrick, D.J.; Bochum, T.M.; Sacha, V.M.; Mook-Vermust, V.; Handgraaf, C.; Klaaen, T.; Melchers, W.J.G. (2009). Prevalence, molecular characterization, and phenotypic confirmation of extended-spectrum beta-lactamases in *Escherichia coli, Klebsiella pneumoniae,* and *Klebsiella oxytoca* at the radboud University Nijmegen Medical centre in the Natherlands, Microbial Drug Res., **16** (1): 55-60.

Pearson, J.; Turnidge, J.; Franklin, C.; Bell, J. and the Australian Group on Antimicrobial Resistance. (2007). Prevalence of antimicrobial resistances in

common pathogenic *Enterobacteriaceae* in Australia, 2004: report from the Australian group on Antimicrob Res., CDI, **31** (1): 106-112.

Perez, F.; Endimiani, A.; Hujer, K.M.; Bonomo, R.A. (2007). The continuing chalenge of ESBLs. Current Opinion in Pharmacology. **7** (5): 459-469.

Pérez-Llarena, F.J. and Bou, G. (2009). Beta-lactamase inhibitors: The story so far. Curr Med Chem s/n, 15006.

Pfaller, M.A. and Segreti, J. (2006). Overview of the epidemiological profile and laboratory detection of extended-spectrum β-lactamases. Clin Infect Dis., **42**: S153-S163.

Philippon, A.; Arlet, G. and Jacoby, G.A. (2002). Plasmid-determined AmpC-type β-lactamases. Antimicrob Agents Chemother., **46**: 1-11.

Piddock, L.J. (2006). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev., **19**: 382–402.

Pitout, J.D.D.; Reisbig, M.D.; Venter, E.C.; Church, D.L.; Hanson, N.D. (2003). Modification of the double-disk test for detection of *Enterobacteriaceae* producing extended-spectrum and AmpC β-lactamases. J Clin Microbiol., **41** (8): 3933-5.

Poirel, L.; Weldhagen, G.F.; Naas, T.; De Champs, C.; Dove, M.G.; Nordmann, P. (2001). GES-2, a class A beta-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. Antimicrob Agents Chemother., **45** (9): 2598-2603.

Poirel, L.; Weldhagen, G.F.; De Champs, C.; Nordmann, P.A. (2002). nosocomial outbreak of *Pseudomonas aeruginosa* isolates expressing the extended-spectrum beta-lactamase GES-2 in South Africa. J Antimicrob Chemother., **49** : 561-5.

Pongpech, P.; Naenna, P.; Taipobsakul, Y.; Tribuddharat, Ch. and Srifuengfung S. (2008). Prevalence of Extended-spectrum beta-lactamase and class 1 Integron integrase gene *INTI1* in *Escherichia coli* from thai patients and

healthy adults southeast asian. J TROP MED Public Health., **39** (3): 425-433.

Poole, K. and Srikumar, R. (2001). Multidrug efflux systems in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. Curr Top Med Chem., **1**: 59-71.

Poole, K. (2004). Resistance to β-lactam antibiotics. Cell Mol Life Sci., **61**:2200-2223.

Pospiech, T.; and Neumann, J. (1995). In Genomic DNA isolation T. Kieser eds. John Innes Center. Norwich NR4 7UH.U.K.

Potz, N.A.C.; Hope, R.; Warner, M.; Johnson, A.P.; Livermore, D.M.; L. on behalf of the and E.P.G. South East. (2006). Prevalence and mechanisms of cephalosporin resistance in Enterobacteriaceae in London and South-East England. J Antimicrob Chemother., **58**: 320-326.

Pratt, R.F. (2002). Functional evolution of the serine β-lactamase active site. J Chem Soc Perkin Trans., **2**: 851-861.

Putman, M.; van Veen, H.W. and Konings, W.N. (2000). Molecular properties of bacterial multi-drug transporters. Microbiol Mol Biol Rev., **64:**672-693.

