Expression of CTX-M Extended Spectrum Beta-Lactamases by *Escherichia coli* and Susceptibility to Antimicrobial Agents

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إنتاج إنزيم CTX-M الواسع المدى في أل *Escherichia coli* ومدى الاستجابة للمضادات الحيوية

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I dedicate this work to my family, my wife Ebtesam, my children Duha, Abdallah, Ahmad and Omar, my father Abdel Fattah, my mother Haliema, my brothers Mustafa, Issa, Jehad and Ahmad and to my sisters, Aydeh, Hana', Hanan and Fatmeh . They provided me with the power to accomplish this work. This work was fulfilled by their support and encouragement.

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LIST OF ABBREVIATIONS

A/A	Acid/Acid
AK	Amikacin
AMC	Amoxicillin/Clavulanate
AMP	Ampicillin
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
ATCC	American Type Culture Collection
ATM	Aztreonam
β	Beta
BAP	Blood Agar Plate
Bla	Beta Lactam
bp	Base Pair
C	Celsius
CAZ	Ceftazidime
CDT	Combination Disk Test
CFU	Colony Forming Unit
CLSI	Clinical Laboratory Standards Institute
cm	Centimeter
CMT	Complex Mutant of TEM

LIST OF ABBREVIATIONS (continued)

CN	Gentamicin
CRO	Ceftriaxone
CTX	Cefotaxime
CTX-M	Cefotaximase
CVA	Clavulanic Acid
DDST	Double Disk Synergy Test
DNA	Deoxyribonucleic Acid
ESBL	Extended Spectrum Beta-Lactamases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
GES	Guiana extended-spectrum beta-lactamase
Gln	Glutamine
Glu	Glutamic Acid
Gly	Glycine
HUS	Hemolytic Uremic Syndrome
IBC	Integron-borne cephalosporinase
ICU	Intensive Care Units
ISE	Insertion Element
KSA	Kingdom of Saudi Arabia
λ	Lambda

LIST OF ABBREVIATIONS (continued)

LVO	Levofloxacin
Lys	Lysine
MDR	Multidrug Resistance
µg	Microgram
MHA	Mueller-Hinton Agar
MIC	Minimum Inhibitory Concentration
MIR	A class C beta-lactamase first discovered from Miriam Hospital in providence, Rhode Island, USA
ml	Milliliter
µl	Microliter
µM	Micromole
µm	Micrometer
mm	Millimeter
MRSA	Methicillin Resistant *S. aureus*
MYSTIC	Meropenem Yearly Susceptibility Test Information Collection
ng	Nanogram
NMC	Not metalloenzyme carbapenemase
No.	Number
OXA	Oxacillinase
Orf	Open Reading Frame
PBP	Penicillin Binding Protein

LIST OF ABBREVIATIONS (continued)

PCR	Polymerase Chain Reaction
PEARLS	PanEuropean Antimicrobial Resistance using Local Surveillance
PER	An ambler class A enzyme first detected in *Pseudomonas aeruginosa* RNL-1
PG	Peptidoglycan
Pro	Proline
PSE	Beta-lactamase first detected in *Pseudomonas*
RNA	Ribonucleic Acid
Ser	Serine
SFO	Beta-lactamase enzyme detected in *Serratia fonticola*
SHV	Sulfhydryl variable
Sme	Serratia marcescens enzyme
spp	Species
SXT	Sulfamethoxazole/Trimethoprim
TAE	Tris-Acetate-EDTA
TEM	An ambler class A enzyme first isolated from a patient 'Temoniera'
TEST	Tigecycline Evaluation and Surveillance Trial
TRC	TEM enzyme that confers resistance to clavulanate
UK	United Kingdom
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary Tract Infection

LIST OF ABBREVIATIONS (continued)

UV	Ultraviolet
V	Voltage
VEB	Vietnamese extended-spectrum beta-lactamase

ABSTRACT

Extended spectrum β-lactamases (ESBLs) are enzymes commonly produced by the family Enterobacteriaceae. They exhibit wide spectrum activities against penicillins, first-, second-, and third-generation cephalosporins, and monobactam, aztreonam (but not the cephamycins or carbapenems), but inhibited by β-lactamase inhibitors. CTX-M β-lactamases were discovered at the beginning of 1990s. They are now replacing TEM and SHV mutants in *E. coli* isolates from both community and hospital sources worldwide. Genes encoding CTX-M β-lactamases are found on plasmids commonly harboring multidrug resistant genes. Multidrug resistant organisms cause profound effects on patients and complicate medical treatment. In Palestine, a paucity of information is available regarding this significant topic. The aim of this study was to determine phenotypic and molecular epidemiology of ESBLs among *E. coli* isolates from various clinical sources. Preliminary screening of ESBL-producers was achieved by utilizing 1 μg/ml cefotaxime containing MacConkey plates and double disk synergy tests. The suspected ESBL-producers were confirmed by Combination disk test and molecular PCR technique. Agar dilution method was used to determine MICs to all ESBL isolates using different antimicrobial agents. Occasionally, faecal carriage of ESBL-producing *E. coli* was evaluated in patients with urinary tract infection caused by the same organisms. A rate of 18.2% (77/423) of *E. coli* isolates were designated as ESBL producers. All ESBL isolates exhibited 100% susceptibility to meropenem, while 30% (23/77) were multidrug resistant to non-β-lactam agents; gentamicin, levofloxacin and sulfa drugs.

CTX-M was detected in all ESBL isolates (100%) while TEM and SHV β-lactamases were found to be 59.7%, (46/77) and 1.3% (1/77) respectively. Analyses of CTX-M amplicons revealed that 80.5%, (62/77) and 19.5%, (15/77) were CTX-M group 1 and group 9 respectively.

ESBL-producing *E. coli* was detected in faecal samples of eight patients with urinary tract infection due to the same organism. These ESBL isolates had similar genotype and susceptibility profile to the third generation cephalosporins except in one case where CTX-M was not detected. Our findings indicate that CTX-M-15 like allele which belongs to group 1 is the most common CTX-M type detected which agree with many studies conducted worldwide. The ESBL detection rate described in this study was comparable to worldwide studies, tends to be towards the upper end of the spectrum, and is therefore a major cause for concern.

الخلاصة

البيتالاكتمات الواسعة المدى (ESBL) عبارة عن إنزيمات تنتج بشكل عام في عائلة الانتيروبكتيريسي. هذه الإنزيمات لها تأثير واسع في مقاومة البنسيلين، الجيل الأول والثاني والثالث من السيفالوسبورينات ، وكذلك المونوباكتام (ولكن ليس السفاميسينات والكاربابنمات) ، إلا أن هذه الأنزيمات تعتبر حساسة وتتأثر بمثبطات البيتالاكتام. البيتالاكتمات من نوع إل CTX-M والتي اكتشفت في أوائل التسعينات من القرن الماضي حلت ألان في انتشارها محل أل TEM وال SHV بيتالاكيمات المطفرة وغدت إل CTX-M تنتج بشكل كبير من بكتيريا الآي كولاي بين مرضى المستشفيات والمجتمع. الجينات المسؤولة عن إنتاج إل CTX-M بيتلاكتيمات غالبا ما تكون محمولة بواسطة بلازميدات تحتوي العديد من الجينات الأخرى المقاومة للمضادات الحيوية ، مما ساهم بشكل كبير في تقليل فرص العلاج للمرضى المصابين بهذه البكتيريا. هنالك القليل من الدراسات المتعلقة بهذا الموضوع في فلسطين. لذلك كان هدف هذه الدراسة تحديد مدى انتشار الطرز الشكلية والجينية لهذه الإنزيمات في بكتيريا الآي كولاي المأخوذة من عينات سريريه مختلفة. تم استعمال صحون المكونكي التي تحوي سيفوتكسيم بتركيز 1 ميكروغرام/مل من اجل الكشف الأولي عن بكتيريا الآي كولاي المنتجة لإنزيمات البيتالاكتمات واسعة المدى. للتأكد من نتائج الفحوصات الأولية، تم استعمال فحص أقراص الحساسية المزدوج وفحص إلPCR. استخدمت طريقة تخفيف الاجار باستخدام عدد مختلف من المضادات الحيوية من اجل تحديد الحد الأدنى اللازم لتثبيط ميكروبات الآي كولاي المنتجة لإنزيم البيتالاكتام واسع المدى. حيثما أمكن، تم أيضا التعرف على بكتيريا الآي كولاي ألمنتجه لإنزيم البيتالاكتام الواسع المدى في عينات البراز لدى المرضى المصابين بالتهاب البول بسبب نفس الميكروب. لقد وجد ما نسبته 18.2% (423/77) من ميكروبات الآي كولاي منتجه لإنزيم البيتالاكتام الواسع المدى. كانت نتائج ميكروبات الآي كولاي المنتجة لهذا الإنزيم، 100% حساسة للميروبينيم، بينما 30% من هذه البكتيريا، كانت متعددة المقاومة للمضادات الحيوية من غير البيتالاكتمات وهي الجنتاميسين، ليفوفلوكسين وعقاقير الصلفه. لقد كانت نسبة إنزيم البيتالاكتام من نوع أل CTX-M 100% ، ومن نوع أل TEM 59.7%، ومن نوع ألSHV 1.3%. بعد تحليل الجينات المنتجة لإنزيم CTX-M، وجد ما نسبته 80.5% لمجموعة CTX-M-1 و 19.5% لمجموعة CTX-M-9. لقد استطعنا تحديد ميكروبات الآي كولاي المنتجة لإنزيم البيتالاكتام واسع المدى في ثمان عينات براز للمرضى المصابين بالتهاب البول بسب نفس الميكروب. كان التطابق عاليا من حيث أنواع الجينات وفحص الحساسية للجيل الثالث من السيفالوسبورينات في الميكروبات المعزولة من عينات البول والبراز، إلا في مريض واحد، لم يكن لديه جين CTX-M في عينة البراز. لقد تم الاستنتاج في هذه الدراسة، أن أشباه جينات CTX-M-15 التي تنتمي لمجموع CTX-M-1 هي الأكثر شيوعا بين إنزيمات CTX-M والذي يتوافق بدوره مع نتائج العديد من الدراسات عالميا. بشكل عام، تعد نسبة إنزيمات البيتالاكتمات المكتشفة في هذه الدراسة عاليه نسبيا وهذا يتوافق مع نتائج العديد من الدراسات العالمية، وهذا يشكل بدوره مسألة عالية الاهتمام.

INTRODUCTION

Members of the family Enterobacteriaceae are Gram-negative bacteria that commonly caused community and hospital-acquired infections. *Escherichia coli* (*E. coli*) is the most commonly isolated organism within this family, and the major cause of urinary tract infections (UTIs). Resistance related to production of β-lactamases is the most common resistance mechanism utilized by the Enterobacteriaceae against β-lactam antibiotics. β-lactamases are bacterial enzymes which protect microorganisms from the lethal effects of β-lactam antibiotics by hydrolyzing the β-lactam ring rendering the drugs inactive [1]. In addition, β-lactamases are either chromosomally encoded or plasmid mediated**.** Chromosomal types are either constitutively produced or inducible**.** *Klebsiella pneumoniae* for example, produces class A β-lactamase constitutively, whereas *Enterobacter cloacae*, *Enterobacter aerogenes, Citrobacter freundii*, *Serratia spp.*, and *Pseudomonas aeruginosa* produce inducible class C β-lactamases [2]. Plasmid-mediated β-lactamases have become prevalent among gram-negative bacteria during the past 50 year. The first plasmid-mediated β-lactamase in Gram-negative bacteria, TEM-1, was described in the early 1960s carried by transposons on plasmids [2]. The TEM-1 genes have spread to several bacterial species and now distributed throughout the world. SHV-1 is another frequently encountered plasmid-mediated β-lactamase among gram-negative bacteria [2]. In the early 1980s, third-generation cephalosporins were introduced in the clinical practice as β-lactam antibiotics that are not affected by the common β-lactamases. However, few years later, hospital-acquired infections were caused by β-lactamase resistant mutants. These new mutants were able to neutralize the activity of expanded-

spectrum cephalosporins, and monobactams and thus designated as extended-spectrum β-lactamases (ESBLs) [2]. SHV-2, the first plasmid-mediated β-lactamase capable of hydrolyzing extended-spectrum cephalosporins, was reported in Germany in 1983 [3]. SHV-2 plasmid was spread among *E. coli* isolates in ICU units via conjugation. These strains carrying SHV-2 plasmid had been known for their resistance to cefotaxime and ceftazidime [3].

In France in 1984 [4], *K. pneumoniae* isolates with the ESBL phenotype carrying TEM-2 β-lactamase plasmid were detected in different hospitals. This enzyme was first named CTX-1 and later TEM-3. Both TEM and SHV ESBL enzymes are now distributed worldwide. They have been associated with Enterobacteriaceae species, particularly among *K. pneumoniae* and *Enterobacter* spp. isolates recovered from ICU patients and recently with *P. aeruginosa* and *Acinetobacter baumannii*. Several epidemics have been reported, and new ESBL variants of TEM and SHV have been identified in Europe and other geographical areas [4].

In 1989, in Germany [5] and Argentina [5] and then in France [5] and Italy [5], a new ESBL family was first reported in *E. coli* isolated from clinical specimen. The first enzyme of the new CTX-M family was designated as CTX-M-1, indicating its hydrolytic activity against cefotaxime [5]. The CTX-M ESBLs have also been detected in many species of Enterobacteriaceae family. The spread of CTX-M producing isolates was limited to specific geographic areas during the 1990s. Recent epidemiological studies on ESBL-producing bacteria demonstrated a dramatic increase in the prevalence of CTX-M enzymes worldwide [6].

In 1991 in Ankara (Turkey) [4], and later in France [4], oxacillinases conferring a phenotype similar to that of ESBLs, but with little inhibition by clavulanate, were identified. They were recognized as mutants of broad-extended-spectrum OXA-type β-lactamases (most are OXA-10). These variants have been found worldwide and are commonly associated with *P. aeruginosa* and, to a lesser extent, with *A. baumannii* or Enterobacteriaceae. Less prevalent ESBL groups such as PER enzymes were mainly found in *P. aeruginosa* and *Acinetobacter* spp., and VEB and GES in *P. aeruginosa* and Enterobacteriaceae. These groups are often transferable and usually inhibited by clavulanate. Other rare types encountered include SFO and IBC enzymes [4].

Genes encoding ESBLs are frequently found on the same plasmid as genes encoding resistance for other classes of antibiotics such as aminoglycosides, tetracyclines, and sulfonamides. In addition, many of the Enterobacteriaceae possess chromosomal changes that confer resistance to fluoroquinolones [2]. As a result, ESBL-producing Enterobacteriaceae detected in hospitals are frequently multidrug resistant, posing particular difficulties in the treatment of infections, especially in critically ill patients.

Features of *Escherichia coli*

Theodor Escherich, a German scientist first described *E. coli* in 1885 [7] in stool specimens of babies with enteritis and called it Bacterium coli commune. *E. coli* are Gram negative, facultative anaerobic and non- sporulating cells belonging to the family Enterobacteriaceae. They are motile, rod-shaped with about 2 µm long and 0.5 µm in diameter, and a cell volume of 0.6-0.7 μm^3 [8].

E. coli strains are predominant in the human colonic flora. The organism normally colonizes the gastrointestinal tract of infants within hours of life, and both *E. coli* and the host derive mutual benefits [9].

Colonies of *E. coli* appear circular, convex, and smooth on agar media with distinct margin. It can be recovered from clinical specimens on MacConkey or Eosin Methylene-Blue selective media at 37° C under aerobic conditions.

E. coli are usually identified via biochemical reactions. These tests can be performed either by conventional or semiautomated methods. Conventional identification can be made by examining selective media for typical growth, decarboxylation of lysine, typical A/A reaction on Triple sugar iron, production of indole, and positive Methyl Red test reaction [10].

E. coli can cause several infections including Hemolytic-Uremic Syndrome (HUS), UTI, gastroenteritis, neonatal sepsis and meningitis [11]. Uropathogenic *E. Coli* (UPEC) are the most common cause of community-acquired UTI and are responsible for 70–90% of the estimated 150 million cases diagnosed annually. UPEC are responsible for 40% of all nosocomial UTIs, emphasizing its significant as major nosocomial pathogens [12]. In ascending infections, fecal bacteria colonize the urethra and spread up the urinary tract to the bladder as well as to the kidneys causing pyelonephritis. Because women have a shorter urethra than men, they are 14-times more likely to suffer from an ascending UTI. UPEC utilize fimbriae to bind urinary tract endothelial cells and colonize the bladder [13].

Antimicrobial Agents

Mechanisms of Action

Antimicrobial agents are the main therapeutic mean to treat bacterial infections. They can be classified bactericidal or bacteriostatic [14]. Bactericidal antibiotics kill bacteria directly while bacteriostatic ones inhibit their growth. However, in practice, both classes are capable of eliminating a bacterial infection. Depending on their origin, antimicrobial agents can be classified into three groups; naturally occurring such as penicillins, semi-synthetic such as third generation cephalosporins, and artificially synthesized agents such as quinolones and their derivatives, the fluoroquinolones as shown in Figure 1 [15].

Further classification of antimicrobial agents can also be made according to their mechanism of action [16]. β-lactams are directed against cell wall synthesis, macrolides inhibit protein synthesis, quinolones interfere with nucleic acid synthesis, trimethoprim and sulfamethoxazole inhibit metabolic pathway while polymyxins disrupt membrane structure. Mechanisms of action of antibiotics are summarized in Table 1.

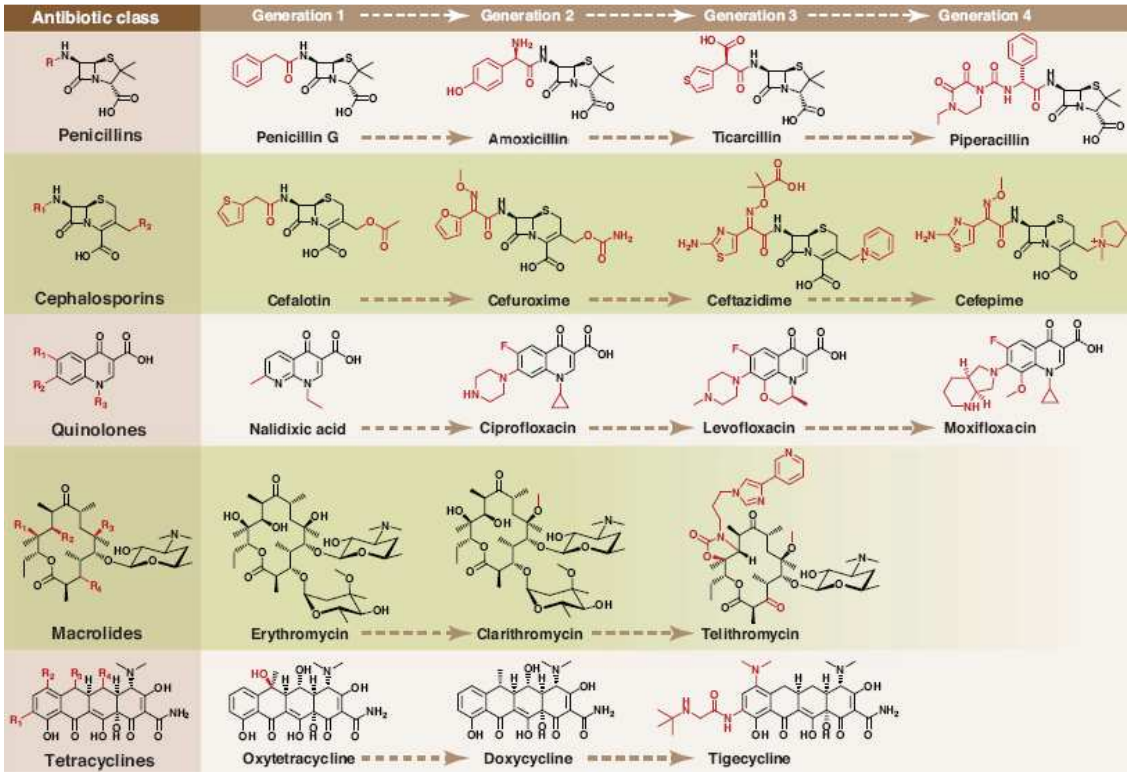

Figure 1. Core structure (scaffold) of each class of antibiotics (black) and their Synthetic derivatives (red). The quinolone scaffold is synthetic, whereas the other scaffolds are natural products [15].

