Characterization and Molecular Epidemiology of Extended-Spectrum-ß-Lactamase-Producing *Escherichia coli* derived from University Hospitals of Egypt and Germany

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1.0. Introduction

The continuous emergence of resistance to antimicrobial agents among the prevalent pathogens is the most dangerous threat for the treatment of infectious disease. The production of ß-lactamases is the major mechanism of bacterial resistance to ß-lactam antibiotics which considered the most widely used class of antibiotic. Curiously, the detection of the first ß-lactamase was reported before the use of penicillin in the medical field. Extended-spectrum-ß-lactams have been introduced in the medical practice in the 1980s for the treatment of serious gram negative bacteria but the resistance to this class of antibiotic has emerged rapidly due to production of a new class of ß-lactamase later termed extended-spectrum ß-lactamase (ESBL) (Al-Jasser, 2006).

In 1983, plasmid born extended-spectrum ß-lactamase, SHV-2, produced by *Klebsiella ozaenae* isolate was discovered in Germany. TEM and SHV types were the predominant ESBL types until the 1990s. In this duration *Klebsiella pneumoniae* was the main ESBL-producer. Later-on, prevalence of TEM and SHV and a new ESBL family, CTX-M which is produced mainly by *E. coli*, has emerged. During the next few years, CTX-M has become the predominant ESBL family and CTX-M-producing *E. coli* has spread globally and has been involved in nosocomial outbreaks and community acquired infections (Canton and Coque, 2006; Marcade *et al*., 2009).

The first $bla_{\text{CTX-M}}$ was detected in clinical *E. coli* isolate in Germany 1990 **(**Bauernfeind *et al.,* 1990) then CTX-M-producing *Enterobacteriaceae* has globally been detected. CTX-M is named after their higher hydrolytic activity against cefotaxime than ceftazidime and the place of first isolation (Munich, Germany). $Bla_{\text{CTX-M}}$ is a 291 amino acids encoding enzyme and the change in any one of them result in a new CTX-M variant (Naseer and Sundsfjord, 2011).

Currently, a total of 130 different $bla_{\text{CTX-M}}$ allele types have been added to Lahey data base for classification and aminoacids sequences of TEM, SHV, CTX-M, OXA and inhibitor resistant ß-lactamase (http://www.lahey.org /Studies accessed for last time in 10th May 2012).

The members of CTX-M family are grouped into five evolutionary groups (clusters) on the basis of their genetic relatedness and their amino acid homology with each group named after their first described member. These groups include CTX-M group 1, 2, 8, 9 and 25 (Naseer and Sundsfjord, 2011).

Currently, CTX-M-15 is the most widely distributed ESBL type. It has been detected almost everywhere in the world among clinical enterobacterial isolates (Bonnet, 2004; Bradford, 2001).

In Egypt, CTX-M-15 is the dominant CTX-M-ESBL type in addition to other less frequent types like CTX-M-14 and CTX-M-27 have been also reported (Mohamed Al-Agamy *et al.*, 2006; Fam *et al*., 2011).

In Germany as well as many other part of Europe, CTX-M-1 and CTX-M-3 in addition to CTX-M-15 are the most prevalent CTX-M variants (Mshana *et al.,* 2009; Cullik *et al.*, 2010).

CTX-M-15 was identified for the first time in 1999 from an isolate from India **(**Karim *et al.*, 2001) and reported for the first time in the African continent 2005 in Tanzania (Blomberg *et al*., 2005).

Among the frequently detected CTX-M-types in Europe are CTX-M-1 and CTX-M-3. The spread of CTX-M-3, the precursor of CTX-M-15, is seemed to be restricted to Eastern Europe but it is sporadically detected in other parts of Europe. CTX-M-15 differs from CTX-M-3 only in single amino acid at the position 240 (Asp240Gly) resulting in enhanced activity against ceftazidime (Poirel *et al., 2002*).

CTX-M-encoding gene has been located on plasmids ranging from 7 to 430 kb in size. It has frequently been located on large conjugative plasmids encoding also genes of resistance to non ß-lactam antimicrobials like tetracycline, aminoglycosides sulphonamide and trimethoprim (Naseer and Sundsfjord, 2011).

Bla$_{\text{CTX-M}}$-types have been associated with certain plasmid replicons like the association of CTX-M-15 with FII either in single or muti-replicons form with FIA and/or FIB. Presence of *bla*$_{\text{CTX-M-15}}$ on Inc FI plasmids mainly FIA and FIB either in a single or multi-replicon form have recently been reported (Gonullu *et al., 2008*; Mshana *et al.*, 2009; Mshana *et al*., 2011).

Inc F plasmids are narrow host range plasmids highly adapted to *Enterobacteriaceae* and frequently carry more than one replicon. These plasmids are a heterogeneous group of plasmids which are variable in size, resistance determinants content and replicon combination (Bergquist *et al*., 1986; Nordstrom, 2006). The other CTX-M-types like CTX-M-1 and CTX-M-3 are frequently associated with broad host range replicon type plasmids as Inc N, Inc I 1 and Inc L/M enabling them to be transferred to a new host of a distantly related or even unrelated species (Novais *et al.*, 2007). Although A/C replicon type plasmid is considered to be rare in *Enterobacteriaceae*, it has been

detected in CTX-M-producing *E. coli* (Marcade *et al.,* 2009). Frequent association of $bla_{\text{CTX-M-15}}$ with $bla_{\text{TEM-1}}$ has been reported previously in 80% of CTX-M-producing *E. coli* (Marcade *et al.*, 2009). Co-existence of $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$ on the same plasmid has previously been reported (Karisik *et al.*, 2006; Marcade *et al.*, 2009).

The horizontal transfer of ESBL-encoding plasmids among clonally related and unrelated, local endemic and international epidemic clones is responsible for the current high prevalence of ESBLs in the different European regions (Coque *et al.,* 2008 B).

The phylogenetic grouping and multi-locus sequence typing are among the most beneficial genotyping way for *E. coli* which can be used for their comparison on the global level. Recently, the simple PCR based method (Clermont method) has been extensively used for determination of the phylogenetic relationship among *E. coli* isolates. CTX-M-15 has been frequently linked to the phylogenetic group B2 and D while CTX-M-14 has been linked to B1, A and D (Naseer and Sundsfjord, 2011).

CTX-M family has been associated with many different MLST some of them displaying a global spread feature while the others have emerged and persisted locally. ST131 is the best representative of the first class being responsible for the global dissemination of CTX-M-15. Another examples are ST405 and ST38, those have been also involved in the global spread of CTX-M-15 (Coque *et al.*, 2008 B; Naseer *et al*., 2009; Oteo *et al*., 2009 A). ST648 has been reported in many different countries. Birds are suspected to be the disseminator of this sequence type (Guenther *et al., 2010*). The remaining sequence types which are detected among the ESBL-producing *E. coli* have emerged and proliferated locally.

1.1. *Enterobacteriaceae*

Members of this family are gram-negative, rod-shaped, facultative anaerobes, most of them are motile (with peri-trichous flagella) grow at 37^{o}C on peptone, meat extract without addition of sodium chloride or other supplements, grow well on MacConkey agar, ferment glucose aerobically and anaerobically, reduce nitrate to nitrite and their G + C DNA content range from 39% to 59%. They are found in soil, water, plants and human and animal intestines. The common genera of this family include: *Escherichia, Klebsiella, Enterobacter, Salmonella, Proteus, Shigella, Citrobacter, Yersinia, Serratia, Morganella, Providencia, and Hafnia.* The most clinically relevant species to ESBL issue are *Escherichia coli* and *Klebsiella pneumoniae* (Irving *et al*., 2005).

1.1.1. *Escherichia coli*

Escherichia coli: is a member of the genus *Escherichia* which contain mostly motile gram-negative bacilli including also *E. blattae, E. fergusonii, E. hermanii and E. vulneris* and belong to the family *Enterobacteriaceae* (Nataro and Kaper, 1998). *E. coli* was identified in 1885 by the German pediatrician Theodor Escherich (Feng *et al*., 2002). *E. coli,* facultative aerobes, is isolated from clinical samples either on general or selective media (such as MacConkey and eosin methylene-blue agar) at 37^{o}C under aerobic conditions (Nataro and Kaper, 1998).

E. coli is considered to be of fecal origin and is regarded as indicator of fecal contamination but sometimes is found temporarily in the other environments such as raw meat and vegetables (Österblad *et al.,* 1999). *E. coli* is widely distributed in the intestine of humans and animals maintaining the physiology of their host and are considered as one of the first bacterial genera (in addition to *Streptococcus*) that colonize the intestine of both human and animal-newborns protecting them against enterotoxigenic *E. coli* and *Salmonella spp*. (Feng *et al.,* 2002; Hudault *et al.*, 2001).

Based on their clinical and genetic characters, *E. coli* strains are classified into commensal strains and pathogenic strains. The pathogenic strains are subdivided into intestinal pathogenic (also termed enteric or diarrheagenic) strains and extraintestinal pathogenic strains (Russo and Johnson, 2000). The great majority of *E. coli* is commensals. But they act as an opportunistic organism causing infections when other factor is implicated like foreign body indwelled in immunocompromised hosts. Most of them belong to phylogenetic group A and are normally devoid of the specialized virulence features that found among pathogenic strains (Feng and Weagant, 2011; Russo and Johnson, 2000).

Intestinal pathogenic strains of *E. coli* are rarely found among the intestinal flora of healthy hosts. Six different pathogenic classes of intestinal pathogenic groups were identified according to their virulence factors namely, enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Russo and Johnson, 2000).

ETEC is the causative agent of travelers' diarrhea (watery diarrhea without fever) that occurs in developing countries due to consumption of soft foods which contain any of their several enterotoxins that are produced by ETEC.

EIEC is the causative agent of invasive, dysenteric form of diarrhea in humans due to the ability to invade the colonic mucosa.

EHEC is the causative agent of hemorrhagic colitis and bloody diarrhea due to the production of Vero or Shiga toxins (Feng and Weagant, 2011).

Extraintestinal pathogenic *E. coli* (ExPEC) strains are not able to cause intestinal disease. However, they can colonize the intestinal tract and predominate to the other strains in 20% of healthy hosts. Entry to extraintestinal anatomical site like urinary tract is an essential prerequisite for infection by such group. In contrary to commensal *E. coli* strains, ExPEC strains are derived either from the phylogenetic group B2 or D and encode various combinations of genes collectively called extraintestinal pathogenicity virulence factors (Russo and Johnson, 2000).

1.2.2. *Klebsiella spp.*

***Klebsiella spp.*:** Gram-negative, non-motile, encapsulated, rod-shaped bacteria belong to the family *Enterobacteriaceae.* According to their medical importance, the genus *Klebsiella* are divided into the following species: *K. pneumoniae*, *K. ozaenae* and *K. rhinoscleromatis* and *K. oxytoca* (Podschun and Ullmann, 1998).

Klebsiella spp. are found in two habitats. The first environment is the surface water, sewage, soil and on the plants, while the other environment is mucosal surfaces of mammals (Bagley *et al.,* 1978; Brown and Seidler, 1973**)**. In humans, *Klebsiella pneumoniae* are found as saprophyte in the nasopharynx and in the intestinal tract of human (Davis and Matsen, 1974).

Klebsiella are opportunistic bacteria that cause nosocomial infection in immunocompromised, hospitalized patients. *Klebsiella* are the causative agent of pneumonia, septicemia and urinary tract infection. *Klebsiella pneumoniae* and *K. oxytoca* regarded as the only pathogenic members of the genus *Klebsiella* (Bennett *et al.*, 1995).

Klebsiella usually develop a prominent capsule of complex acidic polysaccharide (K antigen) which is an essential determinant of the virulence of *Klebsiella*. This capsule protects *Klebsiella* from phagocytosis and from the bactericidal effect of serum factors. Presence of pili, responsible for the adhesive properties, is another contributor of *Klebsiella* virulence (Podschun and Ullmann, 1998).

1.2. Antimicrobial agents

The great majority of antimicrobial agents can be classified on the basis of their mechanism of action into four groups:

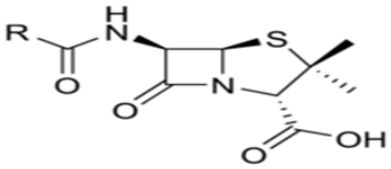
- Cell wall synthesis inhibitors.
- Protein synthesis inhibitors.
- Inhibitors of certain metabolic pathway
- Nucleic acid synthesis inhibitors (Neu, 1992).

ß-Lactams like penicillins, cephalosporins, monobactam and carbapenem belong to the first group which inhibits the cell wall synthesis by interference with the enzymes that are involved in the peptidoglycan cross-linking. Tetracycline, aminoglycosides and macrolides selectively inhibit the bacterial protein synthesis but do not affect that of eukaryotes due to their ribosomal difference. Fluroquinolones interfere with DNA replication, while sulphonamides and trimethoprim block the synthetic pathway for folic acid (Tenover, 2006).

1.2.1. ß-Lactam antibiotics

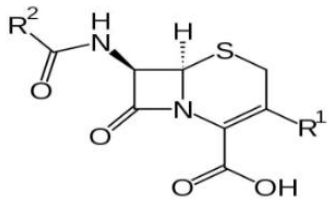
This group of antibiotic includes penicillins, cephalosporins, carbapenems and monobactams. ß-lactam ring is considered as Achilles heel in this group which is easily broken by ß-lactamase enzymes, which are produced by frequent bacterial species, resulting in loss of their anti-bacterial activity.

Penicillins: in these members the ß-lactam ring is fused with thiazolidine ring



Basic penicillin structure (source: Wikipedia)

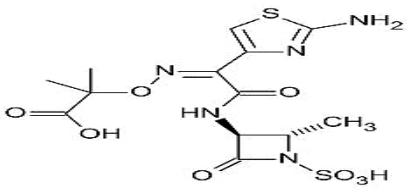
Cephalosporins: In this group ß-lactam ring is fused with dihydrothiazine (six-membered) ring. Cephalosporins are classified into first, second, third and fourth generations.



Basic cephalosporins structure (source: Wikipedia)

The first generation includes the compounds which were available before 1975 as cefalexin and cefalothin, the second generation includes the compounds which are stable to ß-lactamase as cefuroxime and cefoxitin, the third generation includes the compounds which have ß-lactamase (but not ESBLs) stability, higher intrinsic activity and extended spectrum like cefotaxime, ceftazidime, the fourth generation is a group of new compounds like cefepime **(**Greenwood *et al*., 2006).

Monobactams: This class of ß-lactam antibiotics contains non-fused ß-lactam ring. Aztreonam is the only commercially available member of this class. Aztreonam is active against aerobic, Gram negative organisms including *Pseudomonas aeruginosae* but inactive against anaerobic bacilli and the Gram positive organisms. The lack of cross-allergy with the other ß-lactam antibiotics enables it be used as an alternative for penicillin and cephalosporin in case of patient allergy to these agents (Bodey, 1990).



Aztreonam structure

Carbapenems: This group include: imipenem, meropenem and ertapenem. Carbapenems have the widest spectrum of activity compared to any other group of antibiotics which provide broad-spectrum umbrella when multiple and/or unknown organisms are expected otherwise it should be reserved for the treatment of infection caused by ESBL– producing organisms (Bodey, 1990).

ß-Lactamase inhibitors: Are the agents that inhibit ß-lactamase enzymes by irreversible binding to its active site rendering it permanently inactive. The first clinically used ß-lactamase inhibitor was clavulanic acid (isolated from *Streptomyces clavuligaris*). It has a weak antimicrobial activity. But if combined with amoxicillin, it significantly increases the antimicrobial activity of the later. The other ß-Lactamase inhibitors such as sulbactam and tazobactam are combined with ampicillin and piperacillin respectively. ß-lactamase inhibitors are effective against class-A ß-lactamase including CTX-M, TEM and SHV-ESBLs (Drawz and Bonomo, 2010).

1.3. Antimicrobials resistance

The microbiologists and the clinicians regard an organism to be resistant to an antimicrobial agent when it is inhibited in vitro by a concentration greater than the highest achievable concentration in the human body (Hawkey, 1998).

1.3.1. Types of resistance

I-Intrinsic resistance: The resistance of all members of a bacterial species without any genetic extra-modifications. This type of resistance is due to either the lack of the target for the action of drug or inability of drug to enter the bacterial cell (Normark and Normark, 2002; Greenwood *et al*., 2006).

II- Acquired resistance: This type of resistance includes:

- Mutational resistance: Occur either by point mutation, deletion, inversion or insertion in the bacterial genome resulting in a very few individuals, among the huge bacterial populations, exhibiting spontaneous resistance. These resistant mutants proliferate under the action of antibiotic selective pressure to constitute the majority or even the whole population (Normark and Normark, 2002; Greenwood *et al*., 2006).
- Transferable resistance: in which a resistance gene (or genes) transfer from resistant to susceptible bacterial cell. Among the different DNA elements those transfer antibiotic resistances are plasmids, phages, transposons and integrons (Normark and Normark, 2002; Greenwood *et al*., 2006).

1.3.2. Mechanisms of resistance

The ability of an antimicrobial agent to inhibit a bacterial cell requires the following conditions:

- Presence of susceptible vital target in the bacterial cell (frequently enzymes or essential proteins)
- Sufficient and metabolically active concentration reaches to the target site.