Queenan, A.M.; Shang, W.; Kania, M.; Page, M.G.; Bush, K. (2007). Interactions of ceftobiprole with beta-lactamases from molecular classes A to D. Antimicrob Agents Chemother., **51:** 3098-3095.

Rashid, A.; Chowdhury, A.; Rahman, S.; Begum, Sh.; Muazzam, N. (2007). Infections by *Pseudomonas aeruginosa* and antibiotic resistance pattern of the isolates from Dhaka medical college hospital, Bangladesh J Med Microbiol., **01** (02): 48-51.

Ratna, A.K.; Menon, I.; Kapur, I. and Kulkarni, R. (2003). Occurrence & detection of AmpC ß-lactamases at a referral hospital in Karnataka. Indian J Med Res., **118:** 29-32.

Reshedko, G.; Ryabkova, E.; Strachunskaya, E. and RosNet Group. (2004). Antimicrobial resistance in Gram-negative pathogens isolated from patients with nosocomial lower respiratory tract infections in intensive care units, Institute of Antimicrob Chemother, Russia., **98**.

Reslinski, A.; Gospodarek, E.; Mikucka, A. (2005). Prevalence of multidrug-resistant *Proteus* spp. strains in clinical specimens and their susceptibility to antibiotics, Med Dosw Mikrobiol., **57** (2): 175-184.

Roderic, M.; Dale, K.; Schnell, G. and Wong, J.P. (2004). Therapeutic efficacy of "Nubiotics" against burn wound infection by *Pseudomonas aeruginosa*; Antimicrob Agents Chemother., **48** (8): 2918–2923.

Rossi F.; Baquero, F.; Hsueh, P.R.; Paterson, D.L.; Bochicchio, G.V.; Snyder, T.A.; Satishchandran, V.; McCarroll, K.; DiNubile, M.J.; Chow, J.W. (2006). In vitro susceptibilities of aerobic and facultatively anaerobic Gram-negative bacilli isolated from patients with intra-abdominal infections worldwide: 2004 results from SMART (Study for Monitoring Antimicrobial Resistance Trends). J Antimicrob Chemother., **58** (1): 205-210.

Ryoo, NH.; Kim, EC, Hong, SG. (2005). Dissemination of SHV-12 and CTX-M-type extended-spectrum beta-lactamases among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* and emergence of GES-3 in Korea. J Antimicrob Chemother., **56**: 698-702.

Samaha-Kfoury, J.N.; Araj, G.F. (2003). Recent developments in beta-lactamases and extended-spectrum beta-lactamases. BMJ., **327**: 1209-1213.

Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning, 2nd ed. CSH Laboratory Press, New York.

Saurina, G.; Quale, J.M.; Manikal, V.M.; Oydna, E.; Landman, D. (2000). Antimicrobial resistance in *Enterobacteriaceae* in Brooklyn, NY: epidemiology and relation to antibiotic usage patterns. J Antimicrob Chemother., **45**:895-898.

Savas, L.; Duran, N.; Savas, N.; Nlen, Y.; Ocak, S. (2005). The prevalence and resistance patterns of *Pseudomonas aeruginosa* in Intensive Care Units in a University Hospital. Turk J Med Sci., **35**: 317-322.

Schwarz, S. and Nobel, W.C. (1999). Aspects of bacterial resistance to antimicrobials used in veterinary dermatological practice. Veterinary Dermatology, **10**: 163-176.

Shah, A.A.; Hasan, F.; Ahmed, S.; Hameed, A. (2004). Characteristics, epidemiology and clinical importance of emerging strains of gram-negative bacilli producing extended-spectrum beta-lactamases. Res Microbiol., **155**: 409-421.

Shahcheraghi, F. Nikbin, VS. Feizabadi, MM. (2009). Prevalence of ESBLs genes among multidrug-resistant isolates of *Pseudomonas aeruginosa* isolated from patients in Tehran. Microb Drug Resist., **15** (1): 37-39.