Table 1. Mechanisms of action of antimicrobial agents.

Mechanism of action	Antimicrobial agents
1. Interfere with cell wall synthesis	β-lactams: penicillins, cephalosporins, carbapenems, monobactams. Glycopeptides: vancomycin, teicoplanin
2. Inhibit protein synthesis • Bind to 50S ribosomal subunit • Bind to 30S ribosomal subunit	 Macrolides, chloramphenicol, clindamycin. Aminoglycosides, tetracyclines.
3. Interfere with nucleic acid synthesis • Inhibit DNA synthesis • Inhibit RNA synthesis	 Quinolones. Rifampin
4. Inhibit metabolic pathway	Sulfonamides, folic acid analogues
5. Disrupt bacterial membrane structure	Polymyxins, daptomycin.

β-Lactam Antibiotics

Penicillin was discovered accidentally in 1928 by Alexander Fleming [17]. Since then, many different classes of β-lactams were developed. β-lactams are frequently prescribed worldwide due to their efficacy and low toxicity. β-lactams constitute a large group of antibiotics containing the characteristic β-lactam ring (Figure 2) [18]. There are four major groups of β-lactams, penicillins, cephalosporins, carbapenems and monobactams. These groups differ in the nature of the extra ring attached to the β-lactams one as well as the side chains attached to the core rings [19].

The effectiveness of β-lactam antibiotics attributed to their ability to inhibit bacterial growth by inactivation of the penicillin binding proteins (PBPs). PBPs are enzymes located on the outer surface of the cytoplasmic membrane. They catalyze the final stages of peptidoglycan (PG) synthesis, the major component of the bacterial cell wall [20].

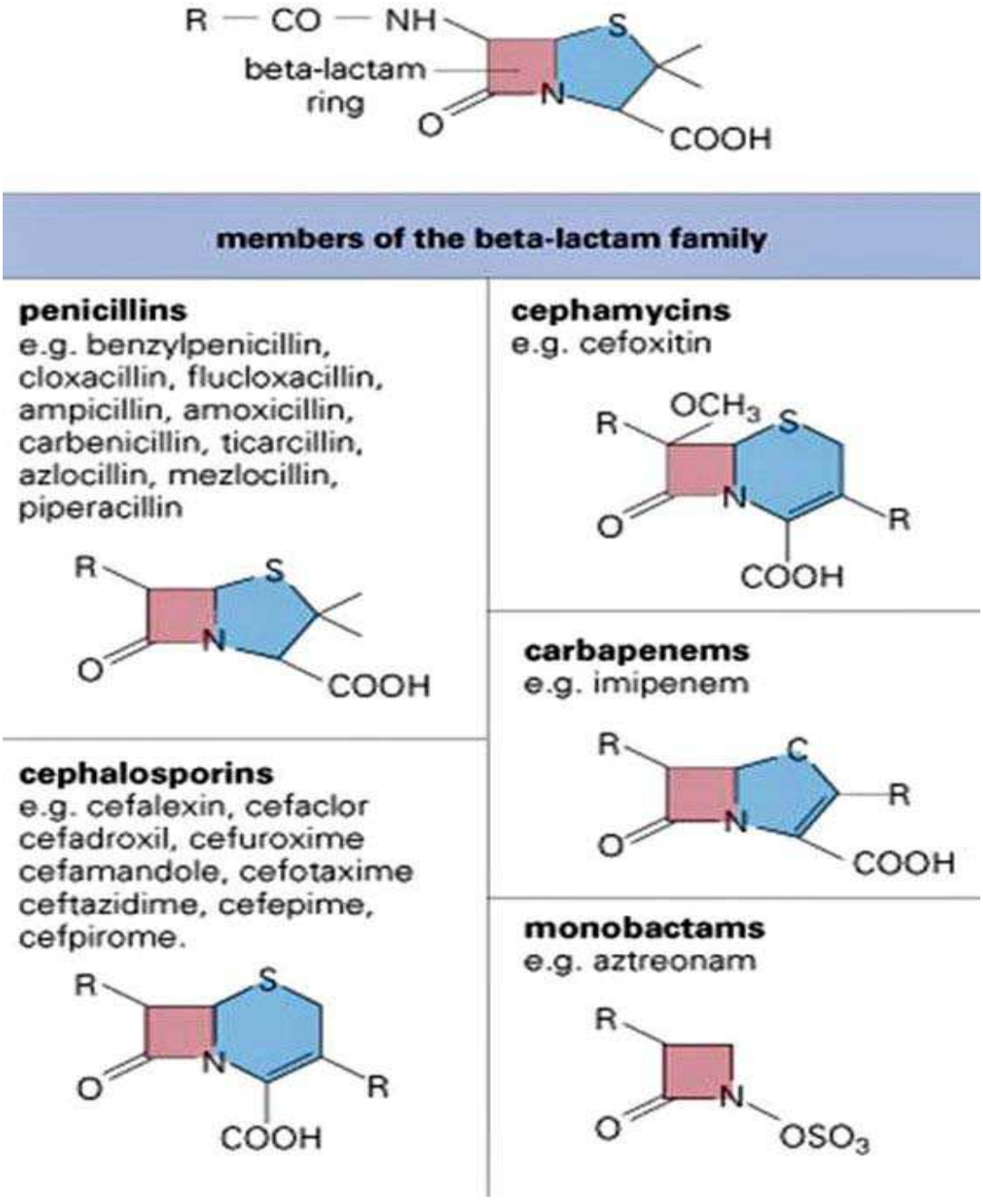

Figure 2. Members of the β-lactam family. The β-lactam ring is marked in red and the ring marked in blue show the different side chains attached to the β-lactam ring [18].

Cephalosporins and Cephamycins

Cephalosporins and cephamycins are β-lactam antibiotic similar to penicillins. These classes exhibit a wider antibacterial spectrum, resist many β-Lactamases, and have improved pharmacokinetic properties. Cephalosporins are subdivided into narrow-spectrum first generation cephalosporins, broad-spectrum second-generation cephalosporins, and extended-spectrum third and fourth generation cephalosporins as shown in Table 2 [21].

Table 2. Cephalosporins and cephamycins antibiotics and their spectrum of activity.

Antibiotics	Spectrum of activity
First generation Cephalexin, cephalothin, cefazolin, cephapirin, cephradine	Activity equivalent to oxacillin against Gram-positive bacteria; some Gram-negative bacteria (*E. coli, Klebsiella spp, Proteus mirabilis*)
Second generation Cefaclor, cefamandole, cefuroxime, cefotetan, cefoxitin	Activity equivalent to oxacillin against Gram-positive bacteria; improved Gram-negative activity against *Enterobacter*, *Citobacter* and additional *Proteus* species
Third generation Cefixime, cefotaxime, Ceftriaxone, ceftazidime	Activity equivalent to oxacillin against Gram-positive bacteria; improved Gram-negative activity including *Pseudomonas*
Fourth generation Cefepime, cefpirome	Activity equivalent to oxacillin against Gram-positive bacteria; marginally improved Gram-negative activity

Bacterial Resistance

Mechanisms of Action

Bacterial resistance to antibiotics can occur by several mechanisms as shown in Table 3 [22].

Table 3. Mechanisms of antimicrobial resistance.

Mechanisms of resistance	Antimicrobial(s) affected
1- Modification of the antimicrobial agent	Aminoglycosides, chloramphenicol, and β-lactams.
2- Alteration or protection of the target site	Aminoglycosides, β-lactams, macrolides, quinolones, rifampicin, trimethoprim, and tetracycline.
3- Decreased antibiotic accumulation • Decreased uptake • Increased efflux	 Many antibiotics (quinolones). Tetracycline, macrolides, quinolones, and chloramphenicol.
4- Alteration of the metabolic pathway	Sulfonamides, trimethoprim.

These resistances are either intrinsically mediated and/or acquired process. Intrinsic resistance is a natural process displayed by all members of a species. Acquired resistance occurs via mutation in the regulatory or structural genes and/or through acquisition of resistance gene. Evidence suggests that most bacterial genomes that have been sequenced contain foreign DNA segments acquired from external sources. Acquired DNA usually encodes functions that are of selective advantage to the organism such as antibiotic resistance [16]. There are three common mechanisms for horizontal gene transfer [16]:

(1) Transformation, a process by which bacteria takes up free DNA directly from their environment.

(2) Transduction, a process by which bacterial DNA is moved from one bacterium to another by a bacteriophage.

(3) Conjugation, a process by which living bacterial cell transfers genetic material through cell-to-cell contact.

A number of different DNA elements have played a main role in the development of resistance in bacteria. Such genetic elements include plasmids, transposons, genomic islands, phage, integrons and gene cassettes.

Resistance to β-lactam antibiotics

Resistance to β-lactam antibiotics in Gram-negative bacteria usually occur by several mechanisms. Alteration of porin proteins in the cell membrane cause reduced permeability and block entry of the antibiotic. Efflux mechanisms to pump out antibiotics from the bacterial cell. Alteration in the target PBPs prevents β-lactam binding and eventually productions of β-lactamase enzymes inactivate the antimicrobial agents [20]. β-lactamases are bacterial enzymes that catalyze the hydrolysis of the β-lactam ring to yield inactive products as shown in figure 3.

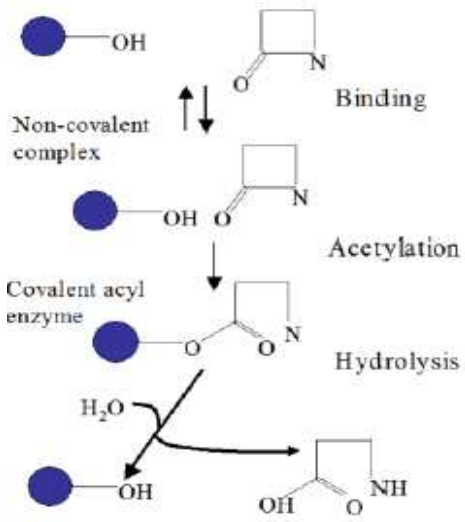

Figure 3. Schematic representation of the activity of a serine β-lactamase (blue) (Ambler classes A, C, and D) [23].

In Gram-negative bacteria, β-lactamases remain in the periplasmic space, where they attack the β-lactam ring before it reaches the PBPs. Since the introduction of β-lactam antibiotics into clinical use, β-lactamases have evolved simultaneously. Hundreds of different β-lactamases have been identified ever since [24]. β-lactamases are either plasmid or chromosomal mediated. The majority of β-lactamases are plasmid mediated. The widespread use of β-lactams is the major factor causing β-lactamase production by pathogens.

Classification of β-lactamases

β-lactamases are a large, heterogeneous group of approximately 300 enzymes [25]. Many attempts have been made to categorize and classify them. Several classification schemes have been proposed according to their hydrolytic spectrum, susceptibility to inhibitors, plasmid or chromosomal origin and amino acid sequence. Richmond and Sykes [26] proposed the classification of β-lactamases in 1973. This scheme was based on β-lactamase function and classifying the β-lactamases into five major groups. In 1980, Ambler [26] presented molecular classification based on the nucleotide sequence and protein homology in these enzymes into four classes (A-D) as shown in Table 4. Classes A, D and C act by a serine-based mechanism while class B is metallo-β-lactamases which requires zinc for their action. A major revision and expansion of the Richmond and Sykes functional scheme was proposed by Bush [26] in 1989 and updated in 1995 [26]. The revised scheme by Bush is based on four major groups (1-4) and subgroups (a-f) and classifies β-lactamases by their substrate preference and by their susceptibility to inhibitors such as clavulanic acid as shown in Table 4 [25]. This functional classification

scheme provides a useful compilation of β-lactamase characteristics. The problem with point mutations that can alter the substrate and inhibitor specificity may change the assigned grouping. The majority of the clinically important β-lactamases belong to Ambler class A and C (Bush group 1 and 2).

Table 4. Classification of β-lactamases [25].

Functional group (bush)	Major subgroup	Molecular class (Ambler)	Referred substrate	Inhibition by clavulanic acid	Representative enzymes
1		C	Cephalosporins	-	AmpC enzymes from Gram-negative bacteria; MIR-1
2	2a	A	Penicillins	+	Penicillinases from Gram-positive bacteria
	2b	A	Penicillins, Cephalosporins	+	TEM-1,TEM-2, SHV-1
	2be	A	Penicillins, narrow- and extended-spectrum cephalosporins, monobactams	+	TEM-3 to TEM-26,CTX-M family, SHV-2 to SHV-6, *K. oxytoca* K1
	2br	A	Penicillins	+/-	TEM-30 to TEM-36, TRC-1
	2C	A	Penicillins, carbenicillins	+	PSE-1, PSE-3, PSE-4
	2d	D	Penicillins, Cloxacillin	+/-	OXA1 to OXA-11, (OXA-10), PSE-2
	2e	A	Cephalosporins	+	Inducible cephalosporinases from *P. vulgaris*
	2f	A	Penicillins, cephalosporins, carbapenems	+	NMC-A from *E*. cloacae, Sme-1 from *S. marcescens*
3	3a, 3b, 3e	B	Most β-lactams including carbapenems	-	*Bacteriodes fragilis*
4		Not determined	penicillins	-	Penicillinase from *Pseudomonas cepacia*

Extended-Spectrum β-Lactamases (ESBLs)

ESBLs are enzymes encoded by genes commonly harbored on plasmids. These enzymes have wide spectrum of activity and capable of hydrolyzing penicillins; first-, second-, third-, and fourth-generation cephalosporins; as well as monobactams. β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam inhibit ESBL enzymes. However, ESBL enzymes are susceptible to carbapenems and cephamycins (cefoxitin and cefotetan) [2]. The ESBL term refer to mutant types of TEM and SHV enzymes that can hydrolyze oxyimino-cephalosporins. These enzymes were classified as group 2be with the Bush functional scheme and class A in the ambler scheme [2].

Structure of β-Lactamases

Beta-lactamases in general are globular proteins composed of alpha–helices and beta–pleated sheets. They share a common overall topology despite their variability in amino acid sequences. All ESBLs have serine at their active sites except metallo-β-lactamases which belonging to class B. They share several highly conserved amino acid sequences with PBPs [27].

The Origin and Genetic Determinants of ESBLs

The evolutionary origin and structural diversity play a significant role in the activity of ESBL enzymes. Substitution mutations of the key amino acid in the parent TEM and SHV enzymes lead to evolution of the most known ESBL types [28]. TEM-1 β-lactamase is commonly produced in Gram-negative bacteria and causing 90% of ampicillin-resistance in *E. coli*. TEM-1 can hydrolyze penicillin and early cephalosporins. TEM-2

evolved as a result of single amino acid substitution in the parent TEM-1 [2]. TEM types producing ESBL phenotypes evolved by substitution mutations in the enzyme active site. They include glutamate to lysine at position 104, arginine to either serine or histidine at position 164, glycine to serine at position 238 and glutamate to lysine at position 240 [2].

The SHV-1 β-lactamase is commonly found in *K. pneumoniae* and responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species. The changes in fewer positions within the structural gene give rise to SHV variants. It was suggested that the origin of bla_{SHV} was chromosomal mediated in *K. pneumoniae*. Evidence shows that IS*26* element played a role in the mobilization of bla_{SHV} to plasmid**.** Some reports illustrated the presence of $bla_{SHV\text{-}5}$ between two IS*26* elements together with the sequence identical to part of the *K. pneumoniae* chromosome [29].

Mutations in the OXA enzymes can give rise to ESBL phenotypes. They are commonly produced by *P. aeruginosa* and can be detected in many other Gram-negative bacteria [29].

In general, the amino acid substitutions in limited positions within the active site of TEM, SHV and OXA enzymes lead to various changes that affect their structures and activities. The active site of these enzymes may expand to hydrolyze the oxyimino-cephalosporins and monobactams. In addition, they can hydrolyze specific oxyimino-cephalosporins, and increase their susceptibility to β-lactamase inhibitors [29].

The selection pressure that drives the emergence of ESBLs has usually been attributed to the intense use of oxyimino-β-lactams, mainly the third generation cephalosporins. ESBL is characterized by highly selective substrate preference [2]. The selection of a particular enzyme variant in a given center has frequently been attributed to the specific profile of

antibiotic use but such a correlation has not always been observed [30]. The strong selective pressure for the use of β-lactam drugs exerted on ESBL producer strains may lead to the selection of strains that hyperproduce ESBL, the emergence of strains expressing different types of ESBLs, the selection of complex mutant enzymes with inhibitor resistant phenotype or porin alteration that lead to the development of resistance to cephamycins and other antimicrobials [29].

Among the non-TEM, non-SHV ESBLs, the CTX-M β-lactamases are the most prevalent. Unlike most (but not all) TEM and SHV-derived ESBLs, CTX-M β-lactamases hydrolyze cefotaxime and Ceftriaxone better than they do ceftazidime. It also appears that CTX-M enzymes are more readily inhibited by tazobactam than they are by clavulanic acid. CTX-M β-lactamases are commonly found in *E. coli* and *K. pneumoniae* [31].

The origin of the CTX-M enzymes is different from the mutated origin of TEM and SHV ESBLs. CTX-M ESBLs were acquired from other bacteria by horizontal gene transfer by conjugative plasmid or transposon. The gene sequences encoding CTX-M enzymes show a high similarity to those of β-lactamases of *Kluyvera* species. In addition, sequences of CTX-M adjacent genes of Enterobacteriaceae are also similar to those flanking the β-lactamase genes on the chromosomes of *Kluyvera* species [6].