The antimicrobial agents enter the bacterial cell either across the cell wall and the outer membrane or are carried by an active transport mechanism. Consequently, the differences in the susceptibilities of the different bacterial species are largely due to the difference in their cell wall structure. Therefore, the complex structure of gram negative bacteria offers a comparatively greater barrier to many antimicrobials than gram positive bacteria (Greenwood *et al.*, 2006).

The mechanisms of drug resistance are divided into the following:

- Inactivation or destruction of the antimicrobial agent.
- Alteration or protection of the target site.
- Blocking the active transport mechanism, decreasing the cell surface permeability or (efflux) removal from the cell.
- Creation of alternative metabolic pathway instead of that was inhibited by antimicrobial agent (McManus, 1997; Tenover, 2006).

1. 3.3. Genetics of resistance

Knowledge of the basic genetics of the microbial resistance enables us to understand the evolution and spread of resistance and hence, it is suitable to start with the recognition of the different DNA elements that play a role in the evolution and spread of resistance.

Plasmids: Extrachromosomal, self-replicating double stranded DNA, present in bacterial host cell in a number of copies ranges from one to hundreds of copies, many of them encode a toxin-antitoxin based system called addiction system that eliminates the daughter cell which did not receive the relevant plasmid during cell division ensuring the survival of the plasmid regardless of the antimicrobial selective pressure. On the other hand, most of the other plasmids are devoid of such system. And hence, their survival depends on the selection of their hosts which encode an antimicrobial

resistance gene under the presence of the relevant antimicrobial selective pressure (Carattoli, 2009). Conjugative plasmids contain *tra* genes which encode all necessary requirements for conjugation. There are also mobilisable (non self-transmissible) plasmids which can transfer in association with the conjugative (self-transmissible) plasmid (Carattoli, 2001).

Bacteriophages: These are viruses infecting only small number of strains of related bacteria (specific host range) and are divided into virulent (lytic) phage and temperate (lysogenic) phage. During transduction, the phage particle can carry exogenous bacterial DNA and transfer it to other recipient bacteria. The narrow host range of the phage greatly limits their role as a vector transferring the resistance genes (Carattoli, 2001).

Transposons: are DNA sequences that have the ability of transposition from one replicon (either plasmid or chromosome) to another and consist of either individual or a group of resistance genes flanked by short (often 40 bp) DNA sequences called direct or inverted repeats. These DNA sequences serve as recognition sites for transposase enzymes that catalyze the transfer of the transposon from one replicon to another. Transposition is an extremely important mechanism for the natural transfer of antibiotic resistance genes from one bacterial replicon and recombination into another. Theoretically, any two similar insertion sequences can bracket any gene and convert it into a transposon. All replicons, at least from the theoretical point of view, are liable to transposition and all genes are transposable and hence transposons and insertion sequences play an important role in the evolution of the resistance explaining how an antibiotic resistance gene can emerge and disseminate over a wide range of non-related replicons (Greenwood *et al.,* 2006).

Integrons: These specialized DNA elements have been frequently observed in multi-drug resistant isolates either located on the chromosome or broad host range plasmids. Integrons are composed of two conserved regions flanking a variable region containing one or more of resistance genes. The essential components of any integron include the integrase gene (*intI*), the attachment site (*attI*) and the promoter (Carattoli, 2001).

1. 3.3.1. Modes of transfer of resistance

Bacterial resistance may develop vertically as a result of chromosomal mutation followed selection under antibiotic selective pressure. Bacterial resistance may also develop horizontally when a susceptible bacterial cell acquires determinants of resistance from a resistant strain through one the following modes of the genetic exchange (Tenover, 2006).

Conjugation: The process in which DNA passes on one direction from a bacterial cell (the donor) to another bacterial cell when the two cells contact one another through an elongated proteinaceous structure called pillus (McManus, 1997).

Transformation: The process in which the bacterial cell acquires naked DNA from the surrounding medium. This process is of a rare occurrence in vivo and depends largely on the competence of the recipient cell to uptake the naked DNA (McManus, 1997).

Transduction: The process in which the phage particle serves as a vector transferring the bacterial DNA that is incorporated into the bacteriophage particle to the next infected cell (McManus, 1997).

The evolution of resistance via mutation and selection and the horizontal transfer of the resistance determinant enable the bacteria to accommodate the introduction of antimicrobial agents quickly and to increase the spectrum of resistance of the bacteria. Single mutation may develop initially to reduce the sensitivity of the host to the present antimicrobial until additional determinants of resistance or further mutations are acquired. In very rare occasion, a single mutation may result in high level of resistance (Tenover, 2006).

1.4. ESBL Definition and classification

Extended-spectrum ß-lactamase (ESBL): This term was used initially to refer to TEM and SHV enzymes that have the ability to hydrolyze oxyimino-cephalosporins. Later on, this term has been widened to include:

- Enzymes derived from other source and have resistance spectra similar to that of TEM and SHV mutants e.g. CTX-M and VEB types.

- Enzymes exhibit wider resistance than their parents but do not belong to 2be group e.g. OXA and Amp C mutants with increased activity against cefepime (Livermore, 2008).

Although there is no consensus on the exact ESBL definition, the currently used definition for ESBL is ß-lactamase that is able to render the bacteria resistant to the penicillins, first, second, and third-generation cephalosporins and aztreonam (but not cephamycins or carbapenems) by hydrolysis (which could be inhibited by ß- lactamase inhibitors) of these antibiotics (Paterson and Bonomo, 2005).

There are two general schemes for classification of ß-lactamases:

- Ambler molecular classification scheme (see Table 1) which is based on the protein sequence similarity. Accordingly, ß-lactamase enzymes are classified into four classes A, B, C and D based on conserved and variable amino acid motifs. Class A, C, and D include the enzymes that hydrolyze their substrates by forming acyl enzymes via the active site serine, while class B (metalloenzymes) utilizes active site zinc to facilitate ß-lactam hydrolysis (Bush and Jacoby, 2010).
- Bush-Jacoby-Medeiros functional classification scheme (see Table 1) which classify these enzymes according to the similarity in their functional (substrates and inhibitors profile) characteristics. Although the molecular classification is the easiest scheme to group these diverse enzymes, the functional classification enables the clinicians and laboratory microbiologists to correlate these enzymes with their clinical roles (Bush *et al.,* 1995; Bush and Jacoby, 2010).

Table 1: Classification of ß-lactamases (Bush *et al.*, 1995).

Bush-Jacoby group	Molecular class	substrate	Inhibitor		Characteristic	Example
			CA or TZB	EDTA		
1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylbenicillin; hydrolyzes cephamycin.	AmpC, ACT-1, CMY-2, FOX-1,MIR-1
1e	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often oxyimino-ß-lactams	CMY-37
2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylbenicillin than cephalosporins.	PC1
2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzyl penicillin and cephalosporins.	TEM-1, TEM-2, SHV-1
2be	A	Extended–spectrum cephalosporins, monobactam	Yes	No	Increased hydrolysis of oxyimino-ßlactams (cefotaxime, ceftazidme, ceftriaxone ,cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, tazobactam	TEM-30, SHV-10
2ber	A	Extended–spectrum cephalosporins, monobactam	No	No	Increased hydrolysis of oxyimino ß-lactamswith resistance to clavulanic acid, sulbactam, tazobactam	TEM-50
2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbinicillin	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, cefpirome	RTG-4
2d	D	Cloxacillin	V	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	D	Extended–spectrum cephalosporins	V	No	Hydrolyzes cloxacillin, oxacillin oxyimino-ß-lactams	OXA-11, OXA-15
2df	D	Carbapenems	V	No	Hydrolyzes cloxacillin, oxacillin, carbapenems	OXA-23, OXA-48
2e	A	Extended–spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins inhibited by clavulanic acid but not aztreonam	CepA
2f	A	Carbapenems	V	No	Increased hydrolysis of carbapenems, oxyimino-ß-lactams,cephamycins	KPC-2, IMI-1, SME-1
3a	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, IND-1
3b	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1

CA : Clavulanic acid.

TZB: Tazobactam.

V : Variable

1.4.1. ESBL types

1.4.1.1. TEM

TEM type ESBLs are derivatives of TEM-1 and TEM-2.

TEM-1 was detected for the first time in 1965 in Greece in an *Escherichia coli* isolate recovered from a patient named Temoneira, and hence the designation TEM (Datta and Kontomichalou, 1965).

TEM-1 hydrolyzes ampicillin at a rate higher than that of carbenicillin, oxacillin, and cephalothin but fail to hydrolyze the extended-spectrum cephalosporins.

TEM-2 has the same hydrolytic activity of TEM-1 but has more active native promotor and a different isoelectric point (5.6 instead 5.4). The plasmid mediated ß-lactamase TEM-3 (an ESBL member) was detected in 1987 in *Klebsiella pneumoniae* isolate in France. It was originally named CTX-1 due to its higher activity against cefotaxime. The amino acid substitutions in the different TEM variants in comparison with TEM-1 are illustrated in the following Figure which is adapted from Bradford (2001).

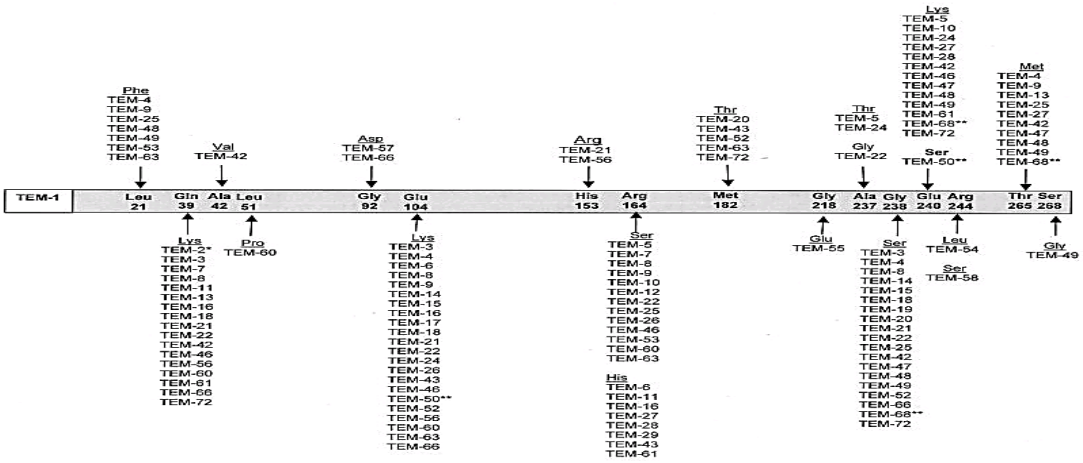


Figure 1: The amino acid substitutions in the different TEM variants in comparison with TEM-1 adapted from Bradford (2001).

1.4.1.2. SHV

SHV-type ESBL was the most frequent ESBL-type that has been found in clinical isolates (Jacoby, 1997). SHV refer to sulf*h*ydryl *v*ariable because it was thought that the inhibition of the enzyme activity by p-chloromercuribenzoate was substrate-dependent and variable according to the substrate used in the assay (Sykes and Bush, 1982).

In1983, a new SHV-ß-lactamase (designated SHV-2) efficiently hydrolyzes cefotaxime and to lesser extent ceftazidime has been detected in *Klebsiella ozaenae* in Germany

(Knothe *et al.*, 1983). SHV-2 differs from SHV-1 by only one amino acid at the 238th position (glycine replaced by serine). This substitution (Gly238Ser) that resulted from a point mutation accounts for the activity of this enzyme against extended-spectrum cephalosporin. SHV-2 spread globally due to the selection pressure exerted by third-generation cephalosporins being detected in a wide range of *Enterobacteriaceae* but mainly *Klebsiella spp.* (Paterson and Bonomo, 2005; Paterson et *al.*, 2003).

The amino acid substitutions in the different SHV variants in comparison with SHV-1 is illustrated in the following Figure

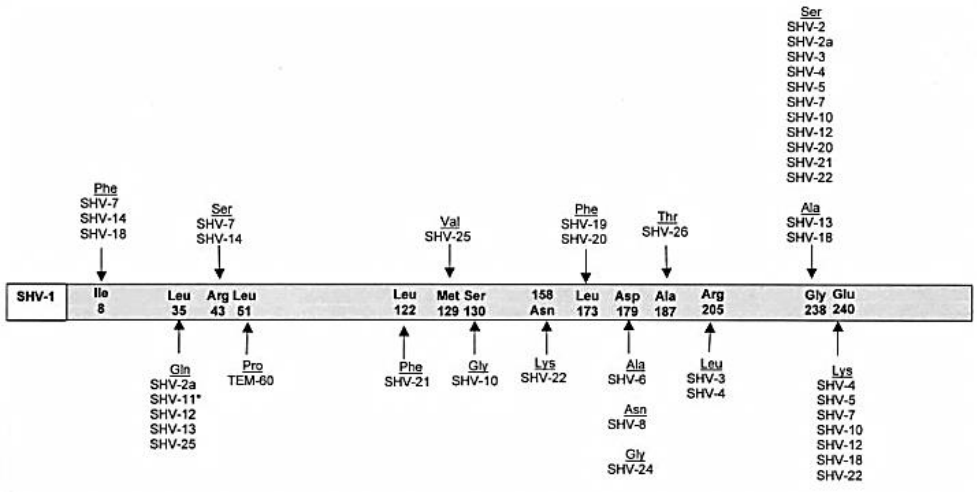


Figure 2: The amino acid substitutions in the different SHV variants in comparison with SHV-1 adapted from Bradford (2001).

1. 4.1.3. CTX-M

The designation CTX refers to the potent hydrolytic activity of these enzymes against cefotaxime. However, some CTX-M-types hydrolyze ceftazidime. CTX-M-types hydrolyze cefepime with high efficiency (Baraniak *et al.*, 2002; Paterson and Bonomo, 2005; Yu *et al.*, 2002). The hydrolytic activity of CTX-M is inhibited by ß-lactamase inhibitors. Tazobactam exhibits 10-fold greater inhibitory activity than clavulanic acid (Bush *et al.*, 1993). CTX-M-type ß-lactamases are related to the chromosomal ß-lactamase of *Kluyvera spp.* (Decousser *et al.*, 2001). The number of CTX-M-ESBL-types is rapidly increasing and worldwide dissemination can be observed (Paterson and Bonomo, 2005).

1. 4.1.4. OXA-ß-lactamase

OXA name refers to the oxacillin-hydrolyzing ability of these ß-lactamases. They hydrolyze oxacillin and cloxacillin at a rate greater than 50% that of benzylpenicillin **(**Bush *et al.,* 1995). OXA-ß-lactamases are mainly found in *Pseudomonas aeruginosae* **(**Weldhagen *et al.,* 2003). The most common OXA-type ß-lactamase is OXA-1. It has been detected in up to 10% of *E. coli* isolates (Livermore, 1995). Most OXA-type ß-lactamases do not hydrolyze the extended- spectrum cephalosporin and hence are not regarded as ESBLs. OXA- ESBLs includes, OXA-10 (weak), -11, -14, -16, -17. -19, -15, -18, -28, -31, -32, -35 and -45 (Paterson and Bonomo, 2005).

1.4.1.5. Other ESBL-types: includes PER-1, PER-2, VEB-1, VEB-2, GES and SFO which share only 25% and 27% homology with TEM and SHV types. PER-1 is widely spread in Turkey and share 86% amino acid homology with PER-2 which is found almost exclusively in South America. VEB-1 discovered in an *E. coli* isolate from Vietnamese patient hospitalized in France (Bradford, 2001). TLA-1 was detected in a clinical *E. coli* isolate in Mexico. PER-1, PER-2, VEB-1 and TLA-1 are related and have some homology to the chromosomal ß-lactamase in *Bacteroides spp.* Therefore, it is likely to be originated from this genus (Bradford, 2001).