Sheng, W.H.; Chen, Y.C.; Wamg, J.T.; Chang, S.C.; Luh, K.T.; and Hsieh, W.C. (2002). Emerging floroquinolone-resistance for commonly clinically important gram-negative bacteria in Taiwan. Diagn Microbiol Infect Dis., **43**:141-147.

Shigemura, K.; Arakawa, S.; Miura, T.; Nakano, Y.; Tanaka, K. and Fujisawa, M. (2008). Significance of floroquinolone-resistant *E. coli* in urinary tract infections. Jpn J Infect Dis., **61**: 226-228.

Siu, L.K.; Lu, P.L.; Chen, J.-Y.; Lin, F. M. and Chang, S.-C. (2003). High level expression of AmpC β-lactamase due to insertion of nucleotides between -10 and -35 promoter sequences in *Escherichia coli* clinical isolates: cases not responsive to extended-spectrum cephalosporin treatment. Antimicrob Agents Chemother., **47**: 2138–2144.

Spencer, R.C.; Wheel, P.F.; Winstanley T.G.; Cox D.M.; Plested, S.J. (1987). Novel β-lactamase in clinical isolate of *Klebsiella pneumoniae* conferring

unusual resistance to β-lactam antibiotics. J Antimicrob chemother., **20**: 919-21.

Srifuengfung, S.; Tribuddharat, Ch.; Yungyuen, Th. and Wensentia Th. (2005). Respiratory tract infection caused by bacteria (non-*Mycobacterium*) and their antibiogram in HIV- positive patients. Southeast Asian J Trop Med Public Health., **36** (3).

Steinke, D. and Davey, P. (2001). Association between antibiotic resistance and community prescribing: a critical review of bias and confounding in published studies. Clin Infect Dis., **33** (3): 193-205.

Stover, C.K.; Pham, X.Q.; Erwin, A.L.; Mizoguchi, S.D.; Warrener, P.; Hickey, M.J.; Brinkman, F.S.L.; Hufnagle, W.O.; Kowalik, D.J.; Lagrou, M.; Garber, R.L.; Goltry, L.; Tolentino, E.; Westbrock-Wadman, S.; Yuan, Y.; Brody, L.L.; Coulter, S.N.; Folger, K.R.; Kas, A.; Larbig, K.; Lim, R.; Smith, K.; Spencer, D.; Wong, G.K.S.; Wu, Z.; Paulsen, I.T.; Reizer, J.; Saier, M.H.; Hancock, R.E.W.; Lory, S. and Olson, M.V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature., **406**: 959-964.

Sun, T.; Nukaga, M.; Mayama, K.; Braswell, E.H. and Knox, J.R. (2003). Comparison of b-lactamases of class A and D: 1.5-Å crystallographic structure of the class D OXA-1 oxacillinase. Protein Science, **12**: 82-91.

Svärd, L. (2007). Evaluation of phenotypic and genotypic extended-spectrum beta-lactamase detection method. MSc. thesis, School of biological sciences, Dublin institute of technology. Uppsala University.

Taneja, N.; Rao, P.; Arora, J. and Dogra, A. (2008). Occurrence of ESBL & Amp-C β-lactamases & susceptibility to newer antimicrobial agents in complicated UTI Indian J Med Res., **127**: 85-88

Taneja, J.; Malik, A.; Malik, A.; Rizvi, M. and Agarwal, M. (2009). Acute lower respiratory tract infections in children Indian pediatrics, JN Marg, India. *E-mail: drjuhitaneja@gmail.com.*

Tantracheewathorn, T.; Vititpatarapak, N.; Phumisantiphong, U. (2007). Epidemiologic study of nosocomial bacterial infection of pediatric patients at BMA Medical College and Vajira Hospital. J Med Assoc Thai., **90** (2): 258-65.