The substrate specificity for hydrolysis in the CTX-M β-lactamases is determined by substitution mutations: Asn104, Asn132, Ser237, and Asp240. Analysis of the crystal structure of CTX-M enzymes has revealed that the active sites of CTX-M enzymes resemble those of narrow-spectrum TEM and SHV enzymes (TEM-1, SHV-1) and are not large enough to recognize ceftazidime, which is larger than cefotaxime. However,

substitution of several amino acids improves the activity of CTX-M enzyme against ceftazidime as seen in CTX-M-14, CTX-M-15, CTX-M-16 and CTX-M-27 [27]. Substitutions of Asp240 and Pro167 are known to lead to such alteration in a hydrolytic profile. Asp240Gly substitution appears to increase the flexibility of B3 β-strand allowing an increase in the activity against ceftazidime. Mutation at Pro167 in the omega-loop modifies the interaction between β-lactams and the binding sites as well [32].

β-Lactam inhibitors such as sulbactam, clavulanate, and tazobactam are generally known as inactivators of class A ESBLs. However, the mutation that affect CTX-M-14 and CTX-M-44 makes these enzymes active against sulbactam, while clavulanate and tazobactam retain their ability to inactivate them [33].

ESBL Types:

SHV

SHV-1 is a β-lactamase capable of hydrolyzing penicillins and narrow-spectrum cephalosporins. Most isolates of *K. pneumoniae* harbor chromosomal SHV-1 β-lactamase. SHV-1 is also common as a plasmid-mediated β-lactamase among Gram-negative bacteria [34]. Plasmid mediated SHV-2 with ESBL phenotype was first reported in clinical isolates of *K. pneumoniae, K. ozaenae,* and *S. marcescens* in Germany, 1983 [34]. SHV-2 was found to be more active against cefotaxime than ceftazidime [2]. SHV-2 differs from SHV-1 by one amino-acid substitution of Gly 238 Ser. Thereafter, several SHV variants with ESBL activity have been described. Most have a Gly238Ser substitution in common. In addition, a number of variants related to SHV-5 also have a Glu240Lys substitution. Ser- 238 is essential for cefotaxime hydrolysis whereas

additional Glu240Lys substitution increases the hydrolytic activity against ceftazidime [34].

TEM

In 1965, the first plasmid-mediated TEM-1 β-lactamase was reported in *E. coli* isolate recovered from *Tem*oniera, a female patient in Athens, Greece [2]. TEM-1 has substrate and inhibition profiles similar to those of SHV-1. Soonafter, TEM-2 was discovered and has hydrolytic activity as TEM-1 but differ from it by single amino acid substitution.

In France 1984 [2], TEM-3 with ESBL phenotype was detected in *K. pneumoniae* isolates harbored a novel plasmid-mediated β-lactamases with enhanced activity against cefotaxime. TEM-3 differs from TEM-2 by two amino acid substitution: Lys for Glu at position 102 and Ser for Gly at position 236 [34]. More than 150 TEM-type β-lactamases have been described eversince. The majority of these enzymes are ESBL types. The amino acid changes in comparison with TEM-1 and TEM-2 are documented at http://www.lahey.org/studies/ temtable.htm [35]. Some of the TEM variants reveal the characteristics of inhibitor-resistant β-lactamases with low activity against oxyimino-cephalosporins [2]. Other TEM variants combined the hydrolytic activity against oxyimino-cephalosporins together with inhibitor resistance [2]. These enzymes are referred to as complex mutants of TEM (CMT). A CMT enzyme possesses both of the amino acid substitutions observed in TEM ESBLs and those observed in inhibitor-resistant TEMs. CMT β-lactamases poses a challenge in detection of ESBLs in clinical practices. Inhibition of ESBLs by β-lactamase inhibitors as a phenotypic methods used in detection of ESBLs is not applicable to CMT β-lactamases.

CTX-M

The CTX-M β-lactamases was first reported in 1989 [34]. It was recovered from clinical *E. coli* isolate. The hydrolytic activity of CTX-M β-lactamases is more potent against cefotaxime than ceftazidime and so called CTX-M-1. In 1992 [34], a new plasmid-mediated cefotaximase, designated CTX-M-2, with an isoelectric point different from that of CTX-M-1, was described from multidrug-resistant *S. enterica* serovar *Typhimurium*. CTX-M-44 (Toho-1) was reported in 1995 [36] with high homology in amino acid sequence to CTX-M-1. Currently, more than 70 CTX-M enzymes have been described [35]. According to homology of their amino acid sequences, CTX-M β-lactamases are divided into five subgroups named CTX-M-1, CTX-M-2, CTXM-8, CTX-M-9, and CTX-M-25 [34].

On agar media, CTX-M producers are often cefotaxime resistant while ceftazidime susceptible. However, some CTX-M types like CTX-M-15 and CTX-M-16 have potent activity against both cefotaxime and ceftazidime. Additionally, most CTX-M enzymes hydrolyze cefepime effectively and MIC values of cefepime for bacteria producing CTX-M enzymes tend to be higher than those producing other types of ESBLs [2]. Organisms producing TEM and SHV-type ESBLs are identified mainly from hospitalized patients. A growing number of infections caused by CTX-M producing organisms have recently been reported in the community [2]. In community acquired UTIs, *E. coli* was the main causative organisms.

GES

GES-1 was first reported in a strain of *K. pneumoniae* isolated from a neonatal patient in France [37]. GES-1 has hydrolytic profile like other ESBL types. However, a Gly170 substitution inside the omega-loop appears to alter the substrate profile of the enzyme. GES-2, which has Gly170Asn substitution compared with GES-1, has an increased activity against imipenem and decreased activity against oxyimino-cephalosporins [38]. In addition, β-lactamase inhibitors only weakly inhibit GES-2. GES-4, which has a Gly170Ser substitution compared with GES-3, is also capable of hydrolyzing carbapenems and weakly inhibited by β-lactamase inhibitors. Furthermore, GES-4 extends its hydrolytic activity towards cephamycins [39].

OXA

The OXA type ESBLs was first reported in *P. aeruginosa* isolates from a single hospital in Ankara, Turkey [2]. They are classified in class D according to the Ambler scheme and in group 2d according to the Bush functional scheme (table 4) [2]. OXA enzymes are commonly produced in *P. aeruginosa* and to a lesser extent in other Gram-negative bacteria. Up to 10% of the *E. coli* isolates, produce OXA-1 type β-lactamase. OXA enzymes have variable inhibition profile by β-lactamase inhibitors [40]. In addition, Most OXA β-lactamases have only minor activity against oxyimino-cephalosporins. However, OXA-10 and its derivatives (OXA-11, OXA-14, OXA-16, and OXA-17), OXA-13 and its derivatives (OXA-19 and OXA-32), and some other OXA enzymes (e.g., OXA-18 and OXA-45) have varying degrees of activity against oxyimino-cephalosporins [2, 41].

These enzymes are regarded as OXA-type ESBLs and have been discovered mainly in *P. aeruginosa* isolates.

Other ESBL Types

A number of other groups of β-lactamases capable of hydrolyzing extended-spectrum cephalosporins have been reported. VEB-1, PER-1 and BES-1 are less commonly encountered genes encode β-lactamases capable of hydrolyzing third generation cephalosporins. Most of these enzymes are plasmid mediated and have remarkable geographic diversity [2].

Risk Factors for Colonization and Infection

Several risk factors associated with the acquisition of ESBL infections are suggested. Serious illness, chronic infection and prolonged hospitalization predispose infections with ESBL producers. Patients with Medical devices such as endotracheal tubes or urinary catheters and extensive use of antibiotic are also at risk [42]. There is a direct relationship between antibiotic use and emerging resistance and it has been suggested that restriction of some antibiotics, particularly third generation cephalosporins, may reduce resistance and improve patient's response [43]. Patients colonized with ESBL organisms are usually elderly, neonates, patients admitted to ICUs are all considered at risk for ESBL infection. Antibiotic treatment as well as colonized and infected patients could be risk factors for transmission of resistant strains to other patients. Many investigators observed an association between ESBL production and mortality rates in patients with prolonged hospital stays [1].

Epidemiology of ESBL-Producing Organisms

The ESBL-producing organisms were primarily in Europe [2], Germany [2] and England [2] at first then in France [2]. Currently, ESBL- producing organisms are spread throughout the world and were reported in all continents.

The majority of ESBL producers are members of the Enterobacteriaceae family, predominantly in *E. coli* and *Klebsiella* spp. However, ESBL β-lactamases in other gram negative bacteria are not as common and mostly reported in *P. aeruginosa* and *Acinetobacter* isolates [2]. The information provided in the Meropenem Yearly Susceptibility Test Information Collection (MYSTIC) study [44] reflects recent global trends in Enterobacteriaceae expressing the ESBL phenotype (Table 5).

Table 5. Percentage of organisms expressing ESBL phenotype in MYSTIC study, 1997–2003 [44].

Isolates with ESBL phenotype			
	E. coli (%)	*K. pneumoniae* (%)	*P. mirabilis* (%)
North America	7.5	12.3	3.9
South America	18.1	51.9	6.2
Northern Europe	16.2	16.7	5.9
Southern Europe	16	24.4	20.5
Eastern Europe	28.9	58.7	21.3
Asia-Pacific	14.2	28.2	23.7

In southeastern Mediterranean region, the resistant rate to third generation cephalosporins in Enterobacteriaceae isolates was found to be 70% in Egypt, 26% in Morocco and 31% in Turkey [45].

The PanEuropean Antimicrobial Resistance using Local Surveillance (PEARLS) study (2001-2002) [46] conducted to evaluate ESBL rate on medical centers in Northern and

Southern European countries, Egypt, Lebanon, Saudi Arabia and South Africa. ESBL production rates among *E. coli*, *K. pneumoniae* and *Enterobacter* spp. was 5.4%, 18.2%, and 8.8% respectively. ESBL production rate for the Enterobacteriaceae was 10.5%. The highest rates were 38.5% in Egypt and 27.4% in Greece while the lowest rates were 2% in the Netherlands and 2.6% in Germany.

In Asia, ESBL producers remained low in Japan [47] and varied from 4.8% in Korea [48] to 12% in Hong Kong [49]. In the Kingdom of Saudi Arabia (KSA) [46], the PEARLS study (2001-2002) showed that the overall ESBL production rate from Enterobacteriaceae was (18.6%). In Riyadh [50], a tertiary care hospital collected blood culture isolates from January 2003 through December 2004. The rates of ESBL producers were 48.4% in *K. pneumoniae* and 15.8% in *E. coli*. Another study was conducted in KSA [51] describing ESBL organisms producing bacteremia. A total of 27% *E. coli* and 17.1% *K. pneumoniae* were found to be ESBL producers.

In Lebanon [52], the prevalence of ESBL-producing isolates was 8% among *E. coli* and 20% among *Klebsiella* spp. In Turkey [53] at Istanbul University Cerrahpasa hospital, ESBL organisms causing bacteremia were 40% *E. coli* and 49% *K. pneumoniae*. In Iran [54], the prevalence of ESBL among 66 Gram negative bacteria isolates from ICUs at Sanandaj's Hospitals was 34.85%. In Gaza strip, Palestine [55], the percentage of ESBLs producer among *E. coli* from community patients was 3.3%. In 2008 [56], a cross-sectional study conducted in Nasser Hospital in Southern Palestine reported a prevalence of ESBLs of 35% and 9% among *K. pneumoniae* and *E. coli* respectively. In the United Arab Emirates [57], a study conducted on hospitalized patients (130 Enterobacteriaceae ESBL isolates) had rates of 32% *E. coli*, 36% *K. pneumoniae* and 4% *K. oxytoca*.

In Israel [58], the rate of ESBLs ranges from 10% to 40% among *Klebsiella* spp. and *E. coli* isolates causing hospital-acquired infections. In a study conducted in Israel, 2003, [59] the rate of ESBLs among *E. coli* and *K. pneumoniae* combined was 25.6% with a rate of 22% (77/250) for *E. coli* and 40.5% (34/84) for *K. pneumoniae*.

The significance ESBL-producing Enterobacteriaceae as pathogens is recognized in both community and hospital acquired infections. In the case of nosocomial infections, it is mostly predominate in intensive care units, general medicine wards, as well as in long-term care facilities [44]. Other hospital units that are at increased risk include surgical wards, pediatrics and neonatology, rehabilitation units and oncology wards. Community clinics and nursing homes have also been identified as a potential reservoir [28].

The transition of ESBL-producing Enterobacteriaceae from the community into the hospital has been attributed to 'home care providers' and other factors that pose a public health threat such as food production and distribution, animal farms and sewage [44].

The ESBL variant may appear in a Center due to *de novo* selection. Selection process occurs usually in ICUs which may lead to patient's colonization or infection. Once selected, the ESBL variant may spread in the center by clonal dissemination of producer strains or horizontal transfer of the ESBL-gene carrying plasmid among non-related strains [28]. Outbreaks usually start in ICUs and then spread to other parts of the hospital. The cross-transmission of the selected resistant strains was mostly attributed to hands contamination of medical and nursing staff. Some reports indicate that contaminated ultrasound gel, thermometers, blood pressure cuffs and bronchoscopes may lead to spread of resistant strains among hospitalized patients [28].

Molecular Epidemiology of ESBL Types

The early detection of ESBLs β-lactamases was TEM and SHV derivatives commonly isolated from *Klebsiella* species. Currently, CTX-M type β-lactamases are largely increased among Gram-negative bacteria. At the same time, *E. coli* has replaced *Klebsiella* as the predominant species of ESBL-producing Enterobacteriaceae in most countries worldwide [60].

More than 300 types of ESBL β-lactamases have been described. Some of the ESBL types are commonly spread among countries while others are variable. Comparing the United States with Europe, TEM-3 ESBL producing strains have only been found in France but not in the United States while TEM-10 is common in both countries [25]. SHV-5 β-lactamase is very common worldwide and has been found in France, Greece, Poland, Hungary, South Africa, the United Kingdom and the United States. SHV-12 is the most common ESBL found in Korea [61]. CTX-M groups as $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-9}}$ are endemic in Spain [62]; *bla*CTX-M-2 predominates in South America, Japan, and Israel [63] while *bla*CTX-M-15 is distributed worldwide and commonly found in Europe (except for Spain), North Africa, the Middle East and Canada [64].

In neighboring countries in Lebanon [65], the prevalence of $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV} in ESBLs producing *E. coli* was 96%, 57% and 67 while among *Klebsiella* isolates was 40%, 82% and 88% respectively. Sequence analysis revealed that CTX-M-15 and TEM-1 and six SHV types are the dominant types. In Egypt [66], 66% of the ESBL producing Gram negative isolates were CTX-M group one, mainly CTX-M-15 type. In Israel [67, 68], CTX-M- 2 and CTX-M-25 were found to be the major ESBL groups detected among CTX-M types produced by Enterobacteriaceae spp. In Algeria [69], CTX-M-15 was

reported as common CTX-M types followed by CTX-M-3. In Kuwait [70], CTX-M-15 was detected as the common CTX-M types followed by CTX-M-9.

Detection Methods for ESBLs

Numerous detection strategies have been developed throughout the world to identify ESBLs producing organisms. National breakpoints regarding ESBL are quite different. According to CLSI criteria, resistance to ceftriaxone, cefotaxime, ceftazidime, cefepime, and aztreonam is defined by MICs $\geq$ 16 μg/ml [71]. Treatment of infections caused by ESBL-producing organisms with extended-spectrum cephalosporins or aztreonam may result in treatment failure even when the causative organisms appear to be susceptible to these antimicrobial agents by routine susceptibility testing [2, 72]. In addition, several ESBL producers have MIC values for extended spectrum cephalosporins and aztreonam below the standard breakpoints for resistance (e.g., between 2 and 8 μg/ml). Revision of cephalosporin breakpoints has been achieved by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and is under way by the CLSI for better prediction of clinical outcome by MIC values [73].

Since the 1980s, several phenotypic tests for detection of ESBL-producing organisms were developed. All methods utilize the characteristics of ESBLs: conferring a reduced susceptibility to extended-spectrum cephalosporins and inhibition by clavulanate. Detection of ESBL production by organisms with inducible chromosomal AmpC β-lactamase is difficult using these methods because AmpC β-lactamase resists inhibition by clavulanate. In addition, clavulanate may act as an inducer of chromosomal AmpC β-lactamases of these organisms [74].

Recommended Methods by CLSI

The CLSI recommends screening *E. coli*, *K. pneumoniae*, and *K. oxytoca* (and isolates of *P. mirabilis* causing bacteremia) for potential production of ESBL. The CLSI method for ESBL detection consists of an initial screening test followed by phenotypic confirmatory test [75].

Initial screening can be performed by several methods. Disk diffusion and broth dilution are common screening methods utilizing third generation cephalosporins like cefpodoxime, ceftazidime, ceftriaxone, cefotaxime, and aztreonam. A decrease in susceptibilities to one or more antibiotics tested may indicate production of ESBLs and warrant performance of the subsequent phenotypic confirmatory test. Other screening method can be applied by using 1 or 2 µg/ml third generation cephalosporins containing MacConkey plates. After overnight incubation, any growth considered as positive screen. In the phenotypic confirmatory test (Figure 4), susceptibilities to cefotaxime and ceftazidime alone, and those with clavulanate are compared using disk diffusion or broth dilution method. If the susceptibility of either antibiotic tested increases significantly (a ≥5 mm increase in a zone diameter or a ≥3 two-fold decrease in an MIC) in the presence of clavulanate, the result indicates confirmation of ESBL production. It is important to perform confirmatory tests using both ceftazidime and cefotaxime to improve the sensitivity of the test. The use of ceftazidime alone sometime can not detect certain ESBLs types particularly CTX-M one [76].

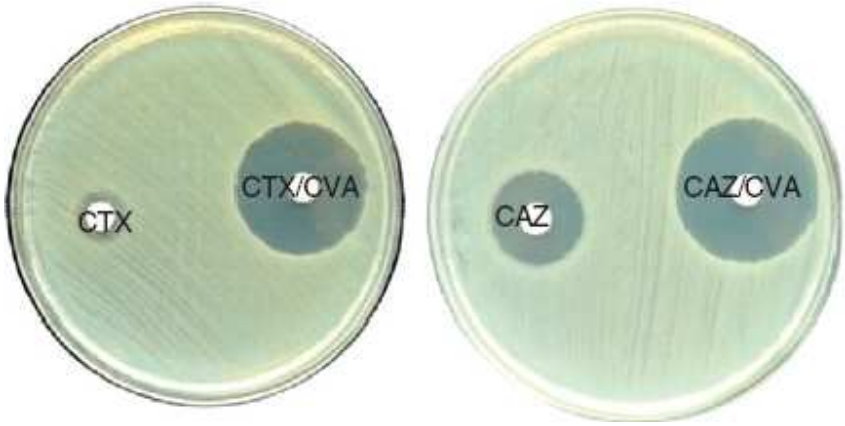

Figure 4. Positive CLSI recommended method for ESBL producing strains. The inhibition zone around the CTX/CVA disk is apparently larger than that around the CTX disk, indicating ESBL production. Comparable result is obtained with CAZ disk and CAZ/CVA disk. Note that the inhibition zone diameter around the CAZ disk is within the susceptible range (≥18 mm). Adherence to the protocol and the use of both CTX and CAZ disks are crucial for the highly sensitive detection of ESBL production [34].
CTX, cefotaxime; CAZ, ceftazidime; CVA, clavulanic acid.