1.5. ESBL Epidemiology

ESBLs currently are a universal problem in hospitalized patients and community settings. Prevalence of ESBLs among clinical isolates is variable from institution to institution and from country to country and from continent to continent (Al-Jasser, 2006). Determination of the prevalence of ESBL-producing organisms at a wider geographical level is difficult and very likely to be under/overestimated. This is attributed to the different methods used for detection, sometimes the difficulty of the detection itself, the difference in the minimum inhibitory concentration (MIC) breakpoints, the variation of the incidence of ESBL among the different hospitals or medical centers located in the same country in addition to discontinuous monitoring and reporting (Sturenburg and Mack, 2003; Al -Jasser, 2006). All these factors complicate the task. Nevertheless, recent studies refer to a significant global increase in the ESBL rate. In North America the ESBL rate in *Klebsiella spp., E. coli* and *Proteus mirabilis* ranges from 4.2% - 44%, 3.3 - 4.7% and 3.1 - 9.5%, respectively. In Latin America the

ESBL rate of *Klebsiella spp.*, *E. coli* and *Proteus mirabilis* lie in the ranges 40% - 47.3%, 6.7 -25.4% and 9.5- 35.5%, respectively. In the Far East-Western Pacific area, the ESBL rate in *Klebsiella spp., E. coli, Salmonella spp.* and *Proteus mirabilis* ranges between 11.3% - 51%, 7.9% - 23.6%, 3.4% and 1.4 -1.8%, respectively (Sturenburg and Mack, 2003). On the national and global levels the overall ESBL production rate for the combined *Enterobacteriaceae* was 10.5%, the highest rate was detected in Egypt (38.5%) and Greece (27.4%) and the lowest rate was in Netherlands (2%) and Germany (2.6%) according to the Pan European Antimicrobial Resistance Local Surveillance (PEARLS) study (2001-2002) (Bouchillon *et al.,* 2004**)**. In the United States the prevalence of ESBL-encoding *Enterobacteriaceae* was around 3% (Bradford, 2001). In Europe, the incidence of ESBL-producing *Enterobacteriaceae* is greatly variable from geographical region to another and from country to country the published studies reflect that lower ESBL prevalence in Northern European countries compared to Southern and Eastern European countries (Bradford, 2001 and Coque *et al* ., 2008 A). In Spain, only 1.5% among1962 invasive *E. coli* isolates in 2001 were found to produce ESBL (Oteo *et al.,* 2002). In France, 11.4% of 6121 *K. pneumoniae* isolates and 47.7% of 2353 *E. aerogenes* were found to produce ESBL in a surveillance covering many medical centers in the period from 1996 to 2000 (Albertini *et al.*, (2002). Northern European countries still have the lowest prevalence of ESBL-producing *Enterobacteriaceae* ranging from <1% in the Netherlands to 3% in Sweden. In 2001, a study accomplished by Paul-Ehrlich-Gesellschaft (PEG) covering many medical centers in Germany revealed that the prevalence of ESBL-producing *K. pneumoniae* 8.2%, *E. coli* 0.8% and *K. oxytoca* 1.3% (Sturenburg and Mack, 2003). Another study carried out by Gröbner *et al* at the University Hospital of Tübingen during the period from 2003 to 2007 reported that the overall ESBL prevalence still low (1.6%), however, the study recorded slight but continuous increase in the percentage of ESBL-producing *Enterobacter spp.* from 0.8% in 2003 to 6.4% in 2007 and in *E. coli* from 0.5% in 2003 to 3.8% in 2007. Percentage of ESBL-producing *Klebsiella spp.* ranged from 1.3% in 2003 to 2.9% in 2007 (Grobner *et al.,* 2009). A previous study carried out in Giessen, Germany by Mshana *et al.* on 63 *E. coli* isolates, collected in the period from August 2006 to April 2007 recorded high prevalence (77.7%) of $bla_{\text{CTX-M}}$ encoding isolates (Mshana *et al.,* 2009). In an earlier study in Dresden, Germany carried out by Schmitt *et al.* on 39 ESBL positive enterobacterial isolates (including 10 *E. coli*) collected in the period

from January to September 2003, 80% of ESBL positive *E. coli* isolates were encoding for bla_{CTX-M}, 50% of the isolates were encoding bla_{TEM} and 20% were encoding bla_{SHV}. One half of the CTX-M-encoding *E. coli* isolates were also encoding bla_{TEM} gene, while the rest were harboring CTX-M gene alone. None of the enterobacterial isolates were found to harbor bla_{CTX-M} together with bla_{SHV} (Schmitt *et al*., 2007). In a relatively recent study carried out by Cullik *et al*. on 22 ESBL-producing *E. coli* isolates collected during 2006 from a German University Hospital, bla_{CTX-M} was the most frequent ß-lactamase-encoding gene being detected in 95.5% of the tested isolates followed by bla_{TEM} (63.6%), while only one isolate (4.5%) was positive for bla_{SHV}. Co-existence of both bla_{CTX-M} and bla_{TEM} was detected in (59.1%), while the presence of bla_{CTX-M} alone was detected in (36.4%) of the tested isolates (Cullik *et al*., 2010).

In a study carried out by Fang *et al*. on 87 ESBL-producing *E. coli* isolates in Stockholm, Sweden collected during the period from 2001 to 2006, 92% of isolates were encoding bla_{CTX-M}, 63% were encoding bla_{TEM} and 6% were encoding bla_{SHV} (Fang *et al*., 2008).

In the United Arab Emirates, five (11.3%) multidrug-resistant enteroaggregative *E. coli* strains demonstrated ESBL production (Sonnevend *et al.*, 2006). The first detection of CTX-M-ESBL-type in Saudi Arabia was documented in 2009 by Al-Agamy *et al*. in a study targeting the estimation of the prevalence of ESBL-producers among 400 *K. pneumoniae* isolates. In which, high ESBL rate (55%) has been detected. Among those 97.3%, 84.1% and 34.1% were positive for bla_{SHV}, bla_{TEM} and bla_{CTX-M}-ß-lactamase genes, respectively. Sixty percent of the bla_{CTX-M} belonged to $bla_{CTX-M-1}$ group and the remainders belonged to the $bla_{CTX-M-9}$ group (Al-Agamy *et al*., 2009).

In Egypt, the first study that referred to the potential high rate of ESBL production that was reflected by the reduced rate of susceptibility of *E. coli*, *Klebsiella* and *Enterobacter* to ceftazidime recording 62%, 40% and 46%, respectively (El-Kholy *et al*., 2003**)**. The first study that aimed at the determination of the molecular basis of ESBL resistance in clinical *E. coli* isolates derived from Egyptian University Hospitals was carried out by Mohamed Al-Agamy *et al.* who reported a very high ESBL rate (60.9%). All isolates were positive for bla_{TEM} and bla_{CTX-M} genes. Bla_{CTX-M} was further differentiated into $bla_{CTX-M-15}$, $bla_{CTX-M-14}$ and $bla_{CTX-M-27}$ (Mohamed Al-Agamy *et al.*, 2006). Another study investigating 85 gram-negative bacterial isolates collected during 2002 from the Intensive Care Unit at Theodor Bilharz Research Institute, Cairo, Egypt

revealed very high overall ESBL rate (65.8%) including *Klebsiella pneumoniae* (55.3%), *E. coli* (35.7%), *Proteus mirabilis* (5.3%), *Enterobacter aerogenes* (1.7%) and *Citrobacter freundii* (1.7%). Furthermore, ten of these ESBL-producing isolates (5 *E. coli* and 5 *K. pneumoniae*) were subjected to molecular analysis revealing that all strains were positive for $bla_{\text{CTX-M}}$ (all of them were molecularly identified as $bla_{\text{CTX-M-15}}$) but were negative for both of bla_{SHV} and bla_{TEM} (Fam and El-Damarawy, 2008). Twenty-nine percent of the organisms that caused nosocomial blood stream infection in Assuit University Hospital in Upper Egypt were gram-negative bacilli of which 10.3% were *K. pneumoniae*, 8.6% were *E. coli*. Moreover, another 135 gram-negative isolates obtained from ICU environment were tested for ESBL production revealing that 18.4% were ESBL-producers. The molecular typing of the *K. pneumoniae* strains (by RAPD method) revealed that two isolates derived from ICU environment and one isolate derived from a patient manifested blood stream infection were identical emphasizing the role of hospital in the dissemination of ESBL-producers (Ahmed *et al.*, 2009). In the same year, another study targeting molecular characterization of CTX-M-ESBLs harbored by five clinical isolates (3 isolates of *K. pneumoniae*, an *E. coli* isolate and an *Enterobacter cloacae* isolate) demonstrated the presence of $bla_{\text{CTX-M-14}}$ in *Klebsiella pneumoniae* and *Enterobacter cloacae* for the first time in Egypt as well as the presence of $bla_{\text{CTX-M-15}}$ in *E. coli*. This study also reported the transferability of $bla_{\text{CTX-M-15}}$ but not $bla_{\text{CTX-M-14}}$ and reported also the association of both types with IS*Ecp*1element (Khalaf *et al.*, 2009).

Recently, a study investigating 520 enterobacterial isolates collected during the period from May 2007 till August 2008 at Theodor Bilharz Research Institute, Cairo, Egypt reported that a total of 16% of all isolates, 19% of *E. coli* and 14% of *K. pneumonae* were ESBL-producers (Fam *et al.*, 2011). Sometimes certain ESBL allele may be restricted to certain country or a certain geographical region. For instance $bla_{\text{TEM-10}}$ has been detected in the United States in several outbreaks for many years before the detection of this allele in Europe (Bradford *et al.*, 1994).

Another example demonstrated by $bla_{\text{TEM-3}}$ which has not been detected in the United States but it is frequently found in France (Nordmann, 1998; Soilleux *et al.*, 1996).

In contrast, there are ESBL alleles which are commonly encountered worldwide like SHV-5 and CTX-M-15 (Bradford, 2001).

Global dissemination of an antibiotic resistance determinant encoded by a bacterial host can be achieved either by human travelers, migrating birds, imported animals or

imported agricultural and meat products (Okeke and Adelman, 2001). Travel plays an important role in the dissemination of antibiotic resistance (Naseer and Sundsfjord, 2011). It was suggested that, the global dissemination of CTX-M-producing / ST131 *E. coli* is attributed to colonization or infection of travelers returning from high risk area like Indian subcontinent and the Middle East (Pitout *et al.,* 2009). CTX-M-15-producing isolates were the most common among ESBL-producing isolates that were recovered from travelers returning from Indian sub-continent and the Middle East, while CTX-M-14-producers were the predominant ESBL-producing *E. coli* isolates that were recovered from travelers returning from Asia confirming the notion that returning travelers are most likely to acquire the most predominant ESBL-determinant in the visited country. Such acquisition can be achieved even without hospitalization or contact with the health care system in the visited country (Pitout *et al.,* 2009).

Migration of the wild birds could contribute to the dissemination of resistance over the globe. CTX-M-type has been detected in the water fowls including gull species in a comparatively high level. CTX-M allele types in the wild bird were the same as those were found in the clinical setting and food-producing animals in the same region (Bonnedahl, 2011). Thirty-five different sequence types have been detected among the avian *E. coli*. The majority of them (ST10, ST90, ST648 and ST69) have been detected among human clinical isolates suggesting interspecies transmission, while the avian ST746 has not been detected among the clinical isolates (Guenther *et al.,* 2011). Other than water fowls, CTX-M-producing *E. coli* has been isolated from Euro-Asian black birds, rock pigeon and white-fronted goose in Germany. All the recovered isolates were assigned to ST648 (Guenther *et al.,* 2010). Therefore, the bird picks up *E. coli* strains derived from human origin and act as reservoir and vector posing potential to re-infect human populations (Bonnedahl *et al.,* 2009). CTX-M-producing *E. coli* are likely to be globally present in chickens. CTX-M variants that have previously been isolated from chickens include CTX-M-1 (the most prevalent variant), CTX-M-2, CTX-M-14 and CTX-M-15. But the pandemic CTX-M-15-producing/ST131 *E. coli* clone has not been isolated from poultry (Randall *et al*., 2010). In addition to their presence in chicken, CTX-M has been recovered from other food- producing animals and has been found in high prevalence in retailed meat representing potential threat to human health (Simões *et al.,* 2010). Imported zoo animals, especially those from developing countries, represent a potential hazard to the native animals and the public health as a source of multidrug resistant bacteria (Sato *et al.,* 2009).

ESBL variants are selected by *de novo* selection then spread by different means such as clonal dissemination of the host strain or by horizontal transmission of the ESBL-gene carrying plasmid either to related strains, in case of narrow–host range plasmids, or to non-related strains in wide host range plasmids **(**Hibbert-Rogers *et al.,* 1994; Gniadkowski, 2001; Palucha *et al.,* 1999; Villa *et al.,* 2000). Epidemic plasmids have been implicated in the dissemination and the high prevalence of bla_{ESBL} in the European region and have been detected among local or international epidemic clones (Canton *et al.,* 2008).

Several outbreaks have been reported, the great majority of which occurred in tertiary hospitals where the transfer of a colonized patient provide a chance for dissemination of the ESBL-producing organism (Patterson, 2001; Palucha *et al*., 1999). Frequently, the exact source of outbreaks has never been detected but many of these resistant bacteria were characterized epidemiologically as illustrated by the following examples:

- In a French Hospital, SHV-5 expressing *K. pneumoniae* were isolated from six peripartum women and two neonates. PFGE profiles of these strains indicated that all of the strains have PFGE-patterns identical to that of a strain isolated from contaminated ultrasonography coupling gel (Gaillot *et al*., 1998).
- In South Africa, an ESBL-producing *K. pneumoniae* carried by cockroaches infesting the neonatal ICU has the same PFGE-type of the strain that were implicated in an outbreak caused high mortality rate among neonates in that hospital (Cotton *et al*., 2000).
- In another outbreak, ESBL-producing *E. coli* and *K. pneumoniae* having different PFGE-types, but carrying identical plasmid encoding TEM-10 have been isolated from many patients in different hospitals in Chicago. The occurrence of the same plasmid in the strains of different PFGE-genotypes is a clue for plasmid transfer (Bradford *et al.,* 1994; Wiener *et al*., 1999).
- More interestingly, $bla_{\text{TEM-24}}$ encoding, 180 kb, conjugative plasmid was detected in four different enterobacterial species *E. coli*, *K. pneumoniae*, *E. aerogenes* and *P. rettgeri* isolated from the same patient suggesting horizontal transfer between the normal flora of the gut (Marchandin *et al.,* 1999).

The first two examples can be regarded as an example of clonal dissemination while the latter two could be considered as an example of horizontal transfer of epidemic plasmid.

During 1990s, infection and colonization with ESBL-producers was mainly hospital-acquired principally in intensive care units (ICU) in addition to surgical, pediatrics, neonatology and oncology wards. Also community clinics and nursing homes are regarded as potential reservoir (Babini and Livermore, 2000; Bermudes *at al*., 1997; Wiener *et al*., 1999). Currently, ESBL-producing bacteria are no longer restricted to hospital environment, but they are circulating in the community setting which plays an essential role in the persistence of ESBL-producing organisms (Andriatahina *et al*., 2010). In general, TEM and SHV type ß-lactamases, produced commonly by *K. pneumoniae*, have spread throughout the hospital setting, while CTX-M enzymes, produced mainly by *E. coli,* has become predominant ESBL-family in the community (Pitout *et al.,* 2008; Mirelis *et al*., 2003).

Recent studies demonstrated significant increase in ESBL-producers in the community setting in many part of the world. Asymptomatic colonization with ESBL-producing organisms has also been described (Hollander *et al.,* 2001). Intestinal carriage is the main reservoir for ESB-producing organism in the hospital setting (Andriatahina *et al*., 2010). The detected rates of ESBL carriage among hospitalized patients was 11.7% in Spain, 16% in Lebanon and 26% in Saudi Arabia (Valverde *et al*., 2004; Moubareck *et al*., 2005; Kader *et al.,* 2007). The existence of ESBL-producing organisms in the intestinal tract increases the risk of transmission to other individuals through human to human transmission or through the environment **(**Andriatahina *et al*., 2010). Emergence of ESBL-producers in the community could be attributed to acquisition of an ESBL-producer by a patient during hospitalization or due to antibiotic overuse by community patients (Kader and Kamath, 2009). Extensive administration of expanded-spectrum ß-lactam antibiotics along with one or more of specific risk factors including the prolonged hospital stay, sever illness, admission to ICU, intubation, mechanical ventilation, urinary or arterial catheterization, undergoing hemodialysis, abdominal surgery, gut colonization, low birth weight and patient transfer between the different units in the hospital are the most common aspects among the hospitals that were infected by ESBL-producing organisms **(**Pena *et al.,* 1997 ; Rice, 1999).

1.6. ESBL detection methods

The detection methods are divided into

- Phenotypic methods.
- Molecular methods.

Phenotypic methods are based on the resistance of ESBL-producers to oxyimino-ß-lactams such as cefotaxime, ceftriaxone, ceftazidime and aztreonam and the ability of ß-lactamase inhibitors to inhibit this resistance. Several tests are recommended including:

1.6.1. Double disk diffusion (double disk approximation/ double disk synergy DDS)

The test was earlier performed by swabbing the organism onto a Muller-Hinton agar plate then a disk containing amoxicillin-clavulanates (20µg/10µg) was placed in the center of the plate and disks containing 30 µg of ceftazidime, ceftriaxone, cefotaxime and aztreonam were placed at a distance of 30 mm (center to center). Enhancement of the zone of inhibition of the oxyimino-ß-lactam caused by the synergy of the clavulanate in the amoxicillin-clavulanate disk was interpreted as positive test indicating the production of ESBL. The use of cefpodoxime as the oxyimino-cephalosporin of choice is recommended. It gives test sensitivity up to 97% and test specificity up to 100%. If the test is negative with an isolate that is highly suspected to be an ESBL-producer the test should be repeated with closer distance (20 mm) but generally the test is reliable, convenient and non-expensive method for screening of ESBL production (Drieux *et al.*, 2008).

1.6.2. Combination disk method

This method is based on measuring the inhibition zone around the disks of cephalosporin (ceftazidime 30µg, cefotaxime 30µg and/or cefpodoxime 30µg) and around the disk of the same cephalosporin plus clavulanate (30 µg/10 µg). A difference of $\geq$ 5 mm between the two diameters or 50% expansion of the inhibition zone is considered a positive indication for ESBL production (Carter *et al.*, 2000; M'Zali *et al.*, 2000). Sensitivity and specificity of this method is up to 96% and 100%, respectively (Linscott and Brown, 2005).

1.6.3. ESBL E-test

The purpose of this test is the assessment of the synergism between extended-spectrum cephalosporin and ß-lactamase inhibitors. E-test is a two-sided strip containing gradient concentrations of cefotaxime (CT) or ceftazidime (TZ) or cefepime (PM) alone at one side of the strip and combined with clavulanate 4 mg/L at the other end giving the designation CT / CTL, TZ / TZL and PM / PML. The reduction of MIC of the tested cephalosporin by more than three doubling dilution i.e. if MIC ratio ≥ 8 and / or if phantom zone appeared just below the lowest concentration of CTL, TZL or PML and /or deformation of the CT, TZ or PM inhibition ellipse at the tapering end the test is interpreted as positive indicating ESBL production (Cormican *et al*., 1996; Leverstein-van Hall *et al*., 2002).