Tenover, F. C.; Raney, P. M.; Williams, P. P.; Rasheed, J. K.; Biddle, J. W.; Oliver, A.; Fridkin, S. K.; Jevitt, L. and McGowan, J. E. Jr. (2003). "Evaluation of the NCCLS extended-spectrum β-lactamase confirmation methods for *Escherichia coli* with isolates collected during project ICARE." J Clin Microbiol., **41**(7): 3142-3146.

Tenover, F.C. (2006). Mechanisms of antimicrobial resistance in bacteria, Am. J. Med., **119:** 3–10.

Tielker, D.; Hacker, S.; Loris, R.; Strathmann, M.; Wingender, J.; Wilhelm, S.; Rosenau, F.; and Jaeger, K. (2005). *Pseudomonas aeruginosa* lectin LecB is located in the outer membrane and is involved in biofilm formation. Microbiol., **151**: 1313-1323.

Tofteland, S.; Haldorsen, B.; Dahl, K.H.; Simonsen, G.S.; Steinbakk, M.; Walsh, T.R.; Sundsfjord, A. and the Norwegian ESBL study group. (2007). Effects of phenotype and genotype on methods for detection of extended-spectrum-beta-lactamase-producing clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* in Norway. J Clin Microbiol., **45** (1): 199-205.

Toleman, M.A.; Rolston, K.; Jones, R.N. and Walsh, T.R. (2003). Molecular and biochemical characterization of OXA-45, an extended-spectrum Class 2d' β-lactamase in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother., **47**: 2859-2863.

Tonkic, M.; Goic-Barisic, I.; Punda-Polic, V. (2005). Prevalence and antimicrobial resistance of extended-spectrum beta-lactamases-producing *Escherichia coli* and *Klebsiella pneumoniae* strains isolated in a university hospital in Split, Croatia. Int. Microbiol., **8**: 119-124.

Tzouvelekis, L.S. and Bonomo, R.A. (1999). SHV type beta-lactamases. Curr Pharm Des. 847-864.

Varotto, F.; Maria, G.D.; Azzaro, R.; Bellissima, P.; Amato, R.; Fogliani, V.; Muscianisi, G.; Vitle, S.; Girbine, G.; Andro, F.; Lagena, P.; Delia, S.; Jacoviello, C.; Maierna, G.; Pezza, A.; Covelli, I.; Magri, M.; Napoletano, G.; Rossi, A.; Marone, P.; Sanguinetti, C.; Pela, R.; Tedeschi, D.; Viola, B.; Cicciarella, S.; Messina, G.; Rizza, S.; Fraschini, F. (2001). An observational study on the epidemiology of respiratory tract bacterial pathogens and their susceptibility to four injectable beta-lactam antibiotics: piperacillin, piperacillin/ tazobactam, ceftazidime and ceftriaxone. J chemother (Florence, Italy), **13** (4): 413-423.

Veena Kumari, H.B.; Nagarathna, S. and Chandramuki, A. (2007). Antimicrobial Resistance Pattern Among Aerobic Gram-negative Bacilli of Lower Respiratory Tract Specimens of Intensive Care Unit Patients in a Neurocentre. Indian J Chest Dis Allied Sci., **49**: 19-22

Villanueva, F.D.; Tupasi, T.E.; Abiad, H.G.; Baello, B.Q. and Cardano, R.C. (2003). Extended-spectrum β-lactamase production among *Escherichia coli* and *Klebsiella* spp. at the Makati medical center: tentative solutions. Phil J Microbiol Infect Dis., **32**:103-108.

Villegas MV.; Correa A.; Perez, F.; Miranda, MC.; Zuluaga, T.; Quinn, JP. (2004). Prevalence and characterization of extended-spectrum β-lactamases in *Klebsiella pneumoniae* and *Escherichia coli* isolates from Columbial hospitals. Diagn Microbiol Infect Dis **49**: 217-222.