Double Disk Synergy Test

Double disk synergy test (DDST) was the first proposed testing method for phenotypic detection of ESBL-producing organisms [77]. DDST is usually performed on an agar plate with a disk containing cefotaxime (30 μg) and a disk containing amoxicillin/clavulanate (20 μg/10 μg, respectively), placed 30 mm apart (center to center). Extension of the inhibition zone around the cefotaxime disk towards the amoxicillin/clavulanate disk indicates production of ESBL (Figure. 5). Disks containing other oxyimino-β-lactams (ceftriaxone, ceftazidime, or aztreonam) can be substituted for cefotaxime disk and performance of this test using multiple oxyimino-cephalosporins improves the sensitivity of DDST in the same way as observed in the CLSI method. If the result of DDST is negative despite the high suspicion of ESBL production, adjustment of disk spacing is recommended [78].

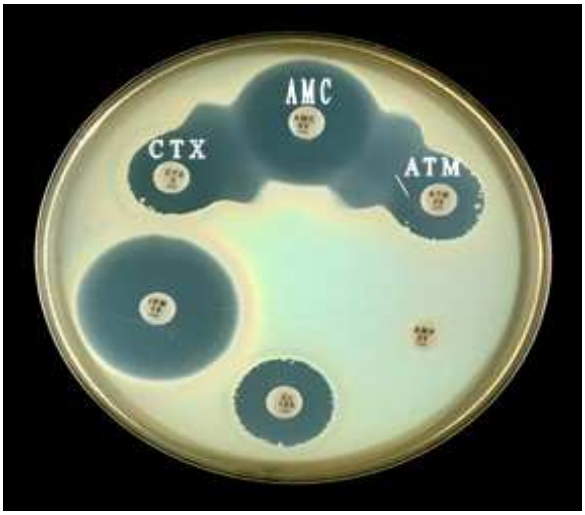

Figure 5. Positive DDST. *K. pneumoniae* producing an ESBL: synergy between AMC (amoxicillin/clavulanate 20 μg/10 μg)/ CTX (30 μg cefotaxime) or ATM (30 μg aztreonam).

In addition, the use of cefepime instead of third-generation cephalosporins improves the sensitivity of DDST when applied for AmpC producing Enterobacteriaceae (e.g., *Enterobacter* spp, *K. pneumoniae* producing plasmid-mediated AmpC β-lactamase) [79]. This phenomenon is reasonable because cefepime is stable against hydrolysis by most AmpC β-lactamases.

Other tests for ESBL detection are described. Among these tests are the Etest and automated VITEK 2 and Phoenix [34].

Molecular Detection of ESBL

The phenotypic ESBL detection methods described above provide only presumptive identification of an ESBL producer. The most common molecular method used to detect the presence of a β-lactamase is polymerase chain reaction (PCR) [29]. In previous studies, oligonucleotide primer sets for TEM-, SHV- and OXA-type β-lactamases were

used in multiplex PCR to amplify internal regions in the target β-lactamase genes. Therefore, because the oligonucleotide primer sets used do not amplify the specific point mutations in the ESBL gene both classical and ESBL types will be amplified and detected. Nucleotide sequence analysis of the resulting amplicons is therefore required to confirm ESBL production. Other PCR-based detection methods involve the design of specific primers targeting the specific ESBL mutations. However, in this case, for each new point mutation that occurs in ESBLs new primers must be designed.

Control of Infections Due to ESBL-Producing Organisms

Organisms producing ESBLs enzymes have caused many local and national outbreaks, mostly within specialized hospital units. Resistance in *E. coli* is important because the organism is the most common Gram-negative pathogen, widespread among populations, and is important in healthcare settings other than hospitals. Occasionally, control procedures are unable to eliminate severe infections and require treatment with carbapenems. Moreover, recent widespread emergence of multiresistant strains of *E. coli* with CTX-M ESBLs and various pathogenecity factors has further complicated control measures creating an important international issue [80].

Termination of outbreaks due to resistant bacteria often involved a previously unused antibiotic class as at the times of introductions of trimethoprim, cephalosporins and quinolones. These agents are potent in modifying the gut flora, reducing carriage of epidemic resistance. However, emergence of multiresistant strains and the fact that no new antibiotic classes are available complicates the control of CTX-M-producing strains [80].

Multiresistant strains can be controlled by reducing selection pressure by avoiding all agents to which the strain or plasmid donor organisms are resistant in the carriage site, mainly the gut for *E. coli*. Antibiotics active against ESBL producers like β-lactam/β-lactamase inhibitor or aminoglycosides (amikacin) may reduce the chance of carriage [81]. Controls of infections are further enforced by minimizing the frequency of procedures that carry a risk of promoting infection from colonization. Urinary catheterization, endotracheal or nasogastric intubations, gut surgery and induction of achlorhydria all increase the risk of infection by transferring resistant strains from colonization to infection sites [82]. Antibiotic prophylaxis in those colonized with ESBL producer organisms should be modified against the colonizing strain**.**

Ensuring adequate hand hygiene and staff／patient ratios minimize the risk of inter patient spread of resistant strains. Admission from other hospitals or from residential care should generate automatic alerts to detect readmitted patients previously found to carry multiresistant ESBL producers. Staff in the transferring units should notify the receiving infection control staff of such transfers. Single-room accommodation, or cohort isolation, for both current and previous cases, may improve control measures and diminish the chances of spread [80].

Treatment

Antibiotic choices for infections caused by ESBL-producing organisms are limited. Treatment of these infections with cephalosporins (except for cephamycins) has been associated with poor clinical outcomes, even if the causative organisms appeared to be susceptible to the antibiotics *in vitro*. Furthermore, ESBL-producing isolates tend to

show a high rate of resistance to various other classes of antibiotics such as fluoroquinolones and aminoglycosides [34].

Carbapenems are regarded as the drugs of choice in treating of infections caused by ESBL-producing organisms. Treatment with carbapenems showed a significant improves in clinical outcome than that with other antibiotics [34].

Cephamycins (e.g., cefoxitin and cefotetan) are active against ESBLs. However, a decrease in the expression of outer membrane protein may occur during the treatment of ESBL-producing organisms and results in resistance to cephamycins. Inducible or constitutive production of AmpC β-lactamase also leads to resistance to cephamycins. Thus, cephamycins are not recommended as first-line therapy for infections caused by ESBL producing organisms [83].

β-Lactam/β-lactamase inhibitor combinations (e.g., amoxicillin/clavulanate and piperacillin/tazobactam) often retain activity against ESBL-producing organisms, but coexistence of other resistance mechanism may lead to resistance to these drugs [34].

Fluoroquinolones may be useful to treat mild infections if the causative organisms are susceptible *in vitro*. Treatment with fluoroquinolones of urinary tract infection without bacteremia is relatively safer than that of bacteremia because of the very high drug concentrations achieved in the urine [34].

Aminoglycosides are effective therapy against ESBL producing pathogens when the organism has a MIC significantly lower than susceptibility breakpoints. Susceptibility to amikacin seems to be preserved, in contrast to gentamicin and tobramycin, thus justifying its use as empiric therapy [84].

Tigecycline can be considered an alternative to carbapenems for treatment of infections due to ESBL-producers. CLSI criteria to interpret susceptibility testing of tigecycline are not yet established .The Food and Drug Administration (FDA) and EUCAST have created temporary resistance breakpoints for tigecycline (MIC $\geq$ 4 µg/ml and $\geq$ 2 µg/ml, respectively). However, clinical experience with tigecycline is still evolving [27].

Statement of the Problem

ESBL-producing *E. coli* strains are implicated in causing hospital-acquired infections comprising a real threat to seriously ill patients. In addition, treatment choices are becoming limited as consequence of multiresistance phenotypes associated with ESBL producers. Moreover, there are rare data concerning molecular epidemiology of ESBL-producing *E. coli* in Palestine.

Objectives

The objectives of this study are:

1. To determine genetic characteristics and molecular epidemiology of ESBL-producing *E. coli* isolated from Al-Makassed Hospital in Jerusalem, Palestine.
2. To evaluate resistance status in ESBL-producing *E. coli* isolates by determining MICs to ampicillin, amikacin, ceftazidime, cefotaxime, ceftriaxone, gentamicin, augmentin, levofloxacin, meropenem and sulfa drugs.
3. To compare molecular characteristics and susceptibility profiles of the third generation cephalosporins in ESBL-producing *E. coli* isolated from urine and feces in the same patients.

MATERIALS AND METHODS

Specimens Collection

Consecutive non-duplicate 423 clinical *E. coli* isolates were screened for ESBL production from patients attended to Al- Makassed Islamic Charitable Hospital at Jerusalem from February to June 2009. Makassed Hospital is a leading medical center in Palestine with 250 beds capacity and providing secondary and tertiary health services to patients from all over Palestine. The majority of samples recovered from hospitalized patients in different hospital wards and rarely from outpatient clinics and emergency room. *E. coli* isolates were recovered from urine, rectal, wound, sputum, nasal, high vaginal swabs, skin, body fluids, blood and catheter devices.

Non-repetitive isolates of *E. coli* were included in this study whether it has come from true infection or patient's colonization. Colonization of *E. coli* was detected either at admission time or during hospital stay by screening patients using swabs. Whenever possible, stool specimen was simultaneously collected with the urine sample from the same patients having clinical signs of UTI. This allowed the evaluation of phenotypes and genotypes of ESBL-producing *E. coli* in both specimens.

Bacterial Identification

Identification of isolates at species level was carried out using Blood, MacConkey and chromogenic media followed by hy.enterotest system (Hy.labs, Israel) and conventional citrate tube methods. All isolates of *E. coli* that grow in the initial ESBL screening

method were preserved in broth media containing 10% glycerol and stored at -70^{o} C until further use.

Susceptibility Detection Methods of ESBL-Producers

Antimicrobial susceptibility tests followed in this study were determined according to the recommendations of the CLSI [75].

Screening Method

The ESBL production was initially screened using 1% cefotaxime (CTX) containing MacConkey plates. ESBL-positive isolates were identified for any growth after overnight incubation. The ESBL-positive screens were further checked by double disk synergy test (DDST) according to guidelines of CLSI [75].

Bacterial suspension equal to 0.5 McFarland (10^{8} CFU/ml) was prepared in normal saline and inoculated on Mueller-Hinton agar plates (MHA) (Oxoid, UK). One disk containing 30µg ceftriaxone (Mast group, UK) and the other containing 20µg/10µg amoxicillin/clavulanate (Oxoid, UK) were placed ≤30 mm apart on Mueller-Hinton plates. After overnight incubation, an extension of the inhibition zone around the ceftriaxone disk towards the amoxicillin/clavulanate disk indicates production of ESBL. The use of more than one third generation cephalosporins disc as cefpodoxime improved the sensitivity of the result.

Confirmation Method

Separated bacterial colonies from overnight growth on 5% blood agar plates (BAP) were emulsified in Mueller-Hinton broth (Oxoid, UK). The inoculum turbidity equivalent to 0.5 McFarland standard (1x 10^8 CFU/ml) was achieved by adjusting the density to 0.08-0.13 absorbance at λ 625 nm. The prepared suspension was inoculated on MHA plates (Oxoid, UK) following CLSI recommendations. Ceftazidime (30µg) disc, (Oxoid, UK) and cefotaxime (30µg) disc, (Oxoid, UK) alone as well as ceftazidime-clavulanate (30µg /10µg) disc (Becton, Dickinson and company Sparks, USA) and cefotaxime-clavulanate (30/10µg) disc, (Becton, Dickinson and company Sparks, USA) were placed apart on inoculated MHA plates. After overnight incubation at 35^o C, an increase in the zone diameter of ≥5 mm of either antibiotic tested in the presence of clavulanate compared to each disc alone was interpreted as confirmatory for ESBL production. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 strains were used as control bacteria.

Minimum Inhibitory Concentration (MICs)

The MICs obtained on all ESBL-producing *E. coli* for different antimicrobial agents are shown in Table 6. MICs were determined according to CLSI guidelines by the Agar Dilution Method using Steers-Replicator. This allows the testing of 37 microorganisms simultaneously on a single agar plate as shown in Figure 6. The antibiotic concentration of the first plate showing ≥99% inhibition is taken as the MIC for the organism. Both *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as control strains for MIC determinations with each antimicrobial agent. The CLSI breakpoints for the MICs of all antibiotics used in this study are listed in Table 7.

Table 6: A list of antimicrobial agents, their potencies and MIC ranges.

Antibiotics	Potency %	Company	MICs rang (two folds)
Ampicillin (AMP)	99.8	Birzeit pharmaceutical Co., Palestine	1-128
Amikacin (AK) as Amikacin sulfate	99.8	Intramed, South Africa	1-128
Ceftazidime (CAZ) as pentahydrate	89.4	GlaxoSmithKline, Italy	1-128
Cefotaxime (CTX)	100	Birzeit Pharmaceutical Co., Palestine.	1-128
Ceftriaxone	100	Birzeit Pharmaceutical Co., Palestine	1-128
Gentamicin	100	Birzeit Pharmaceutical Co., Palestine	1-128
Augmentin	100	GlaxoSmith Kline, UK.	1-128
Levofloxacin	100	AVENTIS, Germany	1-128
Meropenem	74.1	AztraZeneca UK.	0.06-8
Trimethoprim / sulpham-ethoxazole	100%	GlaxoSmithKline, Israel	Trimethoprim/ sulphamethoxazole 1-128

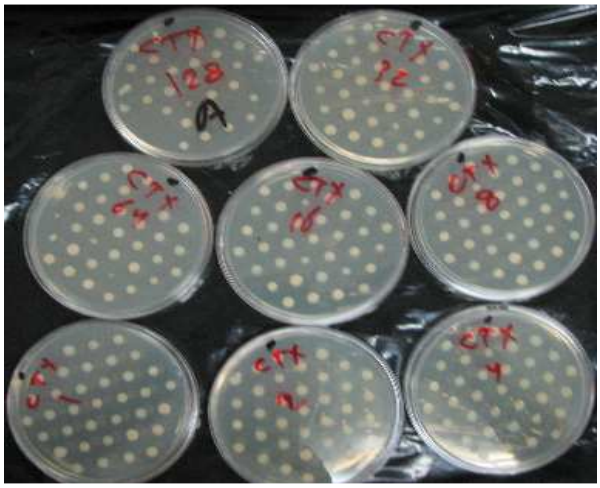

Figure 6: Representative agar dilution method for MICs determination.

Table 7. MICs breakpoints of the antimicrobial agents used in this study according to CLSI 2005.

Antimicrobial agents	MICs breakpoints		
	S≤	I	R≥
Ceftriaxone	8	16-32	64
Cefotaxime	8	16-32	64
Ceftazidime	8	16	32
Ampicillin	8	16	32
Gentamicin	4	8	16
Amikacin	16	32	64
Amoxicillin/clavulanic acid	8	16	32
Trimethoprim/sulfamethoxazole	2	4	8
Levofloxacin	2	4	8
Meropenem	4	8	16

S: Susceptible, I: Intermediate, R: Resistant

Molecular Methods

DNA Extraction

One loopful (10µl) of bacterial colonies taken from an overnight BAP was suspended in 0.5 ml sterile distilled water and heated at 95° C for 10 min. After centrifugation at 13,000 rpm for 2 min at 4° C, the DNA-containing supernatant was used as template for further amplification [85].

Control Strains

bla$_{\text{SHV-18}}$ containing *K. pneumoniae* ATCC 700603 and *bla*$_{\text{CTX-M}}$ containing *K. pneumoniae* strain (kindly provided by Dr. Mousa Hendya, Caritas Ped. Hospital Lab., Bethlehem, Palestine) were used as positive controls for *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M}}$ amplification. *bla*$_{\text{TEM}}$ was recognized throughout the isolates after optimizing the PCR condition and comparing the amplicon size with 100 bp ladder.

Single PCR Amplification for *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX-M}$

A single PCR amplification from genomic DNA was performed on each isolate for the presence of genes encoding SHV, TEM, and CTX-M β-lactamases. The oligonucleotide PCR primers specific for the β-lactamases genes, annealing temperature, and PCR amplicon size are listed in Table 8.

Table 8. Target genes, Primers sequence, annealing temperatures and product size of amplified genes.

Primer target genes	Sequences 5`-3`	Annealing Tem. (°C)	Product size (bp)	Manufacturer	Ref
CTX-M UNV-F CTX-M UNV-R	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG K is G or T, R is A or G, S is G or C, and Y is C or T	58	593	invitrogen	[86]
CTX-M-1 F CTX-M-1 R	AAAAATCACTGCGCCAGTTC AGCTTATTCATCGCCACGTT	57	415	invitrogen	[87]
CTX-M-2 F CTX-M-2 R	CGACGCTACCCCTGCTATT CCAGCGTCAGATTTTTCAGG	57	552	invitrogen	[87]
CTX-M-9 F CTX-M-9 R	CAAAGAGAGTGCAACGGATG ATTGGAAAGCGTTCATCACC	57	205	invitrogen	[87]
CTX-M-8/25 F CTX-M-8/25 R	CTTTGCCATGTGCAGCACC GCTCAGTACGATCGAGCC	57	305	invitrogen	[88]
TEM F TEM R	CGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT	52	444	invitrogen	[86]
SHV F SHV R	ATGCGTTATATTCGCCTGTG TGCTTTGTTATTCGGGCCAA	55	747	invitrogen	[86]

F: Forward, R: Reverse

The amplification was performed in a PCR thermocycler (C1000, Thermocycler, BioRad) using Eppendorf tubes. The reaction components for each single amplicon were 12.5µl Go Taq Green Master Mix, 2X (Promega), 0.5µl forward primer (0.2µM), 0.5µl of reverse primer (0.2µM), 5µl DNA template, and 6.5µl nuclease free water in a final volume of 25µl.

The PCR conditions were initial denaturizing step at 95° C for 6 minutes, 30 cycles of: 94° C for 30 seconds, annealing for 30 seconds at primer set specific annealing temperature (Table 8), and extension at 72° C for 50 seconds. This was followed by a final extension step at 72° C for 6 minutes. A negative (distilled water instead of template) and positive controls were used in each PCR run.

Amplification of *bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{CTX-M}$ by Multiplex PCR

The genes encoding SHV, TEM, and CTX-M β-lactamases were also simultaneously detected by multiplex PCR. Optimization the multiplex PCR conditions were achieved by using an *E. coli* isolate containing TEM, SHV and CTX-M genes.

The reaction mixture was the same as single PCR except that the volume of nuclease free water was adjusted to 4.5µl, and 0.5µl (0.2µM) foreach forward and reverse primers of each gene in a final volume of 25 µl.

The multiplex PCR conditions were initially performed using annealing temperature gradient in 20^{o} range (45-65° C). All subsequent amplifications were carried out at an annealing temperature of 54° C. The other multiplex PCR conditions were the same as those described for single PCR.