1.6.4. Agar supplemented with clavulanate

In this method, two Müller-Hinton agar plates one of which freshly supplemented with 4µg/ml clavulanate are swabbed with the test bacteria and antibiotic disk of ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg) and aztreonam (30 µg) are placed on clavulanate-supplemented and clavulanate-free plates. If the difference in inhibition zone diameter around ß-lactam on the two media is ≥10 mm the test is positive indicating ESBL production. The sensitivity of this test is up to 96% and the specificity is 100% for ceftazidime (Al- Jasser., 2006; Ho, 1998; Vercauteren *et al*., 1997).

1.6.5. Automated method

The automated antimicrobial susceptibility test systems include VITEK test, Phoenix ESBL test.

1.6.5.1. VITEK 2 ESBL test (BioMerieux, France)

This method relies on simultaneous quantification of the antimicrobial activity of cefotaxime, ceftazidime and cefepime with and without clavulanate. A card wells contains 1 mg/L of cefepime or 0.5 mg/L cefotaxime or ceftazidime either alone or with clavulanate 4mg/L. After inoculation with the suspected ESBL- producing organisms, the cards are introduced into the VITEK 2 machine and the turbidity for each antibiotic tested is measured at regular intervals. The growth in the wells containing cephalosporin

with clavulanate compared with that of the cephalosporin alone and the result is interpreted by a computerized system (Drieux *et al*., 2008).

1.6.5.2. The automated Phoenix ESBL test (Becton Dickinson, USA)

This method depends also on the growth response to certain expanded-spectrum cephalosporins and the result also interpreted via computerized system (Drieux *et al*., 2008).

1.6.6. Molecular detection methods

A number of methods can be used for characterization of ESBL. In the past, the determination of the isoelectric point was enough for the identification of ESBL that was present in a clinical isolate. But after the emergence of many ESBL-types that have identical isoelectric points the determination of ESBL type by isoelectric point is no longer feasible (Bradford, 2001).

Detection of earlier ß-lactamase genes was achieved by using specific DNA probes for TEM and SHV-encoding genes but this method is labour intensive (Arlet and Philippon, 1991).

PCR with oligonucleotide primers that are specific for an ESBL-encoding gene is the most convenient and the most widely used method to detect the ESBL family of the variant but will not differentiate among the different variants (Bradford, 2001).

For determination of a specific ß-lactamase gene that is present in a strain, nucleotide sequencing is the gold standard that can detect all variants. But it is labour intensive, technically challenging (Bradford, 1999). So, several molecular methods for detection and differentiation of ESBL without sequencing have been suggested like the oligotyping method that has been used to differentiate between $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-2.}}$ This method is based on the use of oligonucleotide probes that are designed to detect point mutation under strict hybridization conditions. Several bla_{TEM} variants were identified by this method (Bradford, 2001).

Sometimes mutation result in creation or disappearance of restriction sites and hence if this part of the gene is amplified by PCR and the resulting amplicon digested with restriction endonuclease, the analysis of the restriction profile may lead to identification of the new ESBL variant. This lead to another approach combines restriction fragment length polymorphism analysis with PCR (PCR-RFLP). The created pattern by each

restriction enzyme indicates a point mutation within ESBL-encoding-gene variant (Bradford, 2001; Arlet *et al*., 1995).

1.7. Treatment options

Carbapenems are the drugs of choice for life-threating infection with ESBL-producing organisms. The choice between imipenem and meropenem is difficult. The clinical experience is better with imipenem but meropenem has the lower MIC. In nosocomial meningitis meropenem is regarded the drug of first choice. The combination therapy with carbapenem and antibiotic of other classes do not offer an advantage over the use of carbapenem alone. Unfortunately, carbapenem resistance has been observed in organisms that commonly encode ESBLs and hence the use of these agents should be restricted to serious life-threating infection (Al-Jasser, 2006; Segal-Maurer *et al*., 1999).
Third generation cephalosporins should not be used for treatment of infections with ESBL-producing organism; even if *in vitro* susceptibility is detected. Treatment failure may result. Cefepime should not be regarded as the first line therapy against ESBL-producing organisms because cefepime resistance is common in CTX-M-producers (Patterson, 2001; Paterson *et al*., 2000).
In spite of their good (*in vitro*) activity, cephamycins are not regarded as the first line therapy for ESBL-producing organism due to the probable emergence of porin-resistant mutants during the course of the treatment as observed earlier with cefoxitin (Pangon *et al*., 1989).
ß-lactam/ß-lactamase inhibitors also are not regarded as the first line therapy for infections due to ESBL-producing organism. Activity of ß- lactam/ß-lactamase inhibitors depends on the inoculum size. Their effectiveness against the organisms producing multiple ESBL is less as compared to organisms encoding single ESBL. Despite all the above mentioned limitations, amoxicillin/clavulanate still considered as the second line therapy for the urinary tract infections. It is noteworthy that, the use of ß-lactam/ß-lactamase inhibitors has a protective role against infection and colonization with ESBL-producing *K. pneumoniae* (Bradford *et al*., 1994; Thomson and Moland, 2001; Piroth *et al*., 1998).

1.8. Epidemiological techniques for characterization of plasmids harbouring ESBL-encoding genes and their bacterial host

1.8.1. *Escherichia coli* phylogeny and ESBL-type

Determination of *E. coli* phylogenetic type is being of great epidemiological importance because there is a relation between the genetic background and the type of the extended-spectrum ß-lactamase as reported in a study carried out by Branger *et al.* who reported that bla_{SHV} and to lesser extent bla_{TEM}-type were preferentially found in B2 phylogenetic strains, while bla_{CTX-M}-type was associated with D phylogenetic group strains (Branger *et al*., 2005).

Phylogenetic analyses have grouped *E. coli* into four main phylogenetic groups A, B1, B2 and D and there is a relation between the phylogeny and the virulence. The virulent extraintestinal strains belong mainly to B2 and to lesser extent to D group, whereas most commensal strains belong to groups A and B1 (Desjardins *et al.*, 1995).

The reference techniques for phylogenetic grouping are multilocus enzyme electrophoresis and ribotyping. But, these methods are complex and time-consuming (Selander *et al.*, 1986; Desjardins *et al.*, 1995; Bingen *et al*., 1994). To overcome these drawbacks, Clermont *et al*. described a simple and rapid technique for determination of the phylogenetic group of *E. coli* strains based on PCR detection of three specific phylogenetic group markers namely *chu*A, *yja*A, and TSPE4C2. *chu*A is a gene required for heme transport, *yja*A is a gene has been identified after complete genome sequencing of *E. coli* K-12 and its function is still unknown. TSPE4C2 is an anonymous DNA fragment (Clermont *et al*., 2000).

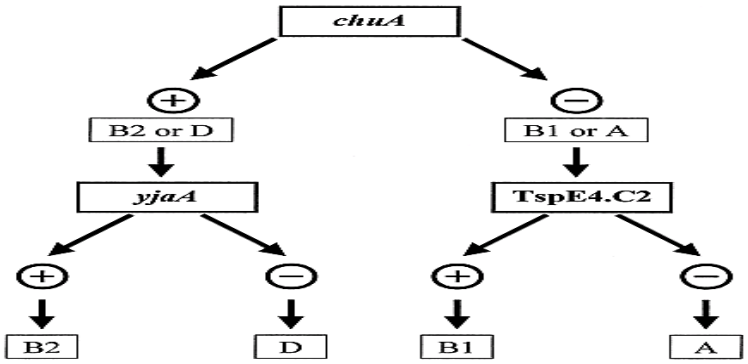


Figure 3: Dichotomous decision tree to determine the phylogenetic group of an *E. coli* strain by using the results of PCR amplification of the *chu*A and *yja*A genes and DNA fragment TSPE4.C2 adapted from, Clermont *et al.* (2000).

1.8.2. Multi-locus sequence typing (MLST) of *E. coli* strains

Multi-locus sequence typing is one of the methods of choice for typing of the isolates of epidemiological or evolutionary importance. This method based on determination of the nucleotide sequences of the internal fragment of a number of a selected housekeeping genes (ranging from seven to nine) that are subjected to very slow and minor changes resulting in polymorphism and diversity. These sequences are then subjected to analysis which determines the genetic relatedness not only to the other strains of the strain collection of study or nation-wide lineage but also to the global ones. The best example reflecting this situation is the detection of the global dissemination of *E. coli* ST131 that was implicated in many worldwide CTX-M-15 epidemics. There are many advantages for MLST include its dependence on the sequence data which are easily comparable, transferable among laboratories in addition to its reproducibility and its assignment of the nucleotide sequence of each of the selected housekeeping gene as a unit of comparison (Tartof *et al*., 2005; Maiden *et al.,* 1998 and Noller *et al.,* 2003).

1.8.3. Pulsed-field gel electrophoresis (PFGE) typing of *Escherichia coli*

PFGE-typing is one of the techniques that determine the relatedness among bacterial isolates by genotypic characterization (Tenover *et al*., 1995). It is based on the use of rare-cutter restriction endonucleases to give few numbers (10-20) of high molecular-weight restriction fragments which are separated by pulsed-field gel electrophoresis resulting in highly specific patterns for each strain. The analysis of the resulting pattern determines accurately the exact relation among the tested isolates (Gautom, 1997).

PFGE is regarded as the gold standard in the epidemiological analysis to discriminate the pathogenic strains and to monitor their dissemination in both clinical and community settings (Yakubu and Pennington, 1995). PFGE gives also reproducibility and the highest discriminatory power that allows it to be the most commonly used method to identify and relate the bacterial pathogens (Gautom, 1997).

1.8.4. Plasmid incompatibility groups (plasmid replicon) typing

The official scheme of plasmid classification relies on incompatibility (Inc) groups. Incompatibility is a feature of plasmids that have the same replicon type and defined as the inability of two related plasmid to propagate in the same host. The incompatibility grouping is based on introduction of a plasmid of unknown Inc group into a host

carrying a plasmid of known Inc group. If the introduced plasmid is eliminated, indicates that it belongs to the same Inc group i.e. has the same replicon type (Novick, 1987; Datta and Hedges, 1971).

Another approach was proposed by Couturier *et al*. based on hybridization with nineteen DNA probes that identify the major replicons of plasmids among *Enterobacteriaceae* (Couturier *et al*., 1988). Among the disadvantages of this method is the difficulty of application to a large number of strains and it is labourious and time consuming. In order to overcome these difficulties, PCR-based replicon typing of plasmids on the basis of published sequences was introduced. But at that time was limited to Inc P, Inc. N, Inc W and Inc Q plasmids (Gotz *et al*., 1996). The urgent need to trace the plasmid that encodes drug resistance motivated Carattoli *et al*. (2005) to develop an Inc/rep PCR-based typing scheme targeting the replicon of the major plasmid families that occur in *Enterobacteriaceae* namely FIA, FIB, FIC, N, L/M, HI2, HI1, I1-y, X, W, Y, P, A/C, T, K, B/O, FrepB and FIIA. The only disadvantage of this scheme is its inability to identify novel replicons. This PCR-based method has been used to follow-up the dissemination and evolution of resistance plasmid especially those carrying ESBL-encoding genes among clinical isolates (Caratolli *et al.*, 2005).

1.8.5. Detection and sizing of large plasmids carrying ESBL genes

When a supercoiled plasmid treated with S1 nuclease, an endonuclease enzyme isolated from the mold *Aspergillus oryzae* attacks only single-stranded DNA, the enzyme will introduce breaks at regions that have a single-stranded character resulting in nicking and loss of supercoiling. Finally, the plasmid will be converted into full-length linear molecule (Barton *et al*., 1995). S1-nuclease digestion followed by pulsed-field gel electrophoresis (S1-PFGE) is a widely used technique for determination of the number and the approximate molecular size of the large plasmids for a given bacterial host.

The principle of (S1-PFGE) is based on: Enzymatic conversion of plasmids from the supercoiled form into full-length linear form, specification of S1 nuclease for restriction of plasmids and subsequent separation of restricted linear form of large plasmids. Each of these bases overcomes a certain difficulty encountered in the usage of the routine plasmid profiling techniques for detection and sizing of large-sized plasmids **(**Barton *et al*, 1995). Preliminary conversion into linear form is due to the fact that, the complex mobility of supercoiled form of plasmid does not show simple relationship with that of

the linear marker in the same gel. Electrophoretic mobility of supercoiled plasmid is also affected by the minor variation in the electrophoresis conditions. Also, the plasmids that have molecular size ≥ 600 kb are not visible in the gel because of their very slow migration that makes them very close to the compression zone (Hightower *et al*., 1989; Mathew, 1988). Hence, supercoiled plasmids were converted into linear form. The selection of S1 nuclease is due to its unique effectiveness on the supercoiled plasmid, its self-limiting action and its very few cleavages in the chromosomal DNA these characters are attributed to its specificity for single stranded DNA. The transient denaturation of supercoiled plasmid (under torsional stress) creates regions sensitive to the action of S1 nuclease. After the cutting of one strand, S1 can cut the intact strand at the opposite site to the initial break (Germond *et al.,* 1974). At this condition further activity of the enzyme on the linear form is extremely reduced (Beard *et al*., 1973). Utilization of PFGE rather than the standard gel electrophoresis is necessary due to inability of the later to separate large plasmids (Goering and Ruff, 1983).

1.9. Motives behind the study

The emergence of ESBL-producing enterobacterial members (mainly *Klebsiella pneumoniae* and *E. coli*) in the hospital setting as nosocomial pathogens leading to increased morbidity and mortality as well as increased treatment failure to the most widely used antimicrobial class with the consequent increase in the cost of the health care services due to the shift to more expensive antimicrobials and to prolonged stay in the hospitals represents an extremely important clinical and public health problem all over the world (Bouchillon *et al.,* 2004; Hoffmann *et al.,* 2006).

The sudden emergence and dissemination of ESBL-producing organisms nearly in the same time in many distant countries over the globe that follow different antibiotic strategies is an intriguing phenomenon and strongly suggests the presence of an epidemiologic link between these countries. Determination of the resistance determinants as well as characterization of ESBL-producers recovered in simultaneous period from two or more distant countries may unravel the mechanism behind the global spread of ESBL and may lead to detection of epidemiological links among these countries. The high number of travelers between Egypt and Germany, together the implication of foreign travel as one of the major risk factors for acquisition of ESBL-

producer as well as the different antimicrobial policies make them ideal countries for such comparison.

Egypt has been reported as one of the countries of the highest occurrence of ESBL (Bouchillon *et al.,* 2004; Kola *et al.,* 2007). High prevalence of nosocomial infection that is either caused by *K. pneumoniae* or *E. coli* in the clinical setting in Egypt has affected many departments particularly ICU, post-operative and pediatrics (Abdel-Hady *et al.,* 2008). Moreover, increasing rates of community acquired ESBL-producing *E. coli* have been detected in Egypt mainly in association with community acquired urinary tract infections (Mohamed Al-Agamy *et al.,* 2006). However, only few studies were reported in this important issue in Egypt and little is known about the genetic background of ESBL-producing organism.

Although the earlier members of SHV and CTX-M family have been detected for the first time in Germany, the prevalence of ESBL in Germany was as low as 8.2% for *K. pneumoniae* and 0.8% for *E. coli* until 2001 (Sturenburg and Mack, 2003). However, the prevalence of ESBL-producing bacteria was increasing in both hospitals and community setting (Sturenburg and Mack, 2003; Pitout, 2005). In the survey of Bouchillon 2004, the rate of ESBL- producing *K. pneumoniae* reached 6% and 1.5% for *E. coli*, while the ESBL-production rate for the entire *Enterobacteriaceae* was 2.6%. Till yet, the number of studies on ESBL in both of Germany and Egypt does not cope with the severity of the current situation. In addition to the urgent need for updated monitoring of ESBL-production as well as characterization of the current ESBL types and their producing organisms.

All the above mentioned factors motivated us to carry out this comparative study utilizing the molecular determination of the genetic determinants of ESBL as well as epidemiological molecular characters of ESBL-producing organisms and their plasmids investigating for an evidence for epidemiologic link between Egypt and Germany (such as shared strain, common epidemic plasmids) and to unravel the molecular mechanisms behind the spread of resistance to extended-spectrum cephalosporins among enterobacterial isolates in both countries and hoping that this study may contributes to the establishment of a modified-therapeutic-antimicrobial strategy to eradicate or even to hamper of ESBL-spread and acts together with the few earlier and current studies on the ESBL problem as a nucleus for a growing cumulative ESBL database in Egypt and considerably contribute to ESBL data in Germany.

1.10. Aims of the study

1- Determination of the prevalence of the most common ESBL-encoding genes and their families (CTX-M, SHV and TEM) among *E. coli* and *K. pneumoniae* among the clinical isolates derived from Egyptian Hospitals.

2- Epidemiological genotypic and phenotypic characterization of some ESBL-producing enterobacterial clinical isolates namely, CTX-M-producing *E. coli* isolates derived from Egyptian and German University Hospitals.

3- Determination of the genetic basis and the molecular mechanisms that have led to the spread of resistance to extended-spectrum cephalosporins among *Enterobacteriaceae* in the clinical setting of both countries.