Vourli, S.; Giakkoupi, P.; Miriagou, V. (2004). Novel GES/IBC extended-spectrum β-lactamase variants with carbapenemase activity in clinical enterobacteria. FEMS Microbiol Lett., **234**: 209-13.

Wachino, J.; Doi, Y.; Yamane, K. (2004). Nosocomial spread of ceftazidime-resistant *Klebsiella pneumoniae* strains producing a novel class A b-lactamase, GES-3, in a neonatal intensive care unit in Japan. Antimicrob Agents Chemother., **48**: 1960–1967.

Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. Nature., **406**: 775-781.

Walsh, C. (2003). Antibiotics: actions, origins, resistance. ASM Press, Washington D.C.

Waterer, G.W. and Wunderink, R.G. (2001). Increasing threat of Gram-negative bacteria. Critical Care Med., **29**: 75-81.

Weldhagen, G.F.; Poirel, L.; Nordmann, P. (2003). Ambler class A extended spectrum β-lactamases in *Pseudomonas aeruginosa*: novel developments and clinical impact. Antimicrob Agents Chemother., **47**: 2385–1392.

Weldhagen, G.F. (2004). "Integrons and β-lactamases a novel perspective on resistance." Int J Antimicrob Agents, **23**: 556-562.

Wiegand, I.; Geiss, H.K.; Mack, D.; Sturenburg, E. and Seifert, H. (2007). "Detection of Extended-Spectrum Beta-Lactamases among Enterobacteriaceae by Use of Semi-automated Microbiology Systems and Manual Detection Procedures." J Clin Microbiol., **45**: 1167-1174.

Wilke, M.S.; Lovering, A.L. and Strynadka, N.C.J. (2005). β-Lactam antibiotic resistance: a current structural perspective. Curr Opin Microbiol. **8**: 525-533.

Wise, R. (1996). β-Lactams: cephalosporins. Antibiotic and Chemother. New York, Churchill Livingstone: 202-255.

Woodford, N.; Ellington, MJ.; Coelho, JM. (2005). Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. J Antimicrob Chemother., **57**: 152–153.

World Health Organization (1995). WHO Global Strategy for Containment of Antimicrobial Resistance. Geneva, Switzerland.

World Health Organisation, (2001). WHO Global strategy for containment of antimicrobial resistance. Available online at http://whqlibdoc.who.int/hq/2001/WHO_CDS_CSR_DRS_2001.2a.

Wright, G.D. (2005). Bacterial resistance to antibiotics: enzymatic degradation and modification. Advanced Drug Delivery Reviews., **57**: 1451-1470.

Yan, J.J.; Ko, W.C.; Tsai, S.H.; Wu, H.M.; Jin, Y.T. and Wu, J.J. (2000). Dissemination of CTX-M-3 and CMY-2 β-lactamases among clinical isolates of *Escherichia coli* in southern Taiwan. J Clin Microbiol., **38:** 4320-4325.

Yan, J.J.; Ko, W.C.; Jung, Y.C.; Chuang, C.L. & Wu, J.J. (2002). Emergence of *Klebsiella pneumoniae* isolates producing inducible DHA-1 beta-lactamase in a university hospital in Taiwan. J Clin Microbiol., **40**: 3121–3126.

Yao, J.D.C. and Moellering, R.C.J. (2003). Antibacterial Agents. Manual of Clinical Microbiology. Washington, DC., American Society of Microbiology**:** 1039-1073.

Zapun, A.; Contreras-Martel, C. and Vernet, T. (2008). Penicillin-binding proteins and beta-lactam resistance. FEMS Microbiol Rev., **32**:361-385.