Detection of CTX-M Groups by Multiplex PCR

More than 70 CTX-M types β-lactamase were determined. These β-lactamases were divided into five phylogenetic groups. The primer pair's sequences for detection of each group, reference, and its amplicon size are described in Table 8. A single primer pair was used to amplify both CTX-M 8 /25 groups.

The amplification mixture was composed of the following: 12.5µl Go Taq Green Master Mix, 2X (Promega), 0.5µl of each CTX-M group forward primers (0.2µM), 0.5µl of each CTX-M group reverse primers (0.2µM), 5µl DNA template, and 3.5µl nuclease free water in a final volume of 25µl.

The PCR conditions were initial denaturizing step at 95° C for 6 minutes, 30 cycles of: 94 ° C for 30 seconds, annealing at 57^{o} C for 30 seconds and extension at 72° C for 50 seconds. This was followed by a final extension step at 72° C for 6 minutes. A negative control (distilled water instead of template) was used in each PCR run. Determination of each CTX-M group amplicon was recognized by comparing the bands obtained with known 100 bp ladder.

Agarose Gel Electrophoresis

PCR products (5µl) were run in an electrophoresis tank (HU6, SCIE-PLAS®, UK) containing 1X TAE buffer (Kibbutz Beit Haemek, Israel) using a 1.7% (w/v) agarose gel (hy.labs, Israel) containing 1µg/10mL ethidium bromide at 90 V (10 V/cm) for a period of 30 minutes. A 100-bp DNA ladder (GeneDirex, USA) (Figure 7) was run in parallel with PCR products to assess the size of amplicons. Thereafter, gels were visualized on a transilluminator and photographed (White/UV model, TMW-20 Transilluminator, CAMLAB, USA).

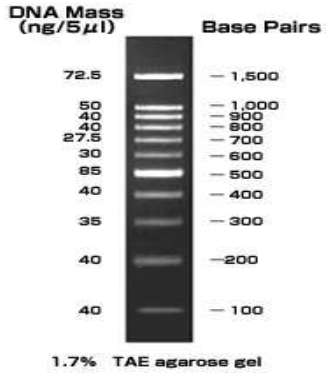

Figure 7. 100bp ladder ready to use (GeneDirex, USA).

RESULTS

Sample Identification

A total of 77(18.2%) ESBL-producers were identified through screening 423 consecutive non-duplicate *E. coli* isolates from patients admitted to Al-Makassed Islamic Charitable Hospital in Jerusalem from February to June 2009. The recovered ESBL-producing *E. coli* isolates from various clinical sources and different hospital wards are demonstrated in Figure 8 and Table 9. The majority of ESBL isolates, 51/77 (66.3%) were from surgical, orthopedic, and pediatric wards. In addition, rectal and urine sources represented 56/77 (72.4%) of the ESBL-producers.

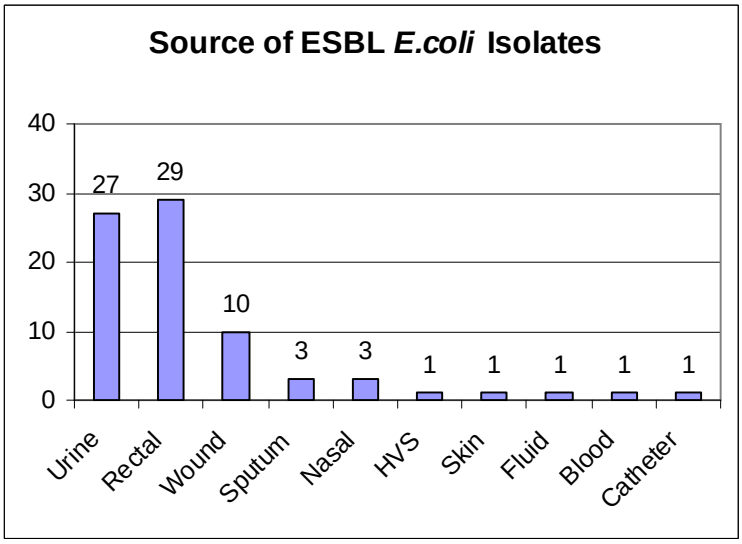

Figure 8. Clinical sources of ESBL-positive *E. coli* isolates.

Table 9. Sources of ESBL producing *E. coli* isolates and its distribution among hospital wards.

wards	Specimens source										
	Urine	Wound	Rectal	Sputum	Nasal	HVS	Skin	Fluid	Blood	Catheter	No. (%)
SW	4	3	6	0	0	1	1	1	0	0	16 (20.8)
OW	6	5	2	0	0	0	0	0	0	1	14 (18.2)
PW	4	1	15	0	1	0	0	0	0	0	21(27.3)
OPC	2	0	0	0	0	0	0	0	0	0	2 (2.6)
MW	4	0	0	1	0	0	0	0	0	0	5 (6.5)
NICU	1	0	0	0	0	0	0	0	0	0	1 (1.3)
CCU	3	0	0	1	0	0	0	0	0	0	4 (5.2)
PICU	1	0	2	0	0	0	0	0	0	0	3 (4)
POH	1	0	3	1	0	0	0	0	0	0	5 (6.5)
AICU	0	1	0	0	1	0	0	0	1	0	3 (4)
ER	1	0	1	0	1	0	0	0	0	0	3 (4)
No. (%)	27 (35.1)	10 (12.4)	29 (37.7)	3 (4)	3 (4)	1 (1.3)	1 (1.3)	1 (1.3)	1 (1.3)	1 (1.3)	77 (100)

SW: Surgical ward, OW: Orthopedic ward, PW: Pediatric ward, OPC: Outpatient clinic, MW: Medical ward, NICU: Neonate intensive care unit, CCU: Cardiac care unit, PICU: Pediatric intensive care unit, POH: Pediatric open heart, AICU: Adult intensive care unit, ER: Emergency room, HVS: High vaginal swab.

Antimicrobial Susceptibility Testing

ESBL Screening. The initial screening of all *E. coli* isolates was performed using 1µg/ml cefotaxime containing MacConkey agar plates. A total of 82/423 (19.4%) of *E. coli* isolates were detected as a positive ESBL by this method.

Double Disc Synergy Test. The ESBL positive screens were further checked by double disk synergy test (DDST) as shown in Figure 9.

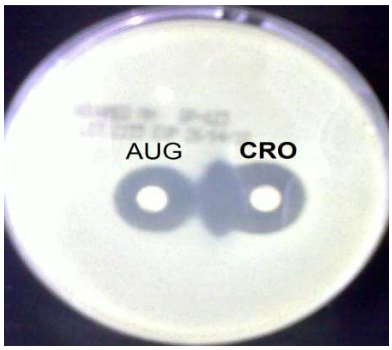

Figure 9. Double disc synergy test for *Klebsiella* ATCC 700603. Ceftriaxone 30µg (right), amoxicillin/clavulanic acid 20/10µg (left).

A total of 75/82 (91.5%) of *E. coli* strains were found to be ESBL-producers as shown in Table 10.

Table 10. Results of DDST for all initial ESBL-positive screens.

No. of all *E. coli* strains	Initial ESBL positive screen No. (%)	Positive ESBL by DDST No. (%)
423	82 (19.4)	75/82 (91.5)

Combination Disc Assay

The combination disc test (CDT) using 30µg discs of the third generation cephalosporins alone and in combination with 10µg clavulanate of both ceftazidime and cefotaxime was also performed on all positive ESBL screens as shown in Figure 10.

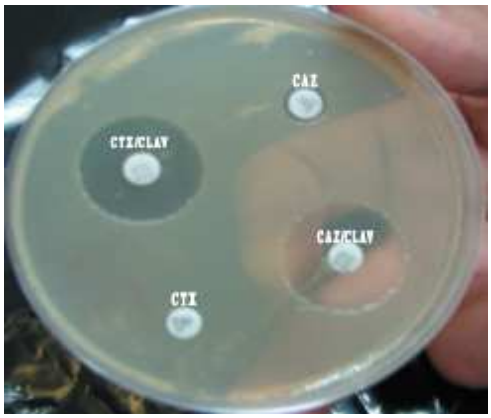

Figure 10: Illustration of combination test assay from this study.
CAZ: ceftazidime, CTX: cefotaxime, CLAV: clavulanic acid.

According to standard criteria of CLSI 2005, an increase in the zone diameter of ≥5 mm in the presence of clavulanate compared to each antibiotic alone is interpreted as confirmation for ESBL production. Of the 82 ESBL positive screens, 77 (93.4%) and 61

(74.2%) were found to be ESBL-producers using both cefotaxime and ceftazidime alone and in combination with clavulanate respectively (Table 11). Five isolates of the positive ESBL initial screens were found to be non-ESBL-producers from which two were found to be resistant to both cefotaxime and ceftazidime and resist inhibition by clavulanate. Accordingly, 77 (18.2%) of the total 423 *E. coli* isolates were found to be ESBL producers. *E. coli* ATCC 25922 (β-lactamase negative) and *K. pneumoniae* ATCC 700603 (ESBL positive) were used as a reference control strains.

Table 11. The combination disc test for the 82 ESBL-positive screens.

Number of ESBL-positive in initial screens (1µ g/ml CTX)	Positive ESBL by combination disc test (CDT)		Total ESBL producers among 423 isolates No. (%)
	CTX/CLAV No. (%)	CAZ/CLAV No. (%)	
82	77 (93.4)	61 (74.2)	77 (18.2)

CTX: Cefotaxime, CLAV: Clavulanate, CAZ: Ceftazidime, CDT: Combination disc test

Susceptibility Tests by Agar Dilution Method

The MICs were determined for the 77 ESBL-producing *E. coli* by the agar dilution method and interpreted following guidelines of CLSI 2005. The results of susceptibility tests to the antimicrobial agents used in this study are shown in Table 12 and Figure 11. According to CLSI, ESBL-producers are considered resistant to all third generation cephalosporins regardless of *in-vitro* susceptibility. Among the β-lactam agents tested, susceptibility to meropenem was 100% (MIC_{90} <0.06). Aminoglycosides revealed 92.2%

Table 12. MIC results for the 77 ESBL-producing *E. coli* isolates to various antimicrobial agents.

MICs							
Antibiotics	[a]MICs Breakpoints	S No. (%)	I No. (%)	R No. (%)	Range	MIC_{50}	MIC_{90}
AMP	S= ≤ 8 I = 16 R ≥ 32	0(0)	0(0)	77(100)	>128	>128	>128
AMC	S= ≤ 8 I = 16 R ≥ 32	0(0)	0(0)	77(100)	>128	>128	>128
CTX	S= ≤ 8 I = 16-32 R ≥ 64	0(0)	4(5.2)	73(94.8)	16 - >128	>128	>128
CRO	S= ≤ 8 I =16-32 R ≥ 64	0(0)	2(2.6)	75(97.4)	32 - >128	>128	>128
CAZ	S= ≤ 8 I = 16 R ≥ 32	23(29.9)	9(10.6)	45(58.4)	<1 - >128	32	128
SXT	S= ≤2 I= 4 R ≥ 8	7(8.2)	1(1.2)	69(89.6)	0.5-32	>32	>32
AK	S= ≤ 16 I = 32 R ≥ 64	71(92.2)	4(5.1)	2(2.5)	1 - 64	8	16
CN	S= ≤ 4 I = 8 R ≥ 16	40(51.4)	1(1.3)	36(46.7)	<1 - >128	4	>128
LVO	S= 2 I = 4 R ≥8	24(31.2)	3(3.5)	50(64.9)	<1 - >128	8	32
MEM	S= 4 I = 8 R ≥16	77(100)	0(0)	0(0)	<0.06 - 1	<0.06	<0.06

AMP: Ampicillin, AMC: Augmentin, CTX: Cefotaxime, CRO: Ceftriaxone, CAZ: ceftazidime, SXT: Sulfamethoxazole/Trimethoprim, AK: Amikacin, CN: Gentamicin, LVO: Levofloxacin, MEM: Meropenem, S: sensitive, R: resistant, I: intermediate, No.: number.
[a]: MICs Breakpoints according to CLSI 2005 [75].

susceptibility for amikacin (MIC_{90} 16), while this rate was low for gentamicin (51.4%). Only 8.2% of the ESBL-positive strains were susceptible to SXT and 32.2% were susceptible to fluoroquinolones (levofloxacin). All the ESBL isolates were 100% resistant to both ampicillin and augmentin. A total of 23/77 (30%) of the ESBL-

producing *E. coli* were resistant to three different classes of antibiotics namely, aminoglycosides (gentamicin), fluoroquinolones (levofloxacin), and sulpha drugs (trimethoprim/sulphamethoxazole) indicating the presence of multidrug resistance.

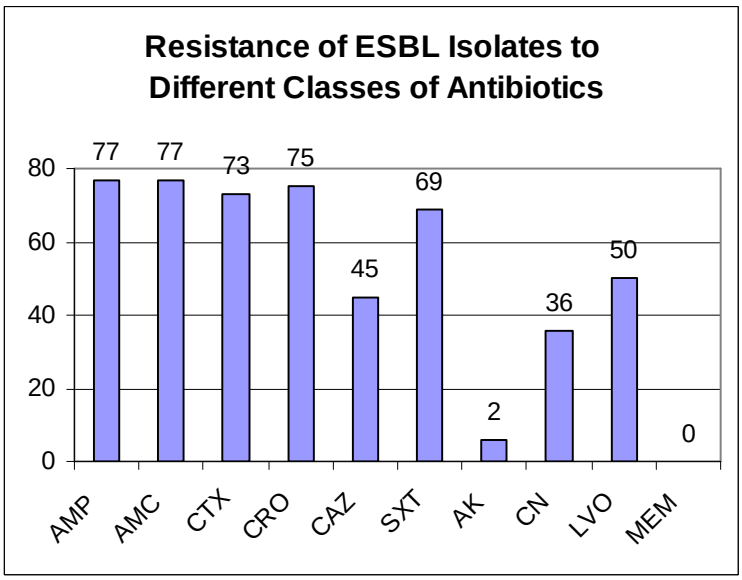

Figure 11. ESBL-positive *E. coli* isolates resistant to antimicrobial agents.
AMP: Ampicillin, AMC: Augmentin, CTX: Cefotaxime, CRO: Ceftriaxone, CAZ: ceftazidime, SXT: Sulfamethoxazole/Trimethoprim, AK: Amikacin, CN: Gentamicin, LVO: Levofloxacin, MEM: Meropenem.

Molecular Detection of β-lactamase Genes

Single PCR

A single PCR amplification from genomic DNA was performed on all 77 ESBL-producing *E. coli* isolates to determine genes encoding SHV, TEM, and CTX-M β-lactamases (Figure 12).

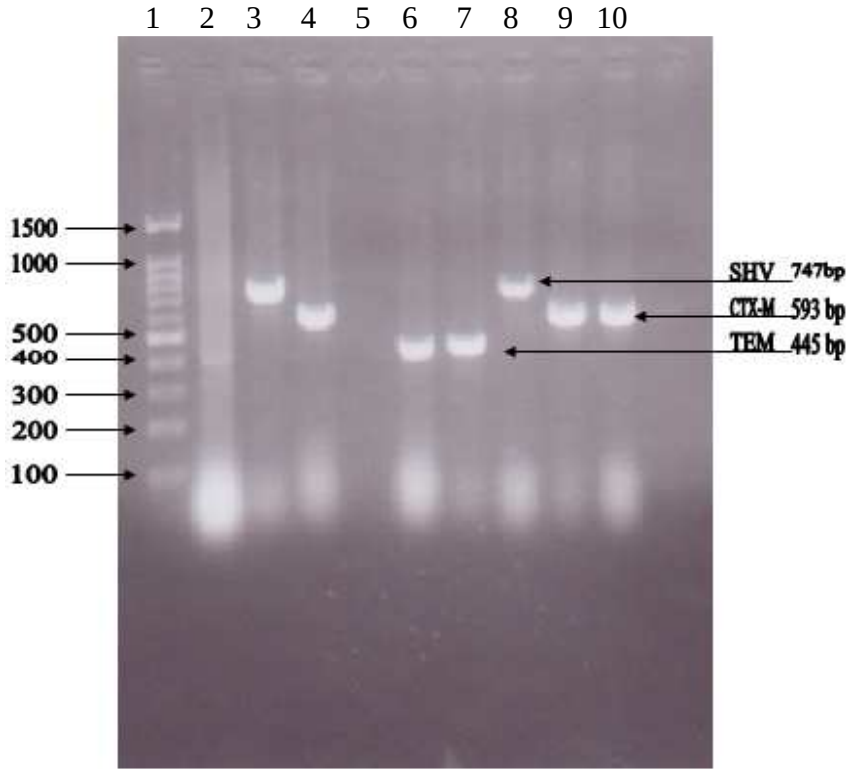

Figure 12. Single PCR for control and *E. coli* isolates. Lane 1 is 100 bp ladder. Lanes 2, 3, and 4 are TEM, SHV, and CTX-M controls respectively. Lane 5 is a negative control. Lane 6 and 7 are bla_{TEM}. Lane 8 is bla_{SHV}. Lane 9 and 10 are $bla_{CTX\text{-}M}$.

The bla_{TEM} was recognized in 46/77 (59.7%) of the isolates while only one bla_{SHV} 1/77 (1.3%) was detected. The predominant ESBL encoding gene among *E. coli* isolates was primarily due to $bla_{CTX\text{-}M}$. All 77 (100%) ESBL-producers encoded $bla_{CTX\text{-}M}$ (Table 13).

Table 13. Number and percentage of bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ recognized by single PCR.

bla_{TEM} NO. (%)	bla_{SHV} NO. (%)	$bla_{CTX\text{-}M}$ NO. (%)
46(59.7)	1(1.3)	77(100)

Multiplex PCR

All 77 ESBL-producing *E. coli* isolates were subjected to amplification utilizing multiplex PCR technique to detect the presence of bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ simultaneously. The conditions were optimized using gradient annealing temperature and a single isolate containing the former three β-lactamase genes (Figure 13).