4- Comparison between the molecular epidemiological features of ESBL-producing isolates derived from Egyptian University Hospitals and those derived from German University Hospital.

5- Investigation for the presence of an evidence for the epidemiologic link between ESBL-producing enterobacterial organisms derived from the clinical setting of Egypt and Germany.

2.0. Materials and Methods

2.1. Bacterial strains

A total of 248 enterobacterial clinical isolates were investigated in this study including:
184 isolates were derived from Egyptian University Hospitals.
64 isolates were derived from a German University Hospital.

2.1.1. Enterobacterial isolates derived from Egyptian University Hospitals

A total of 184 enterobacterial isolates (102 *Klebsiella pneumoniae* and 82 *Escherichia coli*) derived from two distant University Hospitals namely El-Mansoura (located in Lower Egypt) and Assiut (located in Upper Egypt). One hundred thirty three isolates including 77 *K. pneumoniae* isolates and 56 *E. coli* isolates were derived from El-Mansoura University Hospital and 51 isolates including 25 *K. pneumoniae* and 26 *E. coli* were derived from Assiut University Hospital. The strains were isolated and collected (simultaneously in the two hospitals) over a period of six months from October 2008 to March 2009 from different clinical specimens obtained from the different medical wards as illustrated in the following Tables.

Table 2: The clinical specimens from whom the enterobacterial isolates that were derived from Egyptian University Hospitals were isolated.

Specimen type	*K. pneumoniae*	*E. coli*	Total *Enterobacteriaceae*
Urine	34	45	79
Blood	15	5	20
Sputum	11	7	18
Throat swab	7	5	12
ETA	8	2	10
T-tube aspirate	9	4	13
Wound swab	8	10	18
Ascetic fluid	2	1	3
Drains	2	0	2
Chest tube aspirate	1	0	1
Stool	1	0	1
CVP	1	0	1
U-tube aspirate	1	0	1
Suction apparatus	0	1	1
Ventilator	0	1	1
Skin swab	0	1	1
Unknown	2	0	2
Total	102	82	184

Table 3: The different wards from whom the enterobacterial isolates were derived from Egyptian University Hospitals.

Medical ward	***K. pneuomoniae***	***E. coli***	**Total *Enterobacteriaceae***
Oncology	13	22	35
Ped.neonatal	18	5	23
Ped. ICU	3	1	4
Ped. general	5	5	10
Neurosurgery	6	2	8
Chest medicine	6	4	10
GIT	4	2	6
Neurology	5	3	8
MUH	2	3	5
Tropical medicine	2	1	3
Gastroenterology	2	0	2
Surgery	1	4	5
Emergency	2	0	2
Cardio-surgery	1	0	1
Special Medical Hospital	1	1	2
GEC	1	1	2
Urology	25	26	51
Multi-clinics	2	0	2
ENT	1	0	1
Dermatology	0	1	1
Outpatient	0	1	1
Unknown	2	0	2
Total	102	82	184

Ped: pediatric; ENT: Ear, Nose and Throat MUH: Mansoura University Hospital; ICU: Intensive Care Unit.

2.1.2. Clinical isolates derived from Giessen University Hospital, Germany

A total of 64 *E. coli* strains isolated, collected over a period of six months from October 2008 to March 2009 from different clinical specimens obtained from the different wards at Justus-Liebig University Hospital, Giessen, Germany as described in the following Tables.

Table 4: The clinical specimens from which the enterobacterial isolates were derived from a German University Hospital.

Specimen type	*K. pneumoniae*	*E. coli*	*Total Enterobacteriae*
Urine	0	30	30
Anal swab	0	13	13
Stool	0	2	2
Sputum	0	1	1
Nasal swab	0	2	2
Cervical swab	0	2	2
Groin swab	0	1	1
Vaginal swab	0	1	1
Chair swab	0	1	1
Eye swab	0	1	1
Abdomenal swab	0	1	1
Larynx swab	0	1	1
Diabetic foot swab	0	1	1
Leg swab	0	1	1
Oral swab	0	1	1
Catheter- urine swab	0	1	1
Unknown	0	4	4
Total	0	64	64

Table 5: The different Medical Wards from which the clinical *E. coli* isolates derived from German University Hospital were recovered.

Medical ward	*K. pneumoniae*	*E. coli*	*Total Enterobacteriaceae*
Medical Clinic	0	14	14
OB/GY	0	6	6
Int. Surgery	0	6	6
Surgery	0	7	7
Urology	0	9	9
Pediatric	0	5	5
Int.medicine	0	4	4
polyclinic	0	3	3
Oper. Intensive care	0	2	2
Neurology	0	1	1
Orthopedic	0	2	2
Psychiatric	0	1	1
Unknown	0	4	4
Total	0	64	64

OB/Gy: Obstetrics and Gynecology; Int. Medicine: Internal Medicine; Int. Surgery: Internal Surgery.

2.2. Chemicals

The standard chemicals were purchased from Merck (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany), Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany) and Amersham Pharmacia Biotech (Freiburg, Germany).

2.3. Culture media

Luria-Bertani (LB) medium

E. coli strains were grown in Luria-Bertani (LB) medium. LB medium consists of

Tryptone	1.0% [w/v]	10 g
Yeast extract	0.5% [w/v]	5 g
NaCl	0.5% [w/v]	5 g
H_2O	ad	1000 ml

Luria-Bertani (LB) Agar medium

Tryptone	1.0% [w/v]	10 g
Yeast extract	0.5% [w/v]	5 g
NaCl	0.5% [w/v]	5 g
Agar		l5 g
H_2O	ad	1000 ml

Müller-Hinton Agar (Oxoid, UK)

E. coli strains were grown on Muller-Hinton Agar for antibiotic susceptibility testing

Beef dehydrated infusion		300 g
Casein hydrolysate		17.5g
Starch		1.5g
H_2O	ad	1000 ml

Brain-Heart infusion Broth (Merck, Darmstadt, Germany)

Extract of brain heart and peptone		27.5g
(D) Glucose		2g
Sodium Chloride		5g
Di-Sodium hydrogen phosphate		2.5g
H_2O	ad	1000 ml

2.4. Media supplement

For inoculation of culture media by ESBL-producing strains, the following antibiotic was added: cefotaxime (Stock solution1 mg/ml dissolved in H_2O and sterilized by filtration) the final concentration in solid or liquid media was 1µg/ml.

2.5. Buffers and solutions

TE Buffer: used for preparation and washing of plugs in PFGE and for preparation of loading buffer for agarose gel electrophoresis.

Tris -HCl (pH 8.0)		10 mM
EDTA		1.0 mM
H_2O	ad	1000 ml

TAE-Buffer (50X): for agarose gel electrophoresis

Tris		242.3 g
Acetic acid 96% [v/v]		57.1 ml
EDTA 0.5 M (pH 8, 0)		100 ml
H_2O	ad	1000 ml

Loading buffer (5X): for agarose gel electrophoresis

Ficoll Type 400	25.0% [w/v]
Bromophenol-blue	0.25% [w/v]

In TE buffer

10X PCR buffer:

	Taken volume	End concentration
Tris HCl 1 M (pH 8.3)	200 µl	200 mM
$MgCl_2$ 1 M	20 µl	20 mM
KCl 1 M	250 µl	250 mM
Tween20 10% [v/v]	50 µl	0.5% [v/v]
Gelatin 10 mg/ml	100 µl	1 mg/ml
H_2O	ad	1000 µl

Tris 1 M (pH 5):

Tris		121.14 g
H_2O	ad	1000 ml

EDTA 0.5 M (pH 8):

EDTA		186.12g
H_2O	ad	1000 ml

N-Lauryl sarcosyl 10%

SDS 20%

Cell suspension buffer

Tris 1 M, pH 8.0		10 ml
EDTA 0.5 M, pH 8.0		20 ml
H_2O	ad	1000 ml

Cell lysis buffer

Tris 1M, pH 8.0.		25 ml
EDTA 0.5 M, pH 8.0		50 ml
Sarcosyl (N - laurylsarcosyl sodium salt) 10%		50 ml
H_2O	ad	500 ml

Cell lysis -Proteinase K buffer

Cell lysis buffer	50 ml
Proteinase K stock solution (20 mg/ml)	250µl

The final concentration of Proteinase K in lyses buffer is 0.1 mg/ml.

10X TBE buffer: Diluted to 1X which is used as run buffer and for preparation of agarose gel for PFGE.

Tris	108.8 g
Boric acid	55 g
EDTA 0.5M	40 ml
H_2O ad	1000 ml

10X S1 nuclease reaction buffer

Sodium acetate (pH4.6)	300 mM	24.6 g
Zinc acetate	10 mM	2.195g
Glycerol	50% (V/V)	500 ml
H_2O	ad	1000 ml

Sodium chloride (3M)

Sodium chloride		175.3 g
H_2O	ad	1000 ml

S1-nuclease dilution buffer

Tris -HCl (pH7.5)	20 mM	3.152 g
Zinc acetate	0.1 mM	21.95 mg
Sodium chloride	50 mM	2.92 g
Glycerol	5% (V/V)	50 ml
H_2O	ad	1000 ml.

10X PBS:

NaCl		80 g
KCl		2.0 g
Na_2HPO_4		6.1 g
KH_2PO_4		2.0 g
H_2O	ad	1000 ml

pH 7.4 adjusted with 1M NaOH

20 X SSPE

Sodium chloride		175.3 g
Sodium di-hydrogen phosphate		27.6 g
EDTA		7.4 g
H_2O	ad	1000 ml

pH was adjusted to 7.4 with 10 N sodium hydroxide

Pre-hybridization buffer

SDS 10%	100 ml
BSA	2 g
Polyvinylpyrolidone	2 g
Ficoll	2 g

Were added to 600 ml 20X SSPE completed to 2 L with H_2O and dissolved at 60^{o}C for 1 hour.

Depurination solution (0.25 M HCl)

Fuming HCL (37%)		20.66 ml
H_2O	ad	1000 ml

Denaturation solution

Sodium chloride		87.66 g
Sodium hydroxide		20 g
H_2O	ad	1000 ml

Neutralization solution

Tris		121.14g
Sodium chloride		87.66 g
H_2O	ad	1000 ml

Washing buffer: was used for washing of the nylon membrane.

Maleic acid		11.61 g
Sodium chloride		8.77 g
Tween 20		3 ml
H_2O	ad	1000 ml

pH was adjusted to 7.5

Maleic acid buffer: was used for dilution of blocking solution.

Maleic acid		11.61 g
Sodium chloride		8.77g
H_2O	ad	1000 ml

pH was adjusted to 7.5

Detection buffer: was used as buffer for alkaline phosphatase.

Tris –HCl		15.67 g
Sodium chloride		5.84 g
H_2O	ad	1000 ml

pH was adjusted to 9.5

2.6. Biochemicals

2.6.1. Primers

Synthetic oligonucleotides were purchased from Eurofins MWG Operon.

Table 6: Primers used for detection and sequencing of ESBL genes.

Target	Primer name	Primer sequence (5`→3`)	Product size(bp)
Bla$_{TEM}$	TEM-F	TCCGCTCATGAGACAATAACC	931
	TEM-R	TTGGTCTGACAGTTACCAATGC	
Bla$_{SHV}$	SHV-F	TGGTTATGCGTTATATTCGCC	868
	SHV-R	GGTTAGCGTTGCCAGTGCT	
Bla$_{CTX-M}$	CTX-F	TCTTCCAGAATAAGGAATCCC	909
	CTX-R	CCGTTTCCGCTATTACAAAC	

Reference: Kiratisin *et al.* (2008).

Table 7: Primers used for phylogenetic group typing.

Target	Primer name	Primer sequence (5`→3`)	Product size (bp)
*chu*A	*chu*A.1	GACGAACCAACGGTCAGGAT	279
	*chu*A.2	TGCCGCCAGTACCAAAGACA	
yjaA	*yja*A.1	TGAAGTGTCAGGAGACGCTG	211
	*yja*A.2	ATGGAGAATGCGTTCCTCAAC	
TSPE4C2	TSPE4C2.1	GAGTAATGTCGGGGCATTCA	152
	TSPE4C2.2	CGCGCCAACAAAGTATTACG	

Reference: Clermont *et al.* (2000).

Table 8: Primers used for amplification and sequencing of seven housekeeping genes for multilocus sequence typing (MLST).

Gene	Primer name	Primer sequence (5`→3`)	Annealing temperature	Product size (bp)
adk	*Adk F*	ATTCTGCTTGGCGCTCCGGG	54°C	583
	Adk R	CCGTCAACTTTCGCGTATTT		
fumC	*fumC F*	TCACAGGTCGCCAGCGCTTC	54°C	806
	fumC R	GTACGCAGCGAAAAAGATTC		
*gyr*B	*gyrB F*	TCGGCGACACGGATGACGGC	60°C	911
	gyrB R	ATCAGGCCTTCACGCGCATC		
icd	*Icd F*	ATGGAAAGTAAAGTAGTTGT TCCGGCACA	54°C	878
	Icd R	GGACGCAGCAGGATCTGTT		
mdh	*Mdh F*	AGCGCGTTCTGTTCAAATGC	60°C	932
	Mdh R	CAGGTTCAGAACTCTCTCTGT		
purA	*purA F*	CGCGCTGATGAAAGAGATGA	54°C	816
	purA R	CATACGGTAAGCCACGCAGA		
recA	*recA F*	CGCATTCGCTTTACCCTGACC	58°C	780
	recA R	TCGTCGAAATCTACGGACCGGA		

Source: http://mlst.ucc.ie/mlst/dbs/Ecoli/site of University College Cork (UCC)

Table 9: Primers used for plasmid replicon typing.

Target	Primer name	Primer sequence (5`→3`)	Product size(bp)
iterons	FIA FW	CCATGCTGGTTCTAGAGAAGGTG	462
	FIA RV	GTATATCCTTACTGGCTTCCGCAG	
*rep*A	FIB FW	GGATTCTGACACACGATTTTCTG	702
	FIB RV	CTCCCGTCGCTTCAGGGCATT	
*rep*A	FIIS FW	CTGTCGTAAGCTGATGGC	270
	FIIS RV	CTCTGCCACAAACTTCAGC	
RNA I	I1 FW	CGAAAGCCGGACGGCAGAA	139
	I1 RV	TCGTCGTCCGCCAAGTTCGT	
*rep*A	N FW	GTCTAACGAGCTTACCGAAG	559
	N RV	GTTTCAACTCT TGCCAAGTTC	
*rep*A,B,C	L/M FW	GGATGAAAACTATCAGCATCTGAAG	785
	L/M RV	CTGCAGGGGCAGTTCTTTAGG	
*rep*A	A/C FW	GAGAACCAAAGACAAAGACCTGGA	465
	A/C RV	ACGACAAACCTGAATTGCCTCCTT	
*par*A/*par*B	HI1 FW	GGAGCGATGGATTACTTCAGTAC	471
	HI1 RV	TGCCGTTTCACCTCGTGAGTA	
iterons	HI2 FW	TTTCTCCTGAGTCACCTGTTAACAC	644
	HI2 RV	GGCTAACTACCGTTGTCATCCT	
RNAI /*rep*A	FrepB FW	TGATCGTTTAAGGAATTTTG	270
	FrepB RV	GAAGATCAGTCACACCATCC	
iterons	P FW	CTATGGCCCTGCAAACGCGCCAGAAA	534
	P RV	TCACGCGCCAGGGCGCAGCC	
*rep*A	T FW	TTGGCCTGTTTGTGCCTAAACCAT	750
	T RV	CGTTGATTACACTTAGCTTTGGAC	
*rep*A	W FW	CCTAAGAACAACAAAGCCCCCG	242
	W RV	GGTGCGCGGCATAGAACCGT	
ori	X FW	AACCTTAGAGGCTATTTAAGTTGCTGAT	376
	X RV	TGAGAGTCAATTTTTATCTCATGTTTTAGC	
*rep*A	Y FW	AATTCAAACAACACTGTGCAGCCTG	765
	Y RV	GCGAGAATGGACGATTACAAAACTTT	

Reference: Carattoli *et al.* (2005).

2.6.2. Enzymes

- **Proteinase-K stock solution (20 mg/ml)** prepared from the powder in sterile ultrapure water and aliquoted in 500µl amount and kept frozen.
- ***Xba*I-restriction-endonuclease** (*Xanthomonas badrii*, 10 U/µl, Fermentas, Germany) supplied with10 X Tango yellow buffer that should be diluted to 1X dilution for 100 % *Xba*I digestion (Fermentas, Germany).

  1X Tango yellow buffer composition:

Tris-acetate (pH 7. 9)	33 mM
Magnesium acetate	10 mM
Potassium acetate.	66 mM
Bovine serum albumin	0.1 mg/ml

- **S1-Nuclease 100 U/µl (Fermentas, Germany)**
- **DNA polymerase 5 U/µl (Invitrogen, USA)**

2.6.3. DNA Markers

DNA standard marker:

The 1 KbPlus DNA Ladder ™

Aliquots of (1µg/µl) were diluted to be used at an end concentration of 50ng/µl as follows:

1 Kb Plus DNA Ladder TM		20 µl
5X loading buffer		100 µl
TE buffer	ad	400 µl

From this dilution, 7 µl were loaded onto the agarose gel. The ladder contains a total of twenty bands; ten bands ranging in size from 3000bp to 12000bp in 1000bp increments while the other ten bands ranges in size from 100 to 2000 bp was used as a general purpose DNA marker in the ordinary gel electrophoresis such as screening for ESBL genes and PCR-based replicon typing.