Appendix 1: Phenotypic characteristics of GNB isolated from wound and sputum samples of patients

Test	*Ps. aerugenosa* (n= 62)	*E. coli* (n=56)	*Klebsiella* spp.					*Proteus* spp.		
			K. pneumoniae subsp. *pneumoniae* (n= 19)	*K. pneumoniae* subsp. *ozaenae* (n= 15)	*K. pneumoniae* subsp. *rhinoscleromatis* (n= 14)	*K. pneumoniae* subsp. *aerogenes* (n= 8)	*K. oxytoca* (n= 6)	*P. mirabilis* (n= 18)	*P. vulgaris* (n= 5)
Oxidase	+	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+
IMViC tests: Indol	-	+	-	-	-	-	+	-	+
Citrate utilization	+	-	+	+	-	+	+	v	v
Yogas-Proskaures	-	-	+	-	-	-	+	v	-
Methyl red	-	+	-	+	+	+	-	+	+
Urease production	-	-	+	-	-	-	+	+	+
Triple Sugar Iron	K/K	A/A	A/A	A/A	A/A	A/A	A/A	K/A	K/A
H_2S production	-	-	-	-	-	-	-	+	+

Test	*Ps. aerugenosa* (n= 62)	*E. coli* (n=56)	*Klebseilla* spp.					*Proteus* spp.	
			K. pneumoniae subsp. *pneumoniae* (n= 19)	*K. pneumoniae* subsp. *ozaenae* (n= 15)	*K. pneumoniae* subsp. *rhinoscleromatis* (n= 14)	*K. pneumoniae* subsp. *aerogenes* (n= 8)	*K. oxytoca* (n= 6)	*P. mirabilis* (n= 18)	*P. vulgaris* (n= 5)
Acid from: Glucose	-	+	+	+	+	+	+	+	v
Lactose	-	+	+	+	+	+	+	-	-
Sucrose	-	v	+	+	+	+	+	v	+
Glycerol	-	v	+	+	+	+	+	v	v
D- mannose	-	+	+	+	+	+	+	-	-
D- xylose	-	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	v	-	-	-	-	-	+	-
Motility	+	+	-	-	-	-	-	+	+
Growth at 10˚C	-	ND	-	-	-	-	+	ND	ND

ND = Non determine

الخلاصـــة:

تُعد البكتريا السالبة لصبغة غرام والحاملة لإنزيمات البيتا لاكتاميز واسعة الطيف المشكلة الرئيسية للمرضى وخاصة الراقدين في المستشفيات. لذا كان هدف هذه الرسالة هو تحديد انتشار هذه الإنزيمات المقاومة للسيفالوسبورين واسع الطيف وأنواعها.

جمعت 613 عينة سريرية من مستشفى الصدر التعليمي والمركز الاستشاري للأمراض الصدرية في مدينة النجف الأشرف للفترة من شهر تشرين الثاني 2008 ولغاية نهاية شباط 2009، وكانت هذه العينات على نوعين، الأولى مسحات لمرضى الحروق والجروح بواقع 390 عينة والثاني 223 عينة بلغم لمرضى يشكون من أعراض لأمراض الجهاز التنفسي السفلي. ولدراسة أنواع البكتريا السالبة لصبغة غرام، تم زرعها على بيئة الماكونكي الصلب، حيث أمكن عزل البكتريا الأكثر شيوعاً وعددها 203 عزلات.

وبعد دراسة العزلات مظهرياً مع الفحوصات الكيموحيوية ظهر بأن بكتريا الزائفة الزنجارية والكليبسيلا (62 عزلة لكل منهما) كانا الأكثر إنتشاراً ويليهما بكتريا إشيريشيا القولون (56 عزلة) ثم بكتريا المتقلبة (23 عزلة)، وقد شخصت عزلات الكليبسيلا إلى مستوى تحت النوع.

للتحري عن البكتريا المنتجة لإنزيمات البيتا لاكتاميز، تم اختبار مقاومة هذه البكتريا للمضادات الحيوية التابعة إلى تحت الصنف امينوبنيسيلين وقد ظهر بأن 193 عزلة (95.1%) كانت مقاومة للأمبيسيلين والأموكسيسيلين، منها 61 عزلة لبكتريا الكليبسيلا، 59 لبكتريا الزائفة الزنجارية، 52 لبكتريا إشيريشيا القولون، 21 عزلة من بكتريا المتقلبة.