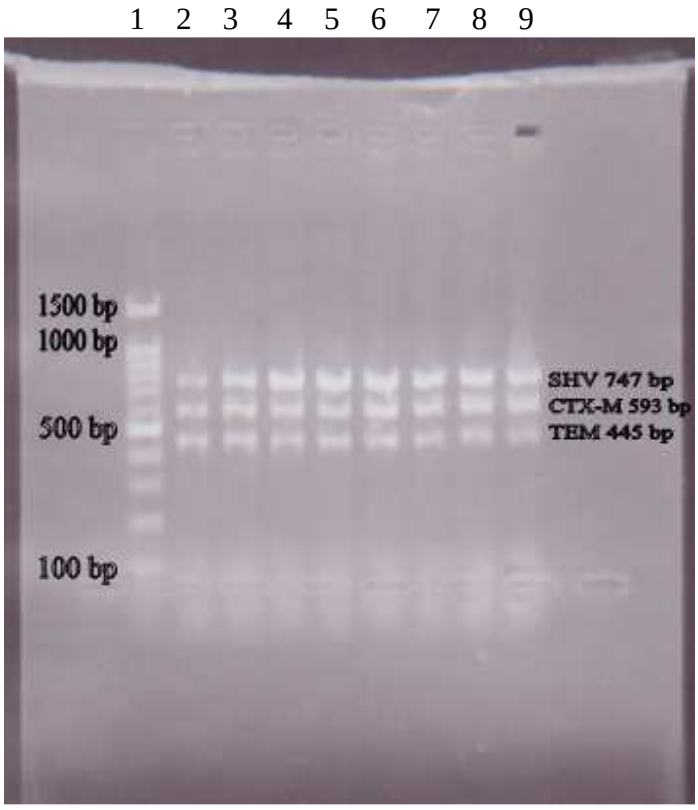

Figure 13. Optimization of annealing temperature for multiplex PCR using SHV, CTX-M and TEM genes. Lane 1 is 100 bp ladder, Lane 2 = 65^{o} C, Lane 3 = 63.8^{o} C, Lane 4 = 61.6 ^{o}C, Lane 5 = 57.6^{o} C, Lane 6 = 52.7^{o} C, Lane 7 = 48.7^{o} C, Lane 8 = 46.2^{o} C, Lane 9 = 45^{o} C. Lane 5 (57.6^{o} C) and lane 6 (52.7^{o} C) appeared as the sharpest bands from which 54^{o} C annealing temperature are selected for the subsequent reactions. The size of each amplicon was indicated on the figure.

After optimization, amplification by multiplex PCR was performed on all ESBL-producing *E. coli* at an annealing temperature of 54° C (Figure 14).

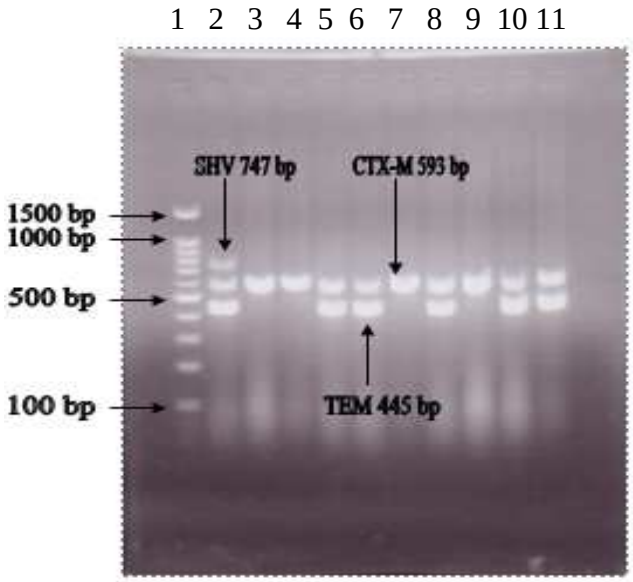

Figure 14. Multiplex PCR for SHV, TEM, and CTX-M genes. Lane 1 is 100 bp ladder. Lane 2 is an *E. coli* isolate containing bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ genes. Lanes 3, 4, 7, and 9 are *E. coli* isolates containing $bla_{CTX\text{-}M}$ gene alone. Lanes 5, 6, 8, 10, and 11 are *E. coli* isolates containing both $bla_{CTX\text{-}M}$ and bla_{TEM} genes.

The major ESBL encoding gene was found to be $bla_{CTX\text{-}M}$ and was detected in 77 (100%) of the isolates. The bla_{TEM} was recognized in 46 (59.7%) of the isolates while only one bla_{SHV} (1.3%) was detected (Table 14 and Figure 15).

Table 14. Number and percentage of bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ detected by multiplex PCR among 77 ESBLs-producing *E. coli*.

No. of ESBL-producing *E. coli*	bla_{TEM} isolates No. (%)	bla_{SHV} isolates No. (%)	$bla_{CTX\text{-}M}$ isolates No. (%)	Isolates containing $bla_{TEM,}$ $bla_{CTX\text{-}M}$ and bla_{SHV} simultaneously No. (%)
77	46(59.7)	1(1.3)	77(100)	1(1.3)

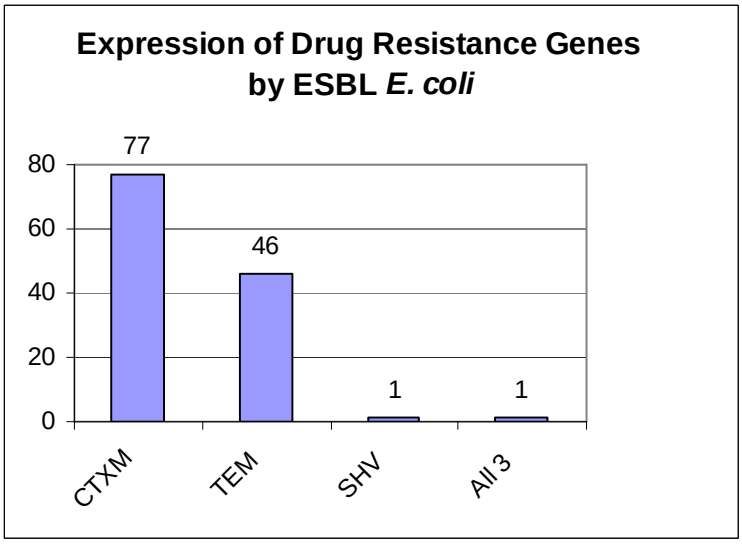

Figure 15. ESBL isolates encoding CTX-M, TEM, SHV β-lactamase enzymes individually and combined.

bla$_{TEM}$ and *bla*$_{CTX-M}$ genes were found in 45(58.5%) of the isolates. Only one isolate (1.3%) was detected to have *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$ simultaneously. *bla*$_{CTX-M}$ alone was found in 31(40.2%) of the isolates (Table 15).

Table 15. Number and percentage of *E. coli* isolates producing one or more of β-lactamases genes.

No. of ESBL-producing *E. coli*	Isolates with *bla*$_{TEM}$ and *bla*$_{CTX-M}$ NO. (%)	Isolates with *bla*$_{TEM,}$ *bla*$_{SHV}$ and *bla*$_{CTX-M}$ NO. (%)	Isolates with *bla*$_{CTX-M}$ alone NO. (%)
77	45(58.5%)	1(1.3%)	31(40.2%)

Detection of *bla*$_{CTX-M}$ Groups by Multiplex PCR

As mentioned in the previous sections, CTX-M β-lactamases were classified in five phylogenetic groups according to homology of their amino acid sequences. To determine

that, the 77 ESBL-producing CTX-M β-lactamase isolates were further subjected to grouping by multiplex PCR (Figure 16).

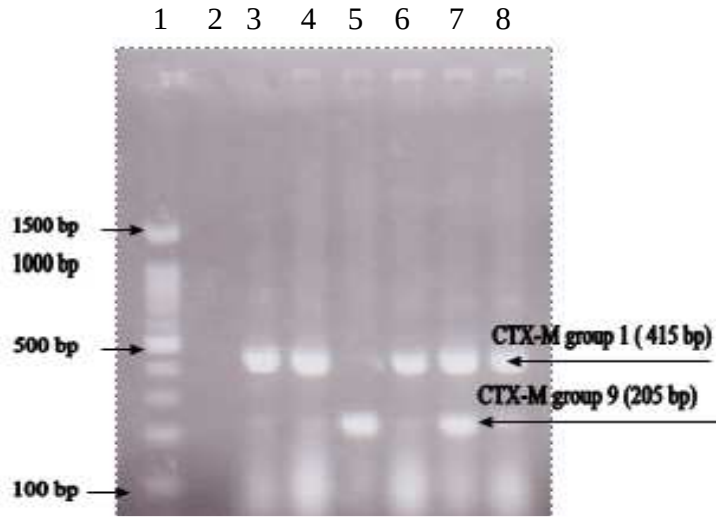

Figure 16. A representative agarose gel electrophoresis for identifying the CTX-M groups by multiplex PCR. Two groups were found: group 1 and group 9. Lane1is100 bp ladder; lane 2 is a negative control; lane 3, 4, 6, and 8 are bands for CTX-M group 1 (415 bp); lane 5 is band for CTX-M group 9 (205 bp) and finally lane 7 (two bands) represents one strain with two *bla*$_{\text{CTX-M}}$ genes, one for CTX-M group 1 and other for CTX-M group 9.

Of the 77 isolates, 62 (80.5%) were found to belong to CTX-M group 1 and 15 (19.5%) to group 9 (Table 16, Figure 17). Furthermore, one isolate (1.3%) was found to be containing two *bla*$_{\text{CTX-M}}$ types belonging to CTX-M group 1 and group 9 (Figure 16 lane 7).

Table 16. Results of CTX-M grouping by multiplex PCR.

Number of *bla*$_{\text{CTX-M}}$ containing isolates	Isolates with *bla*$_{\text{CTX-M}}$ group 1 NO.(%)	Isolates with *bla*$_{\text{CTX-M}}$ group 9 NO.(%)	Isolates with *bla*$_{\text{CTX-M}}$ groups 1 and 9 NO.(%)
77	62(80.5)	15(19.5)	1(1.3)

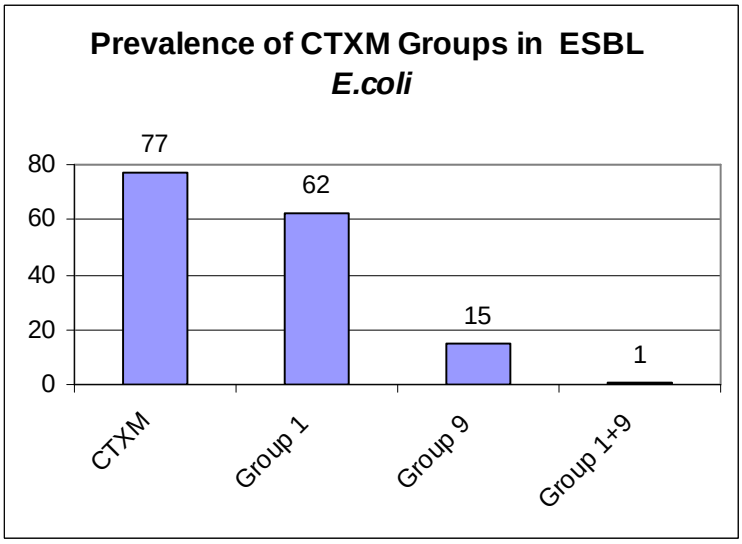

Figure 17. Number of CTX-M groups expressed in ESBL *E. coli*.

CTX-M Types and Susceptibility to Third Generation Cephalosporins

Our results were also interpreted according to the EUCAST breakpoints, with levels lower than those of CLSI but more reliable in detecting ESBL isolates [73].

Among the CTX-M group1, 54/62 isolate demonstrated high hydrolytic activity against third generation cephalosporins and gave positive ESBL results in both CAZ/CLAV and CTX/CLAV tests. This picture was different in CTX-M group 9, where 14/15 isolates were susceptible to ceftazidime and gave a negative ESBL result using CAZ/CLAV test (Table 17, Figure 18).

Table 17. Comparison of CTX-M-1 and -9 isolates to susceptibility profile of 3rd generation cephalosporins and CDT.

CAZ [a]R>8	CTX [a]R >2	CRO [a]R >2	CDT		No. of CTX-M-1 isolates	No. of CTX-M-9 isolates
			CAZ/CLAV	CTX/CLAV		
R	R	R	R	R	54	1
S	R	R	S	R	8	14

R: resistant, S: susceptible, CTX: cefotaxime, CAZ: ceftazidime, CRO: Ceftriaxone, CDT: combination disc test, CLAV: clavulanate, [a] = Resistant breakpoints according to EUCAST recommendation.

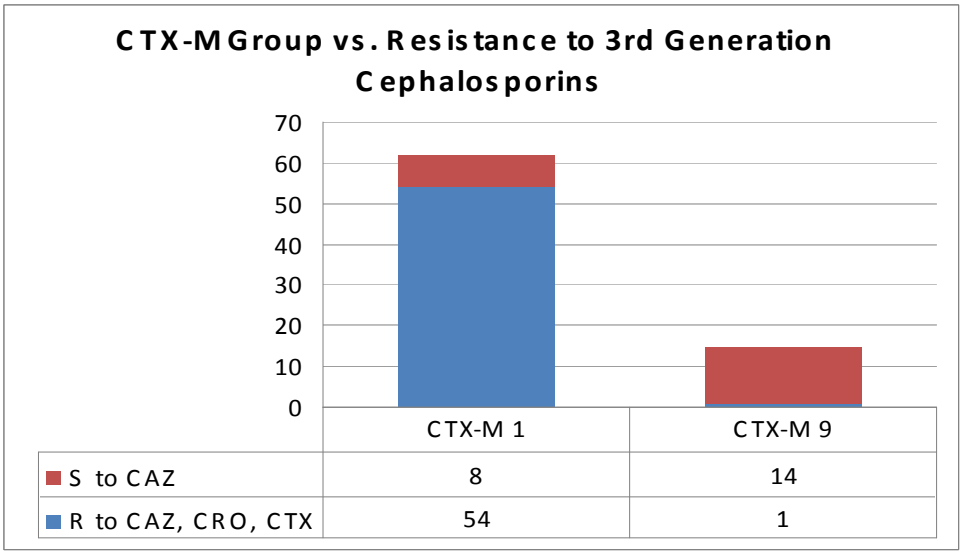

Figure 18. CTX-M groups and susceptibility patterns to cefotaxime, ceftazidime and ceftriaxone.
S: susceptible, R: resistant, CAZ: ceftazidime, CTX: cefotaxime, CRO: ceftriaxone.

Clavulanate-Inhibitor Resistant Isolates

Two isolates tested positive for ESBL using the screening method were negative with the confirmatory tests (resist clavulanate inhibition). One of these isolates showed intermediate susceptibility to ceftazidime (16 MIC) and high hydrolytic activity to

cefotaxime (MIC >128) and Ceftriaxone (MIC 64). The second isolate showed a high hydrolytic activity to ceftazidime, cefotaxime, and Ceftriaxone with MICs >128, 64 and 64 respectively. In addition, these isolates had TEM-type β-lactamases but no CTX-M and SHV types.

UTI with ESBL-producing *E. coli* and Faecal Carriage

Eight ESBL-producing *E. coli* detected from colonized faecal sources were compared with ESBL-producing *E. coli* from urine source in the same patient. The MICs of the third generation cephalosporins and ESBL confirmation tests as well as genotyping were used as the criteria for comparison. Both (urine and fecal) ESBL-producing *E. coli* gave compatible phenotypes and genotypes in all patients except one. This patient had CTX-M-type 1 in urine isolate but non in the faecal strain (Table 18).

Table 18. Phenotypes and genotypes of ESBL-producing *E. coli* from fecal and urine sources from the same patients.

Isolate NO.	PTN NO.	CAZ R≥32	CTX R≥64	CRO R≥64	DDST	CDT		CTX-M-1	CTX-M-9	SHV	TEM
						CAZ/CLAV	CTX/CLAV				
U 39	1	-	+	+	+	-	+	-	+	-	+
F 46		-	+	+	+	-	+	-	+	-	+
U 41	2	+	+	+	+	+	+	+	-	-	-
F 47		+	+	+	+	+	+	+	-	-	-
U 49	3	-	+	+	+	+	+	+	-	-	-
F 51		-	+	+	-	+	+	+	-	-	-
U 50	4	-	+	+	+	-	+	-	+	-	+
F 52		-	+	+	+	-	+	-	+	-	+
U 54	5	+	+	+	+	+	+	+	-	-	+
F 55		+	+	+	+	+	+	+	-	-	+
U 59	6	+	+	+	+	+	+	+	-	-	-
F 62		+	+	+	+	+	+	+	-	-	-
U 87	7	-	-	-	+	+	+	+	-	-	-
F 88		-	-	-	+	+	+	+	-	-	-
U 89	8	+	+	+	+	+	+	+	-	-	+
F 90		+	+	+	+	+	+	-	-	-	+

PTN: patient, No.: number, U: urine, F: faecal, R: resistant, CTX: cefotaxime, CAZ: ceftazidime, CRO: Ceftriaxone, CALV: clavulanate, DDST: double disc synergy test, CDT: combination disk test.
+ = resistant
- = susceptible

DISCUSSION

E. coli has been detected as one of the most common causative agents of both community and nosocomial infections. The worldwide emergence of infections caused by ESBL-producing *E. coli* (particularly isolates producing CTX-M type ESBL) has been recently reported throughout the world [89]. This situation creates a great challenge to clinical practices and further restricts treatment choices.

In Palestine, there is a rare epidemiological data concerning this issue. The results of this study may provide insights into the genetic characteristics and molecular epidemiology of ESBLs among *E. coli* isolates at Al-Makassed Hospital and its associated health care facilities in Jerusalem, Palestine.

Our results revealed that the overall ESBL-producers were 77/423 (18.2%). These results are compatible with results obtained in studies conducted in the same geographical area. In Gaza, Palestine [55], a study conducted in UTI infected community patients in the year 2004, indicated a prevalence of 3.7%, while 9% were detected in different clinical sources from patients (most are in patients) attended Nasser Hospital in 2008 [56]. Several studies were conducted in neighboring Jordan in the years 2000 [90], 2007 [91], 2009 [92]. The prevalence of ESBL producing *E. coli* was found to be 35%, 31%, and 10.8% respectively. Published data from Egypt shows that the prevalence from hospitalized patients is varying from 38% [93] to 66.6 % [94] and from 35.7% [66] up to 80% [95] among isolates from intensive care settings. In Lebanon [96], a study was conducted at Saint George Hospital from 1997 to 2000. The prevalence of ESBL producing *E. coli* was found to be 2%. Another study conducted in 2005 [97] on carriage

of ESBL producing *E. coli* in fecal samples, the prevalence was found to be much higher, 15%. In the Arabian Gulf Region, the lowest ESBL prevalence was described in a report from Kuwait (6.5%) [98]. However, in another study in Kuwait [99], a much higher percentage was reported (37%). In UAE [57], the prevalence of ESBL was found to be 37.5%. Both of these studies in Kuwait and UAE were conducted on hospitalized patients. Data from neighboring Bahrain [100] indicated that the majority of ESBL-producing Enterobacteriaceae was *E. coli*. (52%). In Saudi Arabia in the year 2002 [101], Babay reported that 20% of ESBL-producing Enterobacteriaceae was due to *E. coli* isolated from inpatients in a hospital in Riyadh. Recently in Saudi Arabia, the prevalence of ESBL-producing *E. coli* was 10.3% isolated from specimens from different clinical sources [51] and 31% in blood culture isolates [102]. In Israel, 2004, the prevalence was 12.3% among isolates from different clinical sources [103], while 22% in *E. coli* isolates from UTI [59]. In Turkey, the prevalence of ESBL *E. coli* isolated from community acquired UTI was 21% [104], 40% in blood isolates [53] and 6% from both community and hospitalized patients [105]. Data concerning antimicrobial resistance in Southern and Eastern Mediterranean countries from EARSS [45] revealed that resistance to third generation cephalosporins among *E. coli* isolates varied from 3% in Malta to 72% in Egypt (median 18%).