Lambda ladder PFG marker:

50µg/ml (Bio-Labs, New England) embedded in 1% LMP agarose in a gel syringe dispenser designated to be used as size marker for PFGE with size range from 48.5 to

1018.5kb. Used as DNA marker in PFGE-genotyping and also in S1-nuclease/ PFGE-based plasmid sizing.

Gene Ruler™ Low Range DNA Ladder (Fermentas, Germany)

The ladder has been prepared before loading on agarose gel as follow.

1µl (0.5µg) the ladder

1µl 6X Loading dye (supplied with the ladder)

4µl deionized water.

Size range: from 25-700bp.

Used for the PCR-based phylogenetic typing.

2.7. Susceptibility test discs

Ampicillin (10 µg), ampicillin/sulbactam (10/10 µg), cefepime (30 µg), cefotaxime(30 µg), fosfomycin (200 µg), tetracycline (30 µg), gentamicin (120 µg), sulfamethoxazole/ trimethoprim (23.75/1.25 µg), cefazolin (30 µg), cefpodoxime (10 µg), cefuroxime (30 µg), tobramycin (10 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), moxifloxacin (5 µg), piperacillin/tazobactam (100/10µg), ceftriaxone (30 µg), amoxicillin/clavulanic (20/10 µg), ceftazidime (30 µg), imipenim (10 µg), meropenem (10 µg) supplied from (BD BBL,USA).

2. 8. Kits

2.8.1. QIA quick PCR Purification Kit 250 (Qiagen, Germany)

Used for purification of PCR product for sequencing and for preparation PCR-amplicon generated CTX-M DIG labeled probe.

2.8.2. DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostic GmbH, Germany)

Used for preparation of *bla*$_{\text{CTX-M}}$ probe by labeling of the purified *bla*$_{\text{CTX-M}}$ amplicon with digioxygenin (DIG) and for hybridization with targeted DNA fragments that have been transferred to positively charged polyvinyl-based membrane by southern transfer.

2. 9. Computer softwares, programs and sites

- **Gel Compare II (Applied Maths, Belgium)**

  For comparison of PFGE band patterns and differentiation of strains

- **Lasergene software package (DNASTAR, USA)**

  Was used to align and compare the nucleotide sequences with the published sequences of allele types that proposed by searching for nucleotide sequence homology using BLAST available at the National Center for Biotechnology Information website http://www.ncbi.nlm.nih.gov/BLAST.

- http://mlst.ucc.ie/mlst/dbs/Ecoli/

  Has been used for determination of the allelic profile and the sequence type (ST) and sequence type complex (STC) after feeding with 7 sequences that represent the following house-keeping genes *adk, fum, gyrB, icd, mdh, purA and recA* respectively.

- http://www.cdc.gov/pulsenet/protocols.htmprotocole

  For PFGE typing of *E. coli.*

- http://microbiology.columbia.edu/fidock

  For southern blot protocol.

2.10. Bacterial growth condition

For most purpose, *E. coli* were grown in 3 ml LB-cefotaxime (1µg/ml) broth in 15 ml Greiner tube (Cellstar, Greiner tube) at 37°C with shaking at 180 rpm on a shaking apparatus (GFL3017, Germany) placed in 37°C incubation room for 3-4 hours corresponding to the bacterial exponential growth phase.

2. 11. Bacterial growth measurement

The optical density of bacterial culture was measured with a spectrophotometer (Pharmacia Biotech Ultrospec 3000). One ml of bacterial culture was added to disposable cuvettes (Sarstedt, Nümbrecht, Germany) and the optical density was measured at the wave length of 610 nm. In case of optical density over 0.6 the bacterial culture was diluted (1:10) with the respective buffer or culture medium before measurement.

2.12. Bacterial storage

To keep the bacteria for the routine use over a short period of time, the bacteria were plated on LB agar supplemented with cefotaxime at a final concentration 1µg/ml.
For long-term storage, stock cultures were prepared of all the clinical isolates examined in this study using glycerol as the osmotic protector.
Procedure:
750µl of the bacterial culture (in the exponential phase and under cefotaxime stress) was mixed with 750µl Brain-Heart Infusion broth (BHI) containing 60% glycerol in 2ml Cryo-tube (Sarstedt, Nümbrecht, Germany). The broth was homogenized on a vortex mixer and stored at -80°C.

2.13. Polymerase chain reaction (PCR)

One colony of the bacterial isolates was picked by a tooth pick and placed in a 0.2 ml thermotube (Thermoscientific PCR reaction tubes) then 50 µl of PCR preparation were added in the tube as follows:

	Initial concentration	Taken volume	End concentration
PCR buffer	10X	5.0µl	1X
dNTPs	5mM	2.0µl	200µM
Forward primer	20µM	0.5µl	200nM
Reverse primer	20µM	0.5µl	200nM
Taq -Polymerase	5U/µl	0.25µl	1.25U
H_2O		ad	50.0µl

The PCR product is tested for the right size through agarose gel electrophoresis.

2.14. Agarose gel electrophoresis

Before gel casting, the dried agarose was dissolved in 1X TAE buffer by heating in microwave oven. Ethidium bromide (0.5µg/ml) was added to the warm gel solution to enable fluorescent visualization of the DNA fragments under UV light. The warm gel was poured into a mold which is fitted with a comb. The concentration of agarose in the gel varied from 1% to 2% percent [w/v] depending on the expected size(s) of the amplicons. The agarose gel was submerged in1X TAE electrophoresis buffer in a horizontal electrophoresis apparatus. The amplified DNA samples were mixed with 5X gel tracking dye (1:1 v/v) and loaded into the wells. 1 kb plus or low range marker have been used for fragment molecular size determination.

Electrophoresis conditions were 150V, 250 mA for 30-60 minutes at room temperature depending on the expected molecular size of the amplicon.

The gel was visualized at first on UV detector then by gel documentation system (Bio-Rad, UK) and stored on disks as TIFF files.

2.15. Measurement of DNA concentration with Nano- Drop ND 1000 Spectrophotometer

One microliter of double-distilled water was loaded onto the lower optical surface. The lever arm was closed few times to wash the upper optical surface. The lever arm was then lifted and both optical surfaces were wiped off with soft tissue. The Nano-Drop software was opened and selected for the nucleic acid molecule (DNA 50) setting. Blank measurement was performed by loading 1µl double-distilled water and selecting "blank". When the blank was completed both optical surfaces were cleaned. The nucleic

acid sample was measured by loading 1µl and selecting "measure". Once the measurement was completed both of the optical surfaces were cleaned.

2.16. Testing of antibiotic susceptibility and ESBL production

Antibiotic susceptibility of clinical isolates and transconjugants was determined using the disk diffusion method on Müller-Hinton Agar (Oxoid Basingstock, England) as recommended by Clinical Laboratory and Standard Institute (CLSI). The revealed results were interpreted according to Manufacturer zone diameter interpretive chart (see Appendix IV, Table IV-A) that are adapted in part CLSI Document M100-S17 (M2): Disk Diffusion Supplemental Tables, Performance Standard for Antimicrobial Susceptibility Testing, utilizing *E. coli* ATCC 25922 as a control.

All clinical isolates were tested for ESBL production using the double disk synergy method as described by Drieux *et al*. (2008).

2.17. PCR assays for TEM, SHV and CTX-M-β-lactamase genes

ESBL-producing isolates were tested for the genes encoding TEM, SHV and CTX-M-β-lactamases by PCR using the primers listed in Table (6) and the amplification conditions specified in Table (10).

Table 10: PCR cyclic conditions used for detection of ESBL genes.

Target gene	PCR condition
Bla_{TEM}	Denaturation for 5 minutes at 94ºC; 30 cycles of 94ºC for 30 s, 55ºC for 60 s, 72ºC for 60 s; and final extension of 72˚C for 7 minutes.
Bla_{SHV}	Denaturation for 5 minutes at 94ºC; 30cycles of 94ºC for 30 s, 58ºC for 60 s, 72ºC for 60 s; and final extension of 72ºC for 7 minutes.
Bla_{CTX-M}	Denaturation for 5 minutes at 94ºC; 30 cycles of 94ºC for 30 s, 55ºC for 60 s, 72ºC for 60 s; and final extension of 72ºC for 7 minutes.

2.18. Sequence based allele typing for *bla*$_{\text{CTX-M}}$

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using the same primer used for amplification. Searching for nucleotide sequence homology was performed using BLAST available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).
Nucleotide sequences were aligned and compared with the published sequences of the proposed allele types in the previous step using the Lasergene software package (DNASTAR, USA).

2.19. Pulsed-Field Gel Electrophoresis (PFGE)-based genotyping

PFGE was performed according to the Pulse Net protocol of the Centers for Disease Control and Prevention, Atlanta, USA http://www.cdc.gov/pulsenet/protocols.htm . The agarose-embedded DNA was digested with the restriction endonuclease *Xba*I (Fermentas, Germany) at 37^{o}C for 16 hrs. Electrophoresis was conducted using CHEF Drive II (Bio-Rad, UK) under the following conditions: 6 V, with 2.2 s-54 s pulses for 20 hrs. Strains differentiation by PFGE analysis was achieved by comparison of band patterns using Gelcompar II (Applied Maths, Belgium). The patterns were normalized using the molecular weight marker lambda ladder PFGE (New England Bio-Labs, USA). Dendograms were generated to visualize relationships among the isolates. The similarity coefficient (SAB) of sample pairs was calculated based on band positions by using DICE metric. A similarity coefficient SAB of 0.80 was set as a threshold for defining clusters of genetically similar isolates.

2.20. Phylogenetic group typing

In this study we have used the PCR-based phylogenetic typing method because it is simple, rapid and easily applied for large number of *E. coli* strains as described by Clermont *et al.* (2000).
Escherichia coli phylogenetic grouping was achieved using triplex PCR for *chuA, yjaA* genes and anonymous DNA fragment designated TSPE4.C2. The amplification was performed with the primers listed in Table 7. PCR was performed with (Applied Biosystem 2720) thermocycler under the following conditions:
Denaturation for 5 minutes at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C and final extension step of 7 minutes at 72°C.

2.21. Multi-Locus sequence typing

Multi-locus sequence typing (MLST) is a typing technique utilizes multiple housekeeping gene loci in the characterization of isolates from particular bacterial species. This method takes advantage of the variation present in the nucleotide sequences of these gene fragments. All the unique sequences for a given locus are assigned an allelic number. The combination of the multiple loci numbers defines a particular strains sequence type (ST). Relationships among the isolates are identified by comparing their allelic profiles. The closely related isolates have identical STs, or STCs that differ at a few loci, while unrelated isolates have unrelated STs. *E. coli* MLST scheme has been done using internal fragments of the following seven house-keeping genes: *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase) and *recA* (ATP/GTP binding motif). Table 8 show the primers used for amplification and sequencing of those seven house-keeping genes.
PCR Conditions: 2 minutes at 95°C, 30 cycles of 1 minute at 95°C, 1 minute at annealing temperature (either 54°C, 58°C or 60°C), 2 minutes at 72°C followed by 5 minutes at 72°C. PCR products were sequenced. The sequences were blasted on MLSTs homepage (http://mlst.ucc.ie/mlst/dbs/Ecoli/dbs/Ecoli) to obtain the ST types of the bacterial isolates.

2.22. PCR-based replicon typing (PBRT) of plasmid

PCR-based replicon typing (Inc/rep PCR-based typing) method has been developed for classification of plasmids occurring in the members of *Enterobacteriaceae* (Carattoli *et al*. 2005). In this study PCR-based replicon typing was carried out as described previously (Carattoli *et al.*, 2005). Table 9: shows the primers which were used for plasmid replicon typing.
PCR cycles conditions were: initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 30 s and extension at 72°C for 1 minute followed by a final extension at 72°C for 5 minutes.

2.23. Transfer of antibiotic resistance genes by conjugation

The conjugation experiments were performed as described previously (Mshana *et al.*, 2009) using *E. coli* K-12 CC118 (Rif^R, Lac^{-ve}, plasmid-free and none lysogenized with

lambda phage) as a recipient strain and randomly selected clinical *E. coli* isolates representing different PFGE-based clusters as donor strains. At first, the ability of the donor isolates to grow on 300µg/ml rifampicin-containing LB agar plates was examined. Then the strains were mixed at the ratio of 1:2 (donor / recipient) on LB (without antibiotic) agar followed by an overnight incubation at 37°C. Transconjugants were selected by suspending the growth in 1 ml of PBS and 0.1 ml of 10^{-1} dilutions was plated on LB agar containing 300µg/ml rifampicin and 30µg/ml cefotaxime. The transconjugants were tested for ESBL production using double disk synergy test followed by PCR amplification of $bla_{\text{CTX-M}}$ also the antibiotic susceptibility test was determined for each transconjugant using the disk diffusion method and the resulting new pattern was compared to their parent clinical isolates.

2.24. Detection and sizing of large plasmids

Since the great majority of the currently detected ESBL in question ($bla_{\text{CTX-M}}$) is carried by large-sized plasmids, this study has been restricted to characterization of such types of plasmids. Determination of the number and the approximate molecular size of the large plasmids were carried out for selected transconjugants and clinical isolates by S1 nuclease digestion of the total DNA encased in agarose plug followed by pulsed-field gel electrophoresis (S1-PFGE). The size of the plasmids was identified by lambda phage PFGE-DNA marker (NewEngland Bio-Labs, USA) as described previously (Mshana *et al.*, 2009; Barton *et al*., 1995).

The procedure included:

The plugs were prepared according to Pulse Net protocol but here the antibiotic stress during culture preparation of great importance to retain the plasmid. Each plug was subjected to pre-incubation in 1X S1-nuclease reaction buffer containing 0.15 mM NaCl for 30 minutes at room temperature then the pre-incubation mixture pipetted out. Then, 200µl of the restriction mixture containing 1 unit of S1-nuclease (Fermentas, Germany) 50 mM sodium chloride, 30 mM sodium acetate and 5 mM zinc sulphate were added and incubated at 55°C for 45 minutes and the restriction was stopped with 50µl of 0.5 M EDTA. The electrophoresis chamber was filled with 3000 ml of 0.5X TBE-buffer. The pump was turn on to start the circulation and the cooler was set to 14°C. Agarose gel (1%) was prepared as follow:

One gram of Pulsed-field certified agarose (Bio-Rad, USA) was added to 100 ml 0.5X TBE buffer and dissolved by boiling in the microwave. The gel equipment was assembled and the gel was poured and let to be solidified for 30 minutes at room temperature. The plugs were added after removing the comb. Then, the gel was released from the mold and all excess agarose was removed from the sides and underneath the black support before placing the gel with its supporting black plate in the electrophoresis chamber. PFGE device was programmed and the electrophoresis was run.

Program parameters:

Pulse time: 5-50 sec. (initial pulse-final pulse).

Total run time: 20 hours.

Voltage: 6 v/ cm.

Buffer temperature: 14^{o}C.

Gel running buffer: 0.5 X TBE.

After the run had been finished, the gel stained in ethidium bromide in 0.5X TBE buffer (50µg/L) for 30 minutes and destained in 0.5 X TBE buffer, fast detection has been done in UV detector then the gel was visualized on gel documentation system (Bio-Rad, UK) and stored on disks as TIFF file. TBE-buffer was removed from the chamber and replaced by water. The circulation was run for few minutes then the water was removed and all remaining liquid was wiped off and replaced with Fresh 0.5X TBE buffer when the next run will be done directly or after short period.

2.25. Southern blot

Southern blot is a method used to transfer DNA fragments of different size that were separated by agarose gel electrophoresis to positively-charged nylon membrane. Southern transfer is followed by probe hybridization to detect the presence and location of specific DNA sequence.

In this study the DNA of the large-sized plasmids were linearized by S1- nuclease and separated as discrete, distinct bands and were transferred to positively charged nylon membrane (as described at http://microbiolog.columbia.edu/fidock) which was subjected to hybridization with *bla*$_{CTX-M}$ specific probe to locate the target *bla*$_{CTX-M}$. S1-nuclease digestion and PFGE separation was carried out as mentioned before.

2.25.1. Depurination: The gel was placed in a glass dish, rinsed with double-distilled water, the water replaced by excess volume of depurination solution and rocked slowly on a shaker for 30 minutes at room temperature, rinsed two times with double-distilled water for 10 minutes.

2.25.2. Denaturation: The gel was incubated at room temperature in denaturation buffer for 15 minutes two times and was rinsed with double-distilled water.

2.25.3. Neutralization : The gel was washed two times with excess neutralization solution for 20 minutes then washed once with 2X SSPE washing buffer.

2.25.4. Setting-up of transfer system

Whatman 3MM filter paper wick was soaked in the transfer buffer (10 X SSPE), placed on glass support (glass slide that was longer and wider than the gel) constituting a bridge between two large trays containing excess transfer buffer so that the two edge of the Whatman 3MM filter paper wick were immersed in the transfer buffer. The air-bubbles between the filter paper wick and the glass support were removed with a sterile glass rod.