دُرست حساسية هذه العزلات لـ 22 نوع من المضادات الحيوية بطريقة انتشار القرص لـــ كيربي باور، وقد اعتبرت 174 عزلة ذات مقاومة متعددة للمضادات الحيوية، كما أظهرت هذه العزلات مقاومة عالية للسيفازولين (87.7%) ومقاومة واطئة للإميبينيم (5.1%).

استخدمت طرق النمط الظاهري Phenotype methods للكشف عن إنزيمات البيتا لاكتاميز واسعة الطيف، حيث أظهرت النتائج أن 189 عزلة (97.9%) كانت منتجة لأنزيمات البيتا لاكتاميز واسعة الطيف حسب طريقة اختبار المسح الأولي، بينما أظهرت الطريقة

التأكيدية بأن 10 عزلات فقط (5.2%) كانت تحمل إنزيمات البيتا لاكتاميز واسعة الطيف. ومن الملاحظ بأن الفرق بين النتيجتين كان كبيراً، والسبب المحتمل لهذا الفرق هو إنتاج إنزيم AmpC الذي يعتقد عدد من الباحثين أن وجوده بفعالية عالية سيعمل قناعاً ضد الكشف عن إنزيمات البيتا لاكتاميز واسعة الطيف وهذه احتمالية تدعمها مقاومة هذه العزلات للمضاد الحيوي سيفوكسيتين.

من جانب آخر، تم استخدام طريقة النمط الجيني Genotype method للكشف عن وجود الأنواع الأربعة الأكثر انتشاراً من إنزيمات البيتا لاكتاميز واسعة الطيف وهي إنزيمات TEM, SHV, CTX-M, OXA، وقد استخدمت لأجل ذلك أربعة أنواع من البادئات الخاصة للكشف عنهم وباستخدام جهاز الـــ PCR وجهاز الترحيل الكهربائي، أظهرت النتائج وجود 51 عزلة تحمل واحد أو أكثر من جينات الإنزيمات الأربعة أعلاه. حيث ظهر بأن 17 عزلة منها تحمل الجين *bla* $_{TEM}$ المنتج لإنزيم TEM و 21 عزلة تحمل الجين *bla* $_{SHV}$ المنتج لإنزيم SHV و 24 عزلة تحمل الجين *bla* $_{CTX-M}$ المنتج لإنزيم CTX-M و 17 عزلة تحمل الجين *bla* $_{OXA}$ المنتج لإنزيم OXA. وقد لوحظ بأن هذه العزلات الحاملة لجينات البيتا لاكتاميز واسعة الطيف كانت جميعها ذات مقاومة متعددة للمضادات الحيوية.

من خلال هذه النتائج يظهر لنا بأن نسبة وجود إنزيمات البيتا لاكتاميز واسعة الطيف نسبة متوسطة على الرغم من عدم وجود دراسة سابقة لإنزيمات CTX-M, OXA في المنطقة للمقارنة، ولكن الأخطر بين هذه العزلات هو وجود هذه الأنواع الأربعة من الإنزيمات في بكتريا واحدة. وهذه النتائج قد تساعد في معالجة الإصابات بالبكتريا الحاملة لإنزيمات البيتا لاكتاميز واسعة الطيف.

إنتشار بعض إنزيمات البيتا لاكتاميز واسعة الطيف بين العزلات السريرية العصوية السالبة لصبغة غرام في مدينة النجف الأشرف

رسالة مقدمة إلى

كلية الطب/ جامعة الكوفة جزءاً من متطلبات نيل درجة الماجستير

في علم الأحياء المجهرية الطبية

تقدم بها الطالب

فاضل حسين ناصر المحنّك

بكالوريوس علوم حياة/ جامعة الموصل

بإشراف

الدكتور علي محسن المحنّة

أستاذ مساعد في علم الأحياء المجهرية

1431 هجري

2010 ميلادي