Globally, a recent study based on the Tigecycline Evaluation and Surveillance Trial (TEST) global surveillance database, the prevalence of ESBL-producing *E. coli* collected in Latin America, followed by Asia/Pacific Rim, Europe, and North America was 13.5%, 12.0%, 7.6%, and 2.2%, respectively [106]. Detailed data derived from the TEST database regarding the prevalence of ESBL production among *E. coli* isolates in Europe

has recently been presented. According to data accumulated from 22 European countries for the period from 2004 to 2007, the rate of ESBL production among 794 *E. coli* isolates was 9.8%. Marked differences observed in the country-specific data; the highest rate of ESBL production was in Greece, while the lowest in Denmark [106].

From the aforementioned data, it appears that the epidemiology of ESBL-producing *E. coli* is so variable. These differences are not only seen between continents and countries alone, but also within hospitals in the same country. The variation in the results was mostly attributed to the design of each study. In comparison of our prevalence to the aforementioned regional and international data, the ESBL detection rate described here tends to be towards the upper end of the spectrum and is therefore a major cause for concern.

The ESBL-positive *E. coli* isolates investigated here encoded mainly CTX-M 77(100%) followed by TEM type 47(59.7%) and SHV type 1 (1.3%). Among CTX-M groups, CTX-M group 1 was 62(80.5%) and CTX-M group 9 was 15(19.5%).

CTX-M β-lactamases constitute a novel and rapidly growing family of plasmid-mediated ESBLs that are currently replacing mutant TEM or SHV ESBL families and with much greater expression in *E. coli*. They have become the most prevalent type of ESBLs described during the last 5 years, especially from certain European, Middle Eastern and South American countries. The type of beta lactamase enzyme produced tends to have geographical variation. Specifically, group 9 (CTX-M-9 and -14) are common in Spain and group 1 enzymes (particularly CTX-M-3 and -15) are common in France and UK [107]. The *bla*CTX-M-15 belonging to subgroup one is distributed worldwide and is most commonly encountered in the European countries (except Spain), North Africa, the

Middle East and Canada [60]. In the Middle East and the Mediterranean regions, reports from Lebanon [97], Egypt, and Kuwait [66] pointed that CTX-M15 is the predominant ESBL in *E. coli*. However, $bla_{\text{CTX-M-2}}$ predominates in Israel, South America, and Japan [63].

In our results, CTX-M groups predominated the ESBL producing *E. coli* in agreement with several studies published worldwide. The $bla_{\text{CTX-M}}$ genes were widespread among the ESBL-positive *E. coli* (100%), which was similar to the level reported in Lebanon (96%) [97], Turkey (87%) [105], Sweden (92%), Switzerland (91%), Norway (90%), and Austria (85%) [108]. The majority of the $bla_{\text{CTX-M}}$ belonged to CTX-M group 1 (80.5%), mostly CTX-M-15 like, as reported in Lebanon [97], Egypt [66], Turkey [104, 105] Sweden, Switzerland, France, Austria, Norway, and Amsterdam [108].

The remainder of ESBL *E. coli* isolates in this study encoded CTX-M group 9 (19.5%). CTX-M group 9 (CTX-M-9, -14) is pandemic in Spain, East Asia (notably China) and less frequently in UK [107]. Our results of 19.5% CTX-M group 9 is in agreement with reports from Sweden (16%) [108], UK [109] and Kuwait [70]. Recently, CTX-M-9 like alleles (CTX-M 14) was reported in Egypt [110] in *K. pneumoniae* and *E. cloacae* isolates. Although the high prevalence of CTX-M in our study (particularly CTX-M group 1) is closely related to results obtained from neighboring countries, it is different from that reported in Israel (CTX-M 2 and CTX-M 25/26) [67].

TEM and SHV β-lactamases were investigated in our study and found to be 59.7% and 1.3 % respectively. The rate of SHV type is low here, which is comparable to results

reported in Sweden [108]. Since the specific types of TEM and SHV are not determined in this study, it is necessary to perform sequence analysis for these two types; However, TEM-1 β-lactamase was more commonly detected in ESBL-producing *E. coli* than SHV. In accordance with our results, a study conducted in Sweden in 2008 revealed that 63% of ESBL-positive *E. coli* has TEM encoding genes where TEM1 was the most common β-lactamase found in bla_{TEM} positive strains [108]. Another study conducted in Israel in 2005 revealed that TEM 1 is also the most common β-lactamase found in bla_{TEM} positive strains [67].

Our results emphasize the fact that CTX-M β-lactamase is actually replacing TEM and SHV types and becoming the most prevalent one among ESBL-producing *E. coli* isolates.

The results of ESBL confirmation test (combination disk test), 77/82 (93.4%) were positive ESBL utilizing CTX/CLAV, while only 61/82 (74.2%) were positive for CAZ/CLAV. This confirms the fact that CTX-M β-lactamases have a potent hydrolytic activity against cefotaxime and Ceftriaxone more than ceftazidime. This information was apparent in the results of MIC tests for ceftazidime, cefotaxime, and Ceftriaxone. The MIC results showed that 45/77 (58.4%) of the ESBL isolates were resistant to ceftazidime (MIC_{50} 32), while 73/77 (95%, MIC_{50} >128) and 75/77 (97.4%, MIC_{50} >128) of the ESBL isolates were resistance to both cefotaxime and Ceftriaxone respectively. According to breakpoints implemented by the EUCAST 2007 [73], 54 of the 62 CTX-M group1 isolates demonstrated high hydrolytic activity against the third generation cephalosporins (cefotaxime, ceftazidime and Ceftriaxone) and gave a positive ESBL results in both CAZ/CLAV and CTX/CLAV tests. It is established that CTX-M-15 type

which belongs to CTX-M group 1 was preferentially active against both cefotaxime as well as ceftazidime [111]. Therefore, it is most likely that most of the CTX-M group 1 detected here are CTX-M-15 like allele. CTX-M-15 was first detected on large plasmids isolated from *E. coli*, *K. pneumoniae*, and *E. aerogenes* strains from patients hospitalized in New Delhi, India, in 1999 [112]. However, it should be noted that the first report of the $bla_{\text{CTX-M-15}}$ sequence in the public domain was made in 2001; the sequence, designated *bla*UOE-1, was found on a plasmid from an *E. coli* isolate from Japan (GenBank accession no. AY013478). CTX-M-15 differs from CTX-M-3 by a single amino acid change, Asp240Gly, which results in activity expanding against both cefotaxime and ceftriaxone. (Ambler numbering) [113].

On the other hand, 14/15 of CTX-M groups 1 exhibited low activity against ceftazidime. Analysis of the crystal structure of CTX-M group 9 enzymes (CTX-M-9, -14, -16 and 27) has revealed that the active sites of these enzymes resemble those of narrow-spectrum TEM and SHV enzymes (e.g., TEM-1, SHV-1) and are not large enough to recognize ceftazidime, which is larger than cefotaxime [32]. However, point substitutions leading to specific interactions may be responsible for the improved activity against ceftazidime and cefotaxime. In case of CTX-M-16, the coupled defects in the enzyme's B3 strand, which lines the active site, substitutions Val231/Ala and Asp240/Gly, may be the main cause of an eightfold increase in ceftazidime hydrolytic activity than the pseudo-wild-type CTX-M-14 [32].

In conclusion, the information obtained in this study regarding CTX-M group 9 indicated a low hydrolytic activity against ceftazidime suggesting that it is most likely a CTX-M-9 like allele.

The MICs investigated in this study showed, 7.8%, 48.6%, 91.2%, 67.8%, 100%, and 100% of ESBL-poducing *E. coli* isolates were resistance to amikacin (AK), gentamicin (CN), trimethoprim/sulphamethoxazole (SXT), levofloxacin (LVO), ampicillin (AMP), and augmentine (AMC) respectively. A 100% of the ESBL-poducing *E. coli* isolates were meropenem susceptible (MIC_{90} <0.06). A total of 23/77 (30%) were resistance to non-β-lactams agents such as CN, SXT and LVO and therefore considered as multidrug resistance. Susceptibility results obtained in our study are comparable to those reported by other studies. In Gaza, Palestine 2003 [55], 81.8%, 81.8%, 54.5% and 9.1% of ESBL positive isolates from community patients were resistant to SXT, CN, ciproxin (CIP) and AK respectively. Another study conducted among hospitalized patients in Gaza, Palestine 2008 [56], revealed that 95% of the ESBL-producing *E. coli* were susceptible to Meropenem (MEM), while 80%, 54.2%, 42.8%, 34.2% and 77.2% were resistant to SXT, CN, and ofloxacin (OF), AK and AMC respectively. In Israel 2007 [58], susceptibility rates were also investigated for both β-lactams and non-β-lactam antimicrobial agents. The non-β-lactam agents tested, aminoglycosides and fluoroquinolones, a total of 70%-90% of ESBL-producing *E. coli* isolates exhibited resistance, except for amikacin the susceptibility rate was 77.9%. Susceptibility of all ESBL-producing *E. coli* isolates to Carbapenems was >95%.

In Egypt [66], 90%, 100%, and 90% of CTX-M-positive Gram-negative isolates were resistant to aminoglycosides, Fluoroquinolones, and SXT. Imipenem remained effective on all ESBL-positive multidrug resistant isolates. In Turkey [104], 65%, 88%, 30% and

76% of ESBL-producing *E. coli* isolates were resistant to CIP, SXT, AK and CN respectively.

Our findings agree with the findings of several studies done worldwide indicating the presence of coresistance and multidrug resistance among CTX-M (particularly CTX-M-15) encoding ESBL isolates.

The presence of multidrug resistance in CTX-M isolates was mainly attributed to plasmids harboring MDR genes. Analysis of pC15-1a (belonging to IncF plasmid groups) plasmids from CTX-M-15 encoding *E. coli* implicated for an outbreak in Canada [114] revealed that these plasmids harbored multidrug resistant genes. Among β- lactamase genes $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, and $bla_{\text{TEM-1}}$, the tetracycline resistance gene *tetA*, and aminoglycoside resistance genes *aac*(*6*')-*Ib* and *aac*(*3*)-*II*, are all located in the multidrug resistance region.

A study conducted in Canada dealing with CTX-M-15 encoding *E. coli* isolated from different countries (France, Kuwait, Switzerland, Canada, Portugal and Spain), revealed that most of IncF plasmids detected harbor multidrug resistant genes [64]. Recently, genes encoding CTX-M-15 and quinolone modifying enzyme AAC(6¢)-Ib-cr were found to be carried on 90-kb plasmid of the pC15-1a or pCTX-15 type (IncF group). These plasmids were responsible in transferring genes encoding for both ESBL and quinolone resistance from donors to transconjugant in *K. pneumoniae* and *E. coli*. Analysis of transconjugants confirmed that the genetic determinants of AAC(6¢)-Ib-cr, class I integrons, CTX-M-15 and ISEcp1 were encoded on a large 90-kb plasmid [65].

The ease of horizontal transfer of these plasmids harboring *bla*$_{CTX-M}$ and other resistance genes was largely contributing to dissemination and widespread of MDR among *E. coli* strains worldwide.

In our study, the high rate of MDR (30%) among ESBL-producing *E. coli* isolates is an alarming sign to physicians regarding random prescription of antibiotics in order to minimize selection pressure in this region.

Our results implied that two non-ESBL isolates either be complex mutant of TEM (CMT) or AmpC-producers. These two strains encoded TEM β-lactamases, highly susceptible to meropenem, exhibited high activity against third generation cephalosporins, and resist clavulanate inhibition. Although it is highly indicative that they could be AmpC-producers or CMT, further work must be done to confirm this issue. Sequence analysis, AmpC disk test and molecular detection of AmpC genes are essential to emphasize the presence of one or exclude the other.

A total of 37.7% of ESBL-positive *E. coli* isolates were detected from faecal sources. In addition, ESBL-producing *E. coli* isolated from faecal and urine sources from the same patient revealed that both isolates were phenotypically and genetically identical. An exception to this finding was from patient number 8 (Table 18) where *bla*$_{CTX-M}$ was not detected in faecal isolate. This result indicates that faecal carriage of ESBL-producing *E. coli* may be a risk factor that contributes in dissemination of hospital-acquired infections. However, to generalize this idea, we need larger sample size and clonally related isolates.

Our results are in consistent with study conducted in Spain, 2008 [115]. The study analyzed fecal carriage of ESBL-producing organisms in a group of 40 community patients infected with these organisms (95% were UTIs). Simultaneously, faecal carriage of ESBL-producing organisms of the people living with them (household contact) was also analysed. Twenty-nine out of 40 patients (70%) and 9 out of 54 household contacts (16.7%) presented fecal carriage of ESBL-producing *E. coli* strains. Seventy-two percent (21 of 29) of community patients harbored *E. coli* clinical strains with the same PFGE type as those from their fecal samples. Moreover, PFGE analysis revealed indistinguishable patterns among ESBL-producing *E. coli* isolates from community patients (clinical sample or fecal sample) and their corresponding household contacts for 66% (6 of 9) of the isolates.

Several studies regarding risk factors of faecal carriage to ESBL-poducing *E. coli* were conducted worldwide. A study carried out in Lebanon 2005 [52], revealed that the incidence of strains of the Enterobacteriaceae producing ESβLs in the stools of inpatients was up to 16.1% and the majority are due to ESBL-producing *E. coli* (56/72). Faecal carriage was also evaluated in Belfast, Ireland [116], 120/307 (39%) samples from 13 long-term care facilities yielded at least one ESBL-producing *E. coli* [117]. In France, faecal carriage of ESBL-producing *E. coli* among hospitalized patients was found to be 1.2% in 2002 to 3.9% in 2004.

Faecal carriage with high rates of ESBLs-producing *E. coli* can form a potential reservoir and enhances the opportunity to a variety of infections. Furthermore, Commensal *E. coli* can act as reservoirs of resistance genes that easily transfer to other commensal *E. coli*, as well as other potential pathogenic bacteria [118, 119]. The importance of the detection of

carriers of antimicrobial-resistant bacteria has recently been highlighted not only in inpatient populations but also in healthy people [120]. Although the exact source of hospitals outbreak usually are not identified; however, the lower digestive tract of colonized patients has been recognized as the major source of ESBL-producing organisms and their cross-transmission among patients has been attributed to the hands of medical and nursing personnel [121-123].

Our results indicates, that faecal carriage of ESBL-producing *E. coli* represent a high prevalence specially among patients admitted to pediatric wards 17(20%). This result emphasizes the evidence that abuse of third generation cephalosporins and other antibiotics in hospitals may exert selection pressure and amplify the number of carriers harboring resistant bacteria [124]. This fact could also be responsible for the higher prevalence of faecal carriage of ESβL-producing Enterobacteriaceae in the nosocomial setting than in the community [125].

Our findings indicate that one of the studied strains expressed two CTX-M types, CTX-M-15 and -9 likes, which agree with results from other studies [126, 127]. CTX-M β-lactamases are usually plasmid mediated. Recently, some studies reported that CTX-M types are also chromosomally encoded [64, 127]. Therefore, one of these CTX-M types is most likely chromosomal and the other is plasmid mediated; however, more investigation are required to confirm that.

RECOMMENDATIONS

- Sequence analyses are necessary to determine each ESBL specific type.
- Further studies should be conducted to determine the Phylogenetic tree of ESBL strains.
- Additional analyses are required to determine plasmid types and multidrug resistant region carried by multiresistant strains detected in this study.
- In our region, restrict rules for infection control and antibiotic treatment programs, must be followed to minimize the spread of ESBL-producing organisms in both community and hospital populations.
- In addition to the high rate of ESBL-producing organisms in hospitalized patients, several reports indicated further increasing in community patients. Therefore, conducting another study to evaluate this rate in community patients in our region is a necessary step.

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APPENDIX

Table 1. The MICs of the 77 ESBL-producing *E. coli* isolates

MICs										
Antibiotic	AMP	AK	CN	AMC	CAZ	CTX	CRO	SXT	MEM	LVO
Strain No.										
ATCC 25922	8	2	<1	8	<1	<1	<1	<2	<0.0625	<1
ATCC 700603	>128	<1	16	64	64	32	32	4	<0.0625	8
1	>128	16	2	>128	32	>128	>128	8	<0.0625	16
2	>128	16	128	>128	32	>128	>128	>128	<0.0625	16
3	>128	8	128	64	16	>128	>128	2	<0.0625	16
4	>128	4	1	>128	32	>128	>128	>128	<0.0625	<1
5	>128	16	2	>128	<1	32	32	2	<0.0625	16
6	>128	32	128	>128	32	>128	>128	>128	<0.0625	8
7	>128	16	<1	>128	>128	>128	>128	>128	<0.0625	16
8	>128	8	128	>128	>128	>128	>128	>128	<0.0625	>128
9	>128	8	128	>128	2	>128	>128	>128	<0.0625	>128
10	>128	16	2	>128	64	>128	>128	32	<0.0625	16
11	>128	8	128	64	32	>128	>128	>128	<0.0625	<1
12	>128	4	2	>128	4	>128	>128	>128	<0.0625	<1
13	>128	2	<1	64	16	>128	>128	>128	<0.0625	<1
14	>128	32	>128	>128	32	>128	>128	4	<0.0625	<1
15	>128	4	<1	>128	4	>128	>128	>128	<0.0625	<1
16	>128	8	2	>128	32	>128	>128	>128	<0.0625	16
17	>128	16	>128	>128	128	>128	>128	>128	<0.0625	>128
18	>128	4	128	>128	128	>128	>128	>128	<0.0625	8
19	>128	32	>128	>128	32	>128	>128	>128	<0.0625	<1
20	>128	2	<1	>128	2	>128	>128	>128	<0.0625	<1
21	>128	2	<1	>128	<1	>128	>128	128	<0.0625	<1
22	>128	4	64	>128	32	>128	>128	>128	<0.0625	16
23	>128	4	>128	>128	128	>128	>128	2	<0.0625	8
24	>128	16	2	>128	8	>128	>128	>128	<0.0625	16
25	>128	2	<1	>128	16	>128	>128	>128	<0.0625	<1
26	>128	2	<1	64	<1	>128	>128	16	<0.0625	<1
27	>128	2	2	>128	2	>128	>128	>128	<0.0625	<1
28	>128	16	<1	>128	2	64	>128	32	<0.0625	16
29	>128	16	>128	>128	32	>128	>128	>128	<0.0625	<1
30	>128	8	128	>128	64	>128	>128	>128	<0.0625	<1
31	>128	<1	32	32	4	>128	>128	2	<0.0625	4
32	>128	8	>128	>128	64	>128	>128	64	<0.0625	8
33	>128	2	128	>128	32	>128	>128	>128	<0.0625	16
34	>128	8	128	>128	128	>128	>128	>128	<0.0625	16
35	>128	8	<1	>128	32	>128	>128	128	<0.0625	16
36	>128	16	4	>128	32	>128	>128	128	<0.0625	32
37	>128	2	>128	>128	>128	>128	>128	>128	0.125	32
38	>128	16	8	>128	>128	>128	>128	>128	<0.0625	32
39	>128	2	1	>128	2	>128	>128	>128	<0.0625	<1
40	>128	8	128	>128	64	>128	>128	>128	<0.0625	32
41	>128	16	>128	>128	128	>128	>128	>128	<0.0625	8
42	>128	16	>128	>128	32	>128	>128	>128	<0.0625	<1