The agarose gel was placed with the bottom-side facing wet (impregnated with transfer buffer) Whatman paper wick, the air-bubbles between them were removed, the gel was surrounded with parafilm to prevent the flow of transfer buffer directly from its reservoir to the paper-towel (that have been be stacked in the last step of transfer assembly) by-passing the agarose gel. A piece of positively-charged nylon membrane (1mm larger than the gel) was cut, the orientations was marked by cutting the bottom-left corner of the membrane. The membrane was floated on the surface of double-distilled water until it was completely wetted from underneath then it was placed on the top of the gel followed by the removal of air-bubbles between them. Three-pieces of Whatman 3 MM filter paper (were cut exactly to the size of the gel) damped in 2X SSPE and placed on the top of the nylon membrane followed by the removal of air-bubbles between them. Paper towels stack (just smaller in size than the pieces of Whatman 3 MM filter paper) was placed on it and weighed-down with a proper weight and the transfer was lasted overnight.

2.25.5. Preparation of nylon membrane for hybridization

The membrane was peeled from the gel, rinsed in 2X SSPE for 5 minutes to remove the agarose traces from the gel. The efficiency of transfer was assessed by staining the gel in ethidium bromide followed by UV visualization.

Fixation: The membrane was UV-cross linked for one minute with the DNA-side facing up. After fixation, the membrane was either stored dry at 2-8^{o}C to be worked later on or was used immediately when we wanted to go ahead for pre-hybridization.

2.25.6. Purification and labeling of the probe

Preparation of the probe template: *bla*$_{CTX-M}$ PCR product was purified according to QIA quick PCR Purification Kit 250 (Qiagen, Germany) manufacturer instructions and DNA concentration was determined by Nanodrop (it should be ≥ 62.5 ng/µl to be used as a template).

Template labeling: according to DIG High prime DNA labeling and detection starter Kit II manufacturer instructions as following : template DNA volume equivalent to 1µg was added to autoclaved double distilled water to 16µl final volume (62.5µg/µl) and was denaturated by heating in boiling water bath for 10 minutes and quickly chilled in ice. Then 4µl of DIG-High prime mixture (vial 1) was added to denatured template, mixed and centrifuged briefly and incubated for overnight at 37^{o}C to increase the yield of DIG-labeled DNA. The reaction was stopped by adding 0.2M EDTA.

2.25.7. Prehybridization

To a previously pre-warmed (55^{o}C) fifty ml pre-hybridization solution, 200 µl salmon sperm DNA (10 mg/ml) that was just denatured by heating at 95^{o}C for 5 minutes and quick cooling in ice was added. The mixture was poured in glass dish then the membrane was placed with the DNA-side facing the prehybridization mixture and incubated at 55^{o}C for 2 hour with gentle shaking.

2.25.8. Hybridization

Was carried out according to DIG High prime DNA labeling and detection starter Kit II manufacturer instructions as following: The prehybridization solution was replaced by DIG-easy hybridization solution and incubated at 39^{o}C for 30 minutes with gentle

agitation. Meanwhile, labeled probe/hybridization mixture was prepared as follow: DIG-labeled bla_{CTX-M} probe was denatured by boiling for five minutes and rapid cooling and was added to 35 ml of pre-warmed DIG-easy hybridization solution and mixed throughly (this mixture should not be discarded after the first use and was preserved for next use by storage at -20^{o}C to be freshly denaturated by heating at 68^{o}C for 10 minutes when it was required). The DIG-easy hybridization solution after the end of 30 minutes incubation was replaced by the probe/hybridization mixture and incubated for 4 hours with gentle agitation. After the end of the last incubation, probe/hybridization mixture was stored for the next use, while the membrane was subjected to the stringency wash.
Stringency wash: The membrane was washed two times in excess of 2X SSC 0.1% SDS stringency buffer under constant agitation then washed two times in 0.5X SSC 0.1% SDS pre-warmed to 68^{o}C for 15 minutes under constant agitation.

2.25.9. Detection of hybridization

The membrane was rinsed for 5 minutes in maleic acid washing buffer then incubated for 30 minutes in 200 ml 1X blocking solution to block the unspecific binding sites on the membrane. The working blocking solution was prepared by diluting the 10X blocking solution 1:10 in maleic acid buffer. The blocking solution was replaced by 40 ml of antibody solution and the membrane was incubated with agitation for 30 minutes to bind to DIG-labeled probe. The antibody working solution was prepared by diluting anti-digoxigenin-AB (750U/ ml; vial 4) 1: 10000 (75 mU/ ml) in 1X blocking solution. The membrane was washed two times in 200 ml maleic acid washing buffer for 15 minutes and then was equilibrated in 30 ml of detection buffer for 3 minutes. The membrane was placed with the DNA-side up on the development folder. Two ml of CSPD (Chemiluminescent substrate for alkaline phosphatase) was applied and the membrane and was immediately covered with the second sheet of the development folder to distribute the substrate evenly without formation of air-bubbles over the membrane and it was incubated for 5 minutes at 25^{o} C. The excess liquid was squeezed out and the damp membrane was further incubated at 37^{o}C for 10 minutes to enhance the luminescent reaction. Then, it was exposed to X-ray film for 20 minutes.

3.0. Results

3.1. ESBL production and PCR detection of ß-lactamase-encoding genes among enterobacterial isolates derived from Egyptian University Hospitals

Among the total of 184 tested clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* derived from Egyptian Hospitals, 147(79.9%) were ESBL positive including 84 (45.6%) *K. pneumoniae* and 63 (34.3%) *E. coli*. The proportion of ESBL-producing *K. pneumoniae* was 82.4% (84 /102) while the proportion of *E. coli* was 78.8% (63 /82).

Bla$_{TEM}$ was the most frequently detected ß-lactamase-encoding gene. It was detected in 65.2% (120/184) and 81.6% (120/147) among the total and ESBL-producing *Enterobacteriaceae*, respectively including 68.6% (70 /102) and 83.3% (70 /84) of the total and ESBL-producing *K. pneumoniae* and 61% (50 /82) and 79.4% (50/63) of the total and ESBL- producing *E. coli* , respectively.

Bla$_{CTX-M}$ was detected in 50.5% (93 /184) and 63.7% (93/147) of the total and ESBL-producing *Enterobacteriaceae* including 49% (50 /102) and 59.2% (50/ 84) of the total and ESBL- producing *K. pneumoniae* as well as 52.4% (43 /82) and 68.3% (43 /63) of the total and ESBL-producing *E. coli*, respectively.

Bla$_{SHV}$ was in the third order. It was detected in 20.1% (37 /184) and 25.2% (37/ 147) of the total and ESBL- producing *Enterobacteriaceae*, respectively including 32.6% (33 /102) and 39.3% (33/84) of the total and ESBL-producing *K. pneumoniae* as well as 4.9% (4/82) and 6.3% (4/ 63) of the total and ESBL- producing *E. coli*, respectively.

Table 11: Prevalence of the different types of ß-lactamase-encoding genes among *Klebsiella pneumoniae* and *Escherichia coli* isolates derived from Egyptian University Hospitals.

	Number of strains	**ESBL positive**	***Bla*$_{CTX-M}$**	***Bla*$_{SHV}$**	***Bla*$_{TEM}$**
Total	184	147	93	37	120
K. pneumoniae	102	84	50	33	70
E. coli	82	63	43	4	50

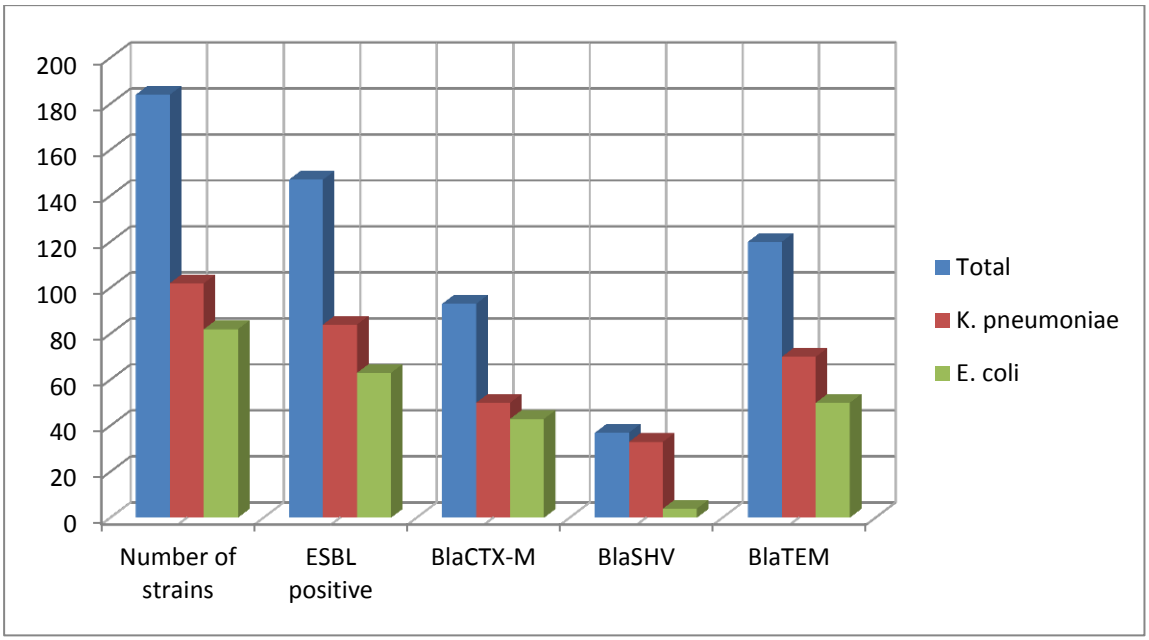


Figure 4: The prevalence of the different types of ß-lactamase-encoding gene among *Klebsiella pneumoniae* and *Escherichia coli* isolates derived from Egyptian University Hospitals.

3.1.1Multiplicity of ß-lactamase-encoding genes among enterobacterial isolates derived from Egyptian University Hospitals

Taking in consideration the number of the different ß-lactamase-encoding genes that were harbored by each enterobacterial isolate, ß-lactamase-producing isolates were distributed into three categories, the first harbored only one type of ß-lactamase encoding gene, the second harbored two types and the third harbored all the three genes. The first and the second categories were further sub-divided into three sub-categories as illustrated in the following Tables and Figures.

Table 12: Multiplicity of ß-lactamase-encoding genes among *K. pneumoniae* and *E. coli* derived from Egyptian University Hospitals.

	One ß-lactamase-encoding gene			**Two ß-lactamase-encoding genes**			**Three ß-lactamase encoding genes**
	***Bla*$_{CTX-M}$**	***Bla*$_{SHV}$**	***Bla*$_{TEM}$**	***Bla*$_{CTX-M}$ + *Bla*$_{SHV}$**	***Bla*$_{CTX-M}$ +*Bla*$_{TEM}$**	***Bla*$_{SHV}$+ *Bla*$_{TEM}$**	***Bla*$_{CTX-M}$ + *Bla*$_{SHV}$ +*Bla*$_{TEM}$**
Total *Enterobacteriaceae*	19	1	39	7	52	14	15
K. pneumoniae	6	1	21	7	24	12	13
E. coli	13	0	18	0	28	2	2

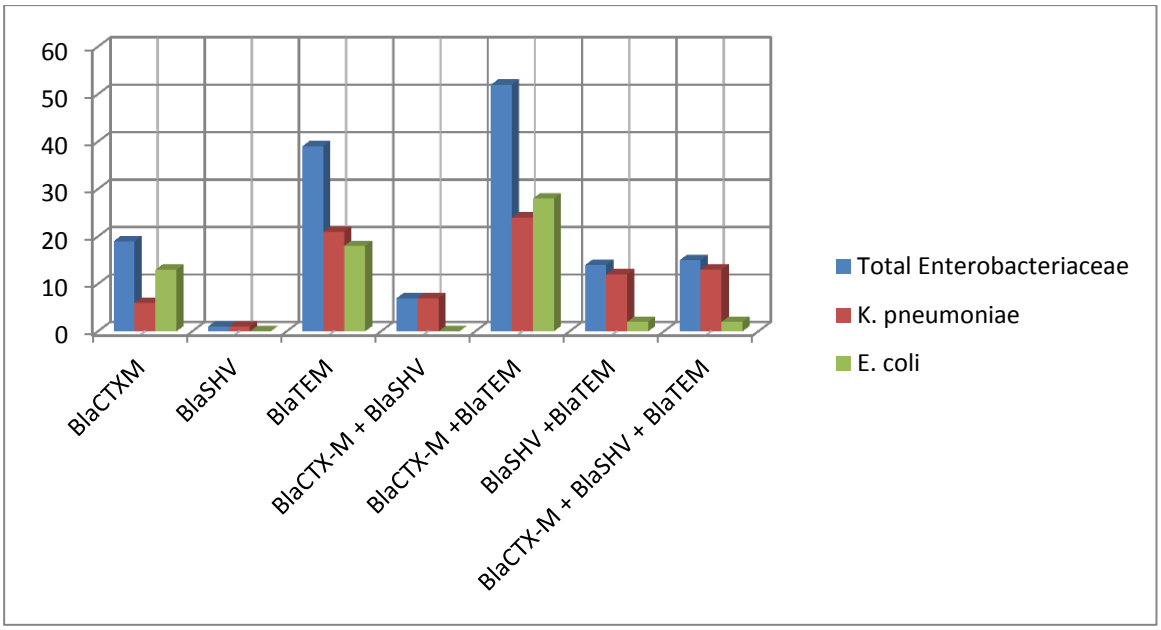


Figure 5: Multiplicity of ß-lactamase-encoding genes among *K. pneumoniae* and *E. coli* derived from Egyptian University Hospitals.

From the previous Table we revealed that:

Fifty-nine (40.1%) of the total ESBL-producing enterobacterial isolates were harboring only one ß-lactamase encoding gene, including 28 (19%) *K. pneumoniae* and 31 (21.1%) *E. coli* isolates. Twenty-seven percent (39/147) were harboring the bla_{TEM} alone distributed nearly equally between *K. pneumoniae* and *E. coli*. Bla_{CTX-M} alone detected in 19 (12.9%) including 13 (8.7%) *E. coli* and 6 (4.2%) *K. pneumoniae*, while bla_{SHV}-encoding gene alone was detected in only one (0.7%) *K. pneumoniae* isolate. A total of 49.7% (73 /147) of the ESBL-producing enterobacterial isolates were found to harbour two different ß-lactamase-encoding genes, the great majority of them (n = 52) were harboring both bla_{CTX-M} and bla_{TEM} including 24 *K. pneumoniae* and 28 *E. coli*, while 14(9.5%) of the total ESBL-producing enterobacterial isolates were harboring bla_{SHV} and bla_{TEM} including 12 *K. pneumoniae* and 2 *E. coli* the remaining 7 isolates in this category were harboring both the bla_{CTX-M} and bla_{SHV} all of them were *K. pneumoniae*. Only 15 (10.2%) of the total ESBL-producing enterobacterial isolates were harboring the three ß-lactamase encoding genes including 13 (8.8%) *K. pneumoniae* and 2 (1.4%) *E. coli* isolates. Among the total ESBL-producing *Enterobacteriaceae*, 41 *E. coli* strains harboring bla_{CTX-M} were selected for further molecular and epidemiological characterization.

3.2. PCR detection of ß-lactamase encoding genes among enterobacterial isolates derived from Giessen University Hospital, Germany

A total of 64 *E. coli* isolates derived from Giessen University Hospital, Germany were screened for bla_{CTX-M}. The bla_{CTX-M}-positive isolates were further screened for bla_{SHV} and $bla_{TEM.}$ The results are summarized in the following Table.

Table 13: Frequency of the different ß-lactamase-encoding genes among *E. coli* isolates derived from Giessen University Hospital, Germany.

ß-lactamase-encoding gene	Bla_{CTX-M}	Bla_{SHV}	Bla_{TEM}
Number of strains	41	-	27

3.3. Characterization of CTX-M-producing *E. coli* clinical isolates derived from Egyptian and German University Hospitals

All forty-one bla_{CTX-M} (64.1%) isolates were subjected to further molecular epidemiological characterization for comparison with an equal number of CTX-M-producing clinical *E. coli* isolates derived from Egyptian University Hospitals.

3.3.1. Distribution of ß-lactamase-encoding genes among the clinical *E. coli* isolates derived from Egyptian and German University Hospitals

Nearly two thirds of the clinical isolates from both countries were harboring both bla_{CTX-M} and bla_{TEM} ß-lactamases, while the remaining isolates were harboring bla_{CTX-M} alone. None of isolates were encoding $bla_{SHV.}$

Table 14: Prevalence of ß-lactamase-encoding genes among CTX-M-producing *E. coli* isolates derived from Egyptian and German University Hospitals.

Source	ß-lactamase-encoding genes		
	***Bla*$_{\text{CTX-M}}$**	***Bla*$_{\text{CTX-M}}$ + *Bla*$_{\text{TEM}}$**	***Bla*$_{\text{CTX-M}}$ + *Bla*$_{\text{SHV}}$**
Egypt	13	28	-
Germany	14	27	-

3.3.2. Sequence based allele typing for *bla*$_{\text{CTX-M}}$

Sequence based allele typing was performed by PCR amplification, purification of amplicons and sequencing using the same primer that has been used for amplification. The analysis of sequences was done using Lasergene software (DNASTAR, USA) together with homology searches using NCBI BLAST algorithm followed by comparison of each sequence with the standard sequences of the corresponding *bla*$_{\text{CTX-M}}$ allele type published at (http: //www.ncbi.nlm.nih.gov) revealing the following results.

Table 15: Frequency of the different *bla*$_{\text{CTX-M}}$ allele types among CTX-M-encoding *E. coli* isolates derived from Egyptian and German University Hospitals.