43	>128	2	>128	>128	2	>128	>128	>128	<0.0625	64
44	>128	2	1	>128	1	64	>128	>128	<0.0625	<1
45	>128	2	1	>128	1	64	64	>128	<0.0625	<1
48	>128	16	2	>128	32	>128	>128	>128	<0.0625	8
49	>128	32	2	>128	8	64	64	>128	<0.0625	16
50	>128	1	1	>128	4	>128	>128	>128	<0.0625	8
54	>128	8	64	>128	128	>128	>128	>128	1	8
56	>128	4	64	>128	32	>128	>128	>128	<0.0625	8
57	>128	8	2	>128	8	>128	>128	>128	0.5	8
59	>128	16	4	>128	128	>128	>128	>128	<0.0625	32
60	>128	4	128	>128	16	>128	>128	>128	<0.0625	4
61	>128	16	2	>128	128	>128	>128	>128	<0.0625	16
63	>128	8	>128	>128	8	>128	>128	1	<0.0625	<1
64	>128	8	1	>128	16	>128	>128	>128	<0.0625	<1
65	>128	8	2	>128	>128	>128	>128	>128	<0.0625	8
66	>128	2	128	>128	4	>128	>128	>128	<0.0625	32
67	>128	8	128	>128	16	>128	>128	4	<0.0625	16
68	>128	2	64	>128	16	>128	>128	>128	<0.0625	4
70	>128	64	1	>128	32	>128	>128	>128	<0.0625	8
71	>128	8	>128	>128	32	>128	>128	>128	<0.0625	8
72	>128	2	128	>128	32	>128	>128	>128	<0.0625	2
75	>128	16	4	>128	8	32	64	>128	<0.0625	32
76	>128	8	>128	>128	64	>128	>128	>128	<0.0625	16
77	>128	2	2	>128	64	>128	>128	>128	<0.0625	64
78	>128	2	2	>128	32	64	>128	>128	<0.0625	16
79	>128	16	4	>128	128	>128	>128	>128	<0.0625	16
80	>128	2	2	>128	64	>128	>128	>128	0.125	32
81	>128	4	2	>128	64	>128	>128	>128	<0.0625	1
82	>128	64	>128	>128	32	>128	>128	>128	<0.0625	1
83	>128	16	128	>128	16	>128	>128	>128	<0.0625	32
84	>128	4	>128	>128	128	>128	>128	>128	<0.0625	32
85	>128	4	2	>128	2	32	>128	>128	<0.0625	16
86	>128	4	2	>128	16	>128	>128	>128	<0.0625	1
87	>128	16	2	>128	2	16	32	>128	<0.0625	16
89	>128	8	>128	>128	64	>128	>128	>128	<0.0625	32

AMP: ampicillin, AK: amikacin, CN: gentamicin, AMC: Augmentin, CAZ: ceftazidime, CTX: cefotaxime, CRO: ceftriaxone, SXT: sulfamethoxazole/trimethoprim, MEM: meropenem, LVO: levofloxacin.

Table 2. The 77 ESBL-producing *E. coli* MICs interpreted according to CLSI 2005.

MICs interpretation according to CLSI 2005										
Antibiotic	AMP	AK	CN	AMC	CAZ	CTX	CRO	SXT	MEM	LVO
Breakpoints	S ≤ 8 I 16 R≥32	S≤16 I=32 R≥64	S≤4 I=8 R≥16	S ≤ 8 I=16 R≥32	S≤ 8 I=16 R≥32	S=≤8 R≥64	S≤ 8 R≥64	S≤2 I = 4 R≥8	S=4 I=8 R≥16	S= 2 I = 4 R ≥8
Strain No.										
**ATCC	S	S	S	S	S	S	S	S	S	S
*ATCC	R	S	R	R	R	I	I	S	S	S
1	R	S	S	R	R	R	R	R	S	R
2	R	S	R	R	R	R	R	R	S	R
3	R	S	R	R	I	R	R	S	S	R
4	R	S	S	R	R	R	R	R	S	S
5	R	S	S	R	S	I	I	S	S	R
6	R	I	R	R	R	R	R	R	S	R
7	R	S	S	R	R	R	R	R	S	R
8	R	S	R	R	R	R	R	R	S	R
9	R	S	R	R	S	R	R	R	S	R
10	R	S	S	R	R	R	R	R	S	R
11	R	S	R	R	R	R	R	R	S	S
12	R	S	S	R	S	R	R	R	S	S
13	R	S	S	R	I	R	R	R	S	S
14	R	I	R	R	R	R	R	S	S	S
15	R	S	S	R	S	R	R	R	S	S
16	R	S	S	R	R	R	R	R	S	R
17	R	S	R	R	R	R	R	R	S	R
18	R	S	R	R	R	R	R	R	S	R
19	R	I	R	R	R	R	R	R	S	S
20	R	S	S	R	S	R	R	R	S	S
21	R	S	S	R	S	R	R	R	S	S
22	R	S	R	R	R	R	R	R	S	R
23	R	S	R	R	R	R	R	S	S	R
24	R	S	S	R	S	R	R	R	S	R
25	R	S	S	R	I	R	R	R	S	S
26	R	S	S	R	S	R	R	R	S	S
27	R	S	S	R	S	R	R	R	S	S
28	R	S	S	R	S	R	R	R	S	R
29	R	S	R	R	R	R	R	R	S	S
30	R	S	R	R	R	R	R	R	S	S
31	R	S	R	R	S	R	R	S	S	I
32	R	S	R	R	R	R	R	R	S	R
33	R	S	R	R	R	R	R	R	S	R
34	R	S	R	R	R	R	R	R	S	R
35	R	S	S	R	R	R	R	R	S	R
36	R	S	S	R	R	R	R	R	S	R
37	R	S	R	R	R	R	R	R	S	R
38	R	S	I	R	R	R	R	R	S	R
39	R	S	S	R	S	R	R	R	S	S
40	R	S	R	R	R	R	R	R	S	R

41	R	S	R	R	R	R	R	R	S	R
42	R	S	R	R	R	R	R	R	S	S
43	R	S	R	R	S	R	R	R	S	R
44	R	S	S	R	S	R	R	R	S	S
45	R	S	S	R	S	R	R	R	S	S
48	R	S	S	R	R	R	R	R	S	R
49	R	I	S	R	S	R	R	R	S	R
50	R	S	S	R	S	R	R	R	S	R
54	R	S	R	R	R	R	R	R	S	R
56	R	S	R	R	R	R	R	R	S	R
57	R	S	S	R	S	R	R	R	S	R
59	R	S	S	R	R	R	R	R	S	R
60	R	S	S	R	I	R	R	R	S	I
61	R	S	S	R	R	R	R	R	S	R
63	R	S	R	R	S	R	R	S	S	S
64	R	S	S	R	I	R	R	R	S	S
65	R	S	S	R	R	R	R	R	S	R
66	R	S	R	R	S	R	R	R	S	R
67	R	S	R	R	I	R	R	S	S	R
68	R	S	R	R	I	R	R	R	S	I
70	R	R	S	R	R	R	R	R	S	R
71	R	S	R	R	R	R	R	R	S	R
72	R	S	R	R	R	R	R	R	S	S
75	R	S	S	R	S	I	R	R	S	R
76	R	S	R	R	R	R	R	R	S	R
77	R	S	S	R	R	R	R	R	S	R
78	R	S	S	R	R	R	R	R	S	R
79	R	S	S	R	R	R	R	R	S	R
80	R	S	S	R	R	R	R	R	S	R
81	R	S	S	R	R	R	R	R	S	S
82	R	R	R	R	R	R	R	R	S	S
83	R	S	R	R	I	R	R	R	S	R
84	R	S	R	R	R	R	R	R	S	R
85	R	S	S	R	S	I	R	R	S	R
86	R	S	S	R	I	R	R	R	S	S
87	R	S	S	R	S	I	I	R	S	R
89	R	S	R	R	R	R	R	R	S	R

AMP: ampicillin, AK: amikacin, CN: gentamicin, AMC: Augmentin, CAZ: ceftazidime, CTX: cefotaxime, CRO: ceftriaxone, SXT: sulfamethoxazole/trimethoprim, MEM: meropenem, LVO: levofloxacin, S: susceptible, I: intermediate, R: resistance.

**: *E. coli* ATCC 25922

*: *K. pneumoniae* ATCC 700603

Table 3. β -lactamase genes detected in 77 ESBL-producing *E. coli*, MIC tests to 3rd generation cephalosporins interpreted according to EUCAST recommendation and confirmatory test results.

Strain No.	MICs			DDST	CDT		CTX-M-1	CTX-M-9	SHV	TEM
	CAZ R>8	CTX R>2	CRO R>2		CAZ/ CLAV	CTX/ CLAV				
-V con	-	-	-	-	-	-	-	-	-	-
+v con	+	+	+	+	+	+	-	-	+	-
1	+	+	+	+	+	+	+	-	-	-
2	+	+	+	+	+	+	+	-	-	-
3	+	+	+	+	+	+	+	-	-	+
4	+	+	+	+	+	+	+	-	-	+
5	-	+	+	+	+	+	+	-	-	-
6	+	+	+	+	+	+	+	-	-	+
7	+	+	+	+	+	+	+	-	-	-
8	+	+	+	+	+	+	+	-	-	+
9	-	+	+	+	-	+	-	+	-	+
10	+	+	+	+	+	+	+	-	-	+
11	+	+	+	+	-	+	+	-	-	+
12	-	+	+	+	-	+	-	+	-	+
13	+	+	+	+	+	+	+	-	+	+
14	+	+	+	+	+	+	+	-	-	+
15	-	+	+	+	-	+	-	+	-	-
16	+	+	+	+	+	+	+	-	-	-
17	+	+	+	-	+	+	+	-	-	+
18	+	+	+	+	+	+	+	-	-	+
19	+	+	+	+	+	+	+	-	-	-
20	-	+	+	+	-	+	-	+	-	+
21	-	+	+	+	-	+	-	+	-	+
22	+	+	+	+	+	+	+	-	-	-
23	+	+	+	+	+	+	+	-	-	-
24	+	+	+	+	+	+	+	-	-	-
25	+	+	+	+	+	+	+	-	-	-
26	-	+	+	+	-	+	-	+	-	+
27	-	+	+	+	-	+	-	+	-	+
28	-	+	+	+	-	+	+	-	-	-
29	+	+	+	+	-	+	+	-	-	+
30	+	+	+	+	+	+	+	-	-	+
31	-	+	+	+	+	+	+	-	-	+
32	+	+	+	+	+	+	+	-	-	+
33	+	+	+	+	+	+	+	-	-	+
34	+	+	+	+	+	+	+	-	-	-
35	+	+	+	+	+	+	+	-	-	-
36	+	+	+	+	+	+	+	-	-	-
37	+	+	+	+	+	+	+	-	-	+
38	+	+	+	+	+	+	+	-	-	-
39	-	+	+	+	-	+	-	+	-	+
40	+	+	+	+	+	+	+	-	-	-
41	+	+	+	+	+	+	+	-	-	-

42	+	+	+	+	+	+	+	-	-	+
43	-	+	+	+	-	+	-	+	-	+
44	-	+	+	+	-	+	-	+	-	+
45	-	+	+	+	-	+	-	+	-	+
48	+	+	+	+	+	+	+	-	-	-
49	-	+	+	+	+	+	+	-	-	-
50	-	+	+	+	-	+	-	+	-	+
54	+	+	+	+	+	+	+	-	-	+
56	+	+	+	+	+	+	+	-	-	-
57	-	+	+	+	+	+	+	-	-	+
59	+	+	+	+	+	+	+	-	-	-
60	+	+	+	+	+	+	+	-	-	+
61	+	+	+	+	+	+	+	-	-	-
63	-	+	+	+	+	+	+	-	-	-
64	+	+	+	+	+	+	+	-	-	+
65	+	+	+	+	+	+	+	-	-	-
66	-	+	+	+	-	+	-	+	-	+
67	+	+	+	+	+	+	+	-	-	-
68	+	+	+	+	+	+	+	-	-	+
70	+	+	+	+	+	+	+	-	-	-
71	+	+	+	+	+	+	+	-	-	+
72	+	+	+	+	+	+	+	-	-	+
75	-	+	+	+	+	+	+	-	-	-
76	+	+	+	+	+	+	+	-	-	-
77	+	+	+	+	+	+	-	+	-	+
78	+	+	+	+	+	+	+	-	-	+
79	+	+	+	+	+	+	+	-	-	+
80	+	+	+	+	+	+	+	-	-	+
81	+	+	+	+	+	+	+	-	-	+
82	+	+	+	+	+	+	+	-	-	+
83	+	+	+	+	+	+	+	-	-	-
84	+	+	+	+	+	+	+	-	-	+
85	-	+	+	+	+	+	-	+	-	+
86	+	+	+	+	+	+	+	-	-	+
87	-	+	+	+	+	+	+	-	-	-
89	+	+	+	+	+	+	+	-	-	+

CAZ: ceftazidime, CTX: cefotaxime, CRO: ceftriaxone, DDST: double disk synergy test, CDT: combination disk test, CLAV: clavulanate.
+ve con: *K. pneumoniae* ATCC 700603
-ve con: *E. coli* ATCC 25922
+ : resistant to 3rd generation cephalosporins, positive DDST or CDT, and presence of β-lactamase gene
- : susceptible to 3rd generation cephalosporins, negative DDST or CDT, and absence of β-lactamase gene
R : resistance to 3rd generation cephalosporins according to EUCAST interpretation.
EUCAST: European Committee on Antimicrobial Susceptibility Testing

Table 4. MICs Comparison of ESBL-producing *E. coli* isolated from urine and faecal sources of same patients and interpreted according to CLSI 2005.

MICs											
	Antibiotic	AMP	AK	CN	AMC	CAZ	CTX	CRO	SXT	MEM	LVO
	Breakpoints	R≥32	R≥64	R≥16	R≥32	R≥32	R≥64	R≥64	R≥8	R≥16	R≥8
Strain no.	Patient no./ Sample source										
39	1/Urine	>128	2	1	>128	2	>128	>128	>128	<0.0625	<1
		R	S	S	R	S	R	R	R	S	S
46	1/Faecal	>128	4	1	>128	1	>128	>128	>128	<0.0625	8
		R	S	S	R	S	R	R	R	S	R
41	2/Urine	>128	16	>128	>128	128	>128	>128	>128	<0.0625	8
		R	S	R	R	R	R	R	R	S	R
47	2/Faecal	>128	16	>128	>128	>128	>128	>128	>128	<0.0625	8
		R	S	R	R	R	R	R	R	S	R
49	3/Urine	>128	32	2	>128	8	64	64	>128	<0.0625	16
		R	I	S	R	S	R	R	R	S	R
51	3/Faecal	>128	16	1	>128	4	>128	64	>128	<0.0625	16
		R	S	S	R	S	R	R	R	S	R
50	4/Urine	>128	1	1	>128	4	>128	>128	>128	<0.0625	8
		R	S	S	R	S	R	R	R	S	R
52	4/Faecal	>128	1	1	>128	4	>128	>128	>128	<0.0625	<1
		R	S	S	R	S	R	R	R	S	S
54	5/Urine	>128	8	64	>128	128	>128	>128	>128	1	8
		R	S	R	R	R	R	R	R	S	R
55	5/Faecal	>128	8	2	>128	128	>128	>128	>128	1	16
		R	S	S	R	R	R	R	R	S	R
59	6/Urine	>128	16	4	>128	128	>128	>128	>128	<0.0625	32
		R	S	S	R	R	R	R	R	S	R
62	6/Faecal	>128	16	2	>128	32	>128	>128	>128	<0.0625	2
		R	S	S	R	R	R	R	R	S	S
87	7/Urine	>128	16	2	>128	2	16	32	>128	<0.0625	16
		R	S	S	R	S	I	I	R	S	R
88	7/Faecal	>128	16	4	>128	2	16	32	>128	<0.0625	16
		R	S	S	R	S	I	I	R	S	R
89	8/Urine	>128	8	>128	>128	64	>128	>128	>128	<0.0625	32
		R	S	R	R	R	R	R	R	S	R
90	8/Faecal	>128	16	>128	>128	32	>128	>128	>128	<0.0625	32
		R	S	R	R	R	R	R	R	S	R

AMP: ampicillin, AK: amikacin, CN: gentamicin, AMC: Augmentin, CAZ: ceftazidime, CTX: cefotaxime, CRO: ceftriaxone, SXT: sulfamethoxazole/trimethoprim, MEM: meropenem, LVO: levofloxacin.
R : resistance according to CLSI 2005 breakpoints
S : susceptible according to CLSI 2005 breakpoints

Table 5. Comparison of urine and faecal ESBL-producing *E. coli* from same patients for their β-lacamase genotypes and susceptibility to 3rd generation cephalosporins. MICs here interpreted according to EUCAST breakpoints.

Strain No.	Patient no	Sample source	CAZ R>8	CTX R>2	CRO R>2	DDST	CAZ/CLAV	CTX/CLAV	CTX-M-1	CTX-M-9	SHV	TEM
39	1	Urine	-	+	+	+	-	+	-	+	-	+
46		Faecal	-	+	+	+	-	+	-	+	-	+
41	2	Urine	+	+	+	+	+	+	+	-	-	-
47		Faecal	+	+	+	+	+	+	+	-	-	-
49	3	Urine	-	+	+	+	+	+	+	-	-	-
51		Faecal	-	+	+	-	+	+	+	-	-	-
50	4	Urine	-	+	+	+	-	+	-	+	-	+
52		Faecal	-	+	+	+	-	+	-	+	-	+
54	5	Urine	+	+	+	+	+	+	+	-	-	+
55		Faecal	+	+	+	+	+	+	+	-	-	+
59	6	Urine	+	+	+	+	+	+	+	-	-	-
62		Faecal	+	+	+	+	+	+	+	-	-	-
87	7	Urine	-	+	+	+	+	+	+	-	-	-
88		Faecal	-	+	+	+	+	+	+	-	-	-
89	8	Urine	+	+	+	+	+	+	+	-	-	+
90		Faecal	+	+	+	+	+	+	-	-	-	+

CAZ: ceftazidime, CTX: cefotaxime, CRO: ceftriaxone, CLAV: clavulanate.
+ : resistant to 3rd generation cephalosporins, positive DDST or CDT, and presence of β-lactamase gene
- : susceptible to 3rd generation cephalosporins, negative DDST or CDT, and absence of β-lactamase gene
R : resistance to 3rd generation cephalosporins according to EUCAST interpretation.
EUCAST: European Committee on Antimicrobial Susceptibility Testing