Source	***Bla*$_{\text{CTX-M}}$ allele type**			
	***Bla*$_{\text{CTX-M-15}}$**	***Bla*$_{\text{CTX-M-1}}$**	***Bla*$_{\text{CTX-M-3}}$**	***Bla*$_{\text{CTX-M-61}}$**
Egypt	41	0	0	0
Germany	34	5	1	1

Bla$_{\text{CTX-M-15}}$ was the most predominant allele type among German isolates (82.9%) and the only *bla*$_{\text{CTX-M}}$ allele type that was detected among Egyptian strains.

German isolates were slightly more diverse harboring *bla*$_{\text{CTX-M-1}}$ with a frequency rate of 12.2% (n = 5) and *bla*$_{\text{CTX-M-3}}$ at a rate 2.4% (n = 1) as well as *bla*$_{\text{CTX-M-61}}$ at the same rate. It was noteworthy that, all the detected *bla*$_{\text{CTX-M}}$ allele types in both countries were belonging to the same CTX-M cluster namely *bla*$_{\text{CTX-M-1}}$ group.

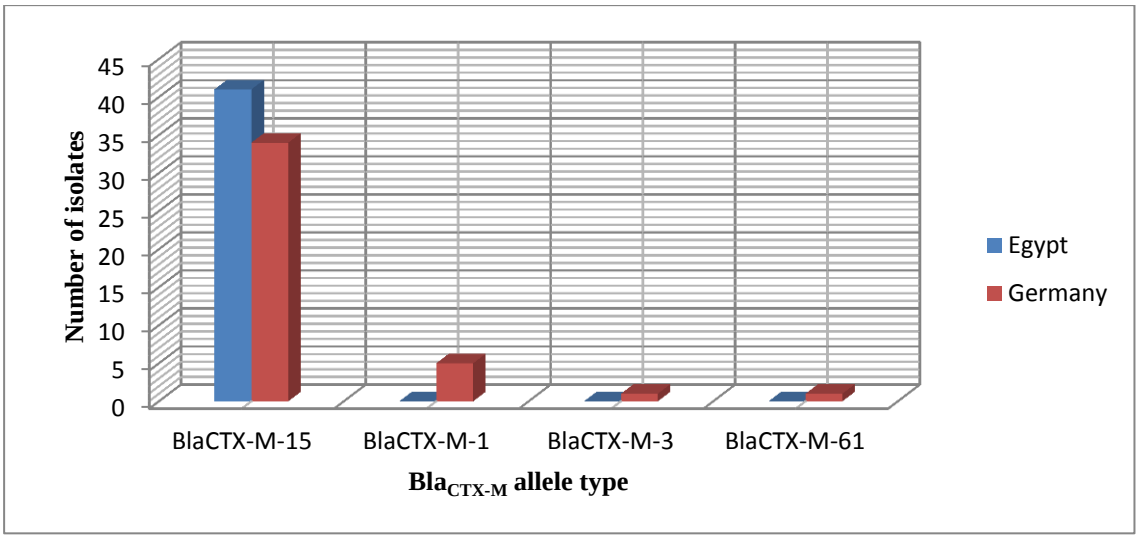


Figure 6: Frequency of the different *bla*$_{CTX-M}$ alleles among CTX-M-encoding *E. coli* isolates derived from Egyptian and German University Hospitals.

3.3.3. Antibiotic susceptibility

All isolates were sensitive to imipenem and meropenem and resistant to the other tested ß-lactams but exhibited variable susceptibility towards ß-lactamase inhibitors/ß-lactam combinations namely amoxicillin/clavulanic acid and piperacillin/tazobactam. Although the great majority of the isolates derived from German University Hospital were resistant to amoxicillin/clavulanic acid and piperacillin/tazobactam combinations only 63.4% (n = 26) and 29.3% (n = 12) of the isolates derived from Egyptian Hospitals were resistant to amoxicillin/clavulanic acid and piperacillin/tazobactam combinations, respectively. On the other hand, all Egyptian isolates and the great majority of the German ones were sensitive to fosfomycin but exhibited variable susceptibility towards the other non ß-lactam antimicrobials. Concerning to the susceptibility to non ß-lactam antimicrobials, fifty-four percent (n = 22) of Egyptian isolates were resistant to all the tested non ß-lactam antimicrobials (other than fosfomycin) versus 41.7% (n = 17) of German isolates. The highest rate of resistance 95.1% (n = 39) showed by Egyptian isolates against tetracycline followed by 92.7% (n = 38) against tobramycin, 90.2% (n = 37) against sulfamethoxazole/trimethoprim combination, 87.8% (n = 36) against the quinolones while the lowest resistance level was 70.7% (n = 29) against gentamicin. On the other hand, the highest rate of resistance showed by German isolates 85.4% (n = 35) against quinolones followed by 73.2% (n=30) against sulfamethoxazole/trimethoprim combination, 58.5% (n = 24) against tobramycin, 61% (n = 25) against tetracycline, 48.8% (n = 20) against gentamicin, while the lowest resistance rate (12.2%) was against

fosfomycin. And hence, Egyptian isolates showed slightly higher resistance to total and individual tested non ß-lactam antimicrobials (other than fosfomycin which was affective in vitro against all tested isolates) than German isolates.

Table 16: Result of antimicrobial susceptibility test of CTX-M-encoding *E. coli* isolates derived from Egyptian and German University Hospitals towards non ß-lactam antimicrobials.

	Tetracycline	Gentamicin	SXT	Tobramycin	Quinolones	Fosfomycin	All tested non ß-lactams
No.(%) of resistant strains (Egypt)	39 (95.1%)	29 (70.7%)	37 (90.2%)	38 (92.7%)	36 (87.8%)	0 (00.0%)	22 (53.7%)
No (%) of resistant strains (Germany)	25 (61%)	20 (48.8%)	30 (73.2%)	24 (58.5%)	35 (85.4%)	5 (12.2%)	17 (41.5%)

SXT: sulfamethoxazole/trimethoprim

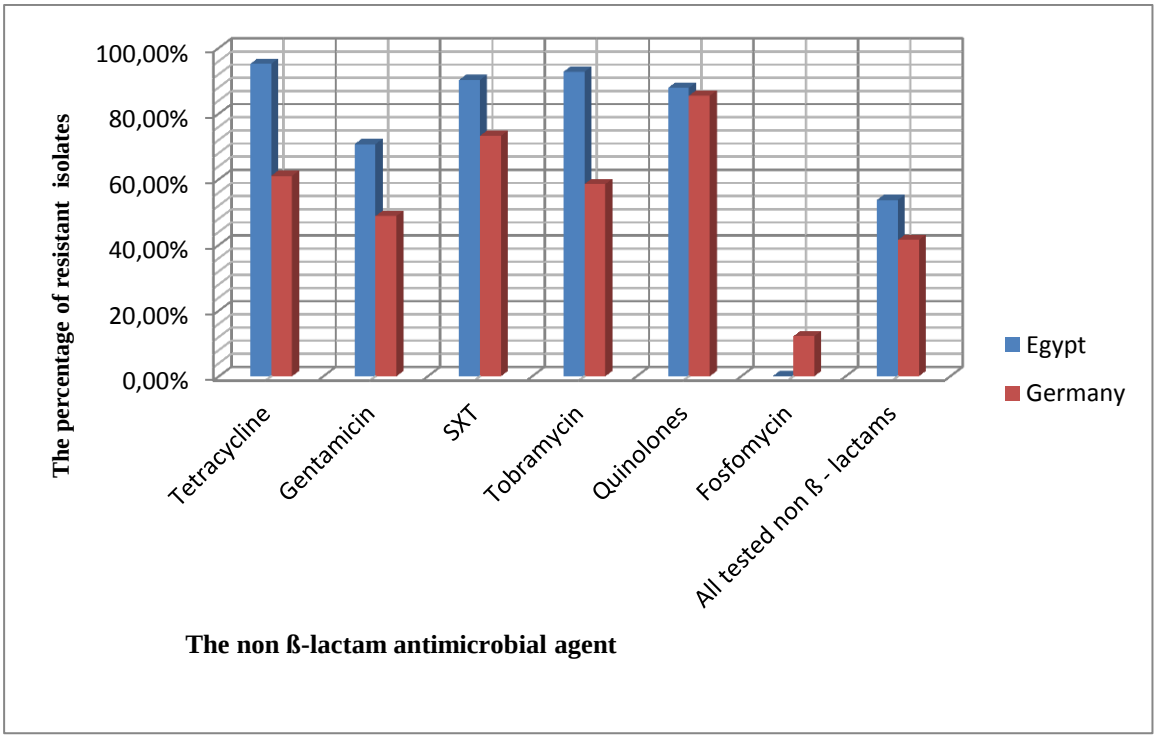


Figure 7: Antimicrobial susceptibility test of CTX-M-encoding *E. coli* isolates derived from Egyptian and German University Hospitals against non ß-lactam antimicrobials.

3.3.4. Pulsed-field gel electrophoresis (PFGE)-based genotyping

A total of eighty-two CTX-M-producing *E. coli* isolates were subjected to *Xba*I-PFGE genotyping using a similarity level SAB of 0.80. *Xba*I-PFGE patterns were obtained for 78 isolates, while DNA of the remaining isolates (455, 615, 616 and 632) was auto-digested. The 78 typeable isolates represented 73 different PFGE genotypes including 12 regional clusters of related isolates (>80% similarity) each cluster were composed of a number of isolates ranged from 2 to 16 (A, B, C to L) and 8 unique genotypes did not belong to any cluster (with Latin number designation). There were ten isolates representing five clonal pairs distributed onto the following clusters B, C, E, F and H, three pairs of them derived from Egyptian University Hospitals, one pair derived from German University Hospital.

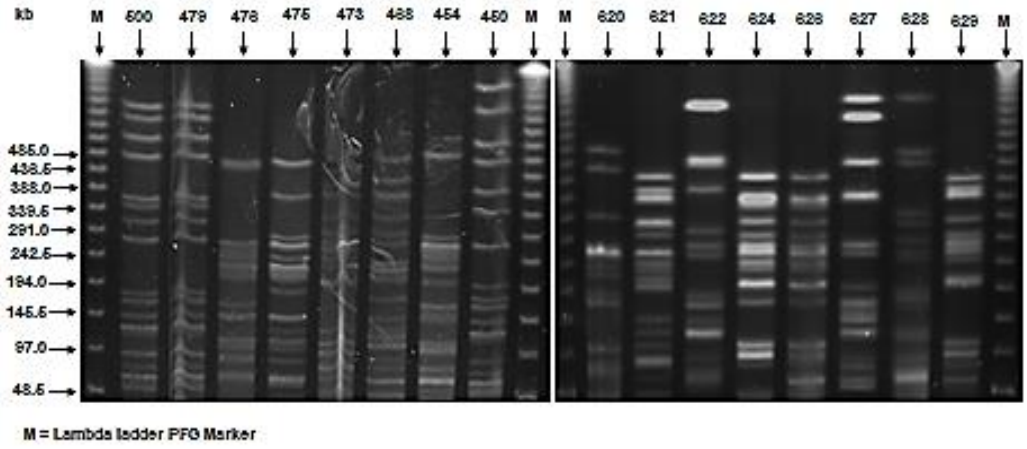


Figure 8: PFGE patterns for *E. coli* isolates derived from Egyptian (left hand side) and German (right hand side) University Hospitals.

The most surprising finding was that, the last clonal pair was composed of a German isolate namely 649 and an Egyptian isolate (476) as shown in Figure 9.

A, B and C were the major clusters. Cluster A was the largest one and was formed mainly of B2 phylogenetic type followed by cluster B which was formed mainly of D phylogenetic type then cluster C which was formed mainly of A phylogenetic type. Analysis of the distribution of the different ß-lactamase-encoding genes among the different clusters and single unique PFGE-genotypes revealed the occurrence of $bla_{\text{CTX-M-15}}$ in the majority of cases in combination with bla_{TEM} in all of the 12 clusters and in 6 of the 8 PFGE unique genotypes. $Bla_{\text{CTX-M-3}}$ detected only in an isolate of a single PFGE genotype III. $Bla_{\text{CTX-M-61}}$ was detected in association with bla_{TEM} in an isolate of the genotype G3 which was belonging to cluster G. $Bla_{\text{CTX-M-1}}$ was detected either alone in

three isolates belonging to the clusters A, L and the unique genotype IX or in combination with bla_{TEM} in an isolate belonging to cluster K.

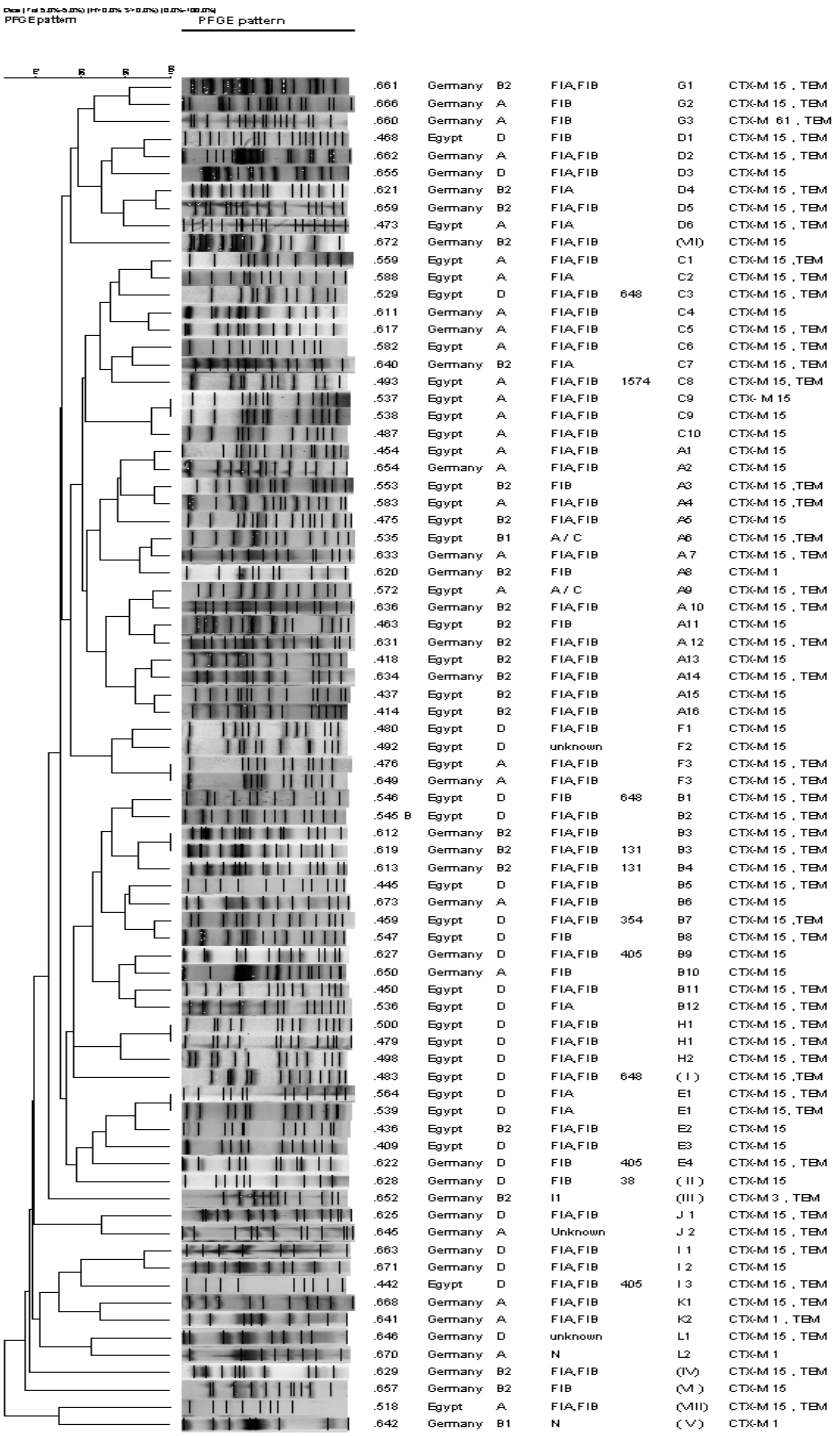


Figure 9: Dendogram illustrating the genetic relatedness of 78 typeable, CTX-M-producing, Egyptian and German *E. coli* isolates examined by pulsed-field gel electrophoresis PFGE, created by using the band based similarity coefficient and unweighted pairs geometric–matched analysis (UPGMA) with 5% position tolerance for band comparison. The major clusters were defined by similarity co-efficient of ≥ 80%.

To determine the clonality among of the clinical isolates derived from the University Hospitals of each country separately, PFGE-genotyping analysis of 40 PFGE-pattern representing the typeable isolates of Egyptian University Hospital revealed their distribution onto 5 clusters and 4 single unique genotypes with 3 non distinguishable pairs belonged to three different clusters. The threshold of similarity among all isolates ≥ 63% which jump to ≥ 73% if we excluded one (corresponding to 518) of the four single unique genotypes which is distantly related to the other strains as shown in Figure (10). On the other side, a total of 38 genotypes corresponding to the typeable isolates obtained from German University Hospital distributed into 10 clusters and 6 unique genotypes using ≥ 80% similarity as the threshold for defining the cluster. The similarity threshold was ≥ 65% as shown in Figure 10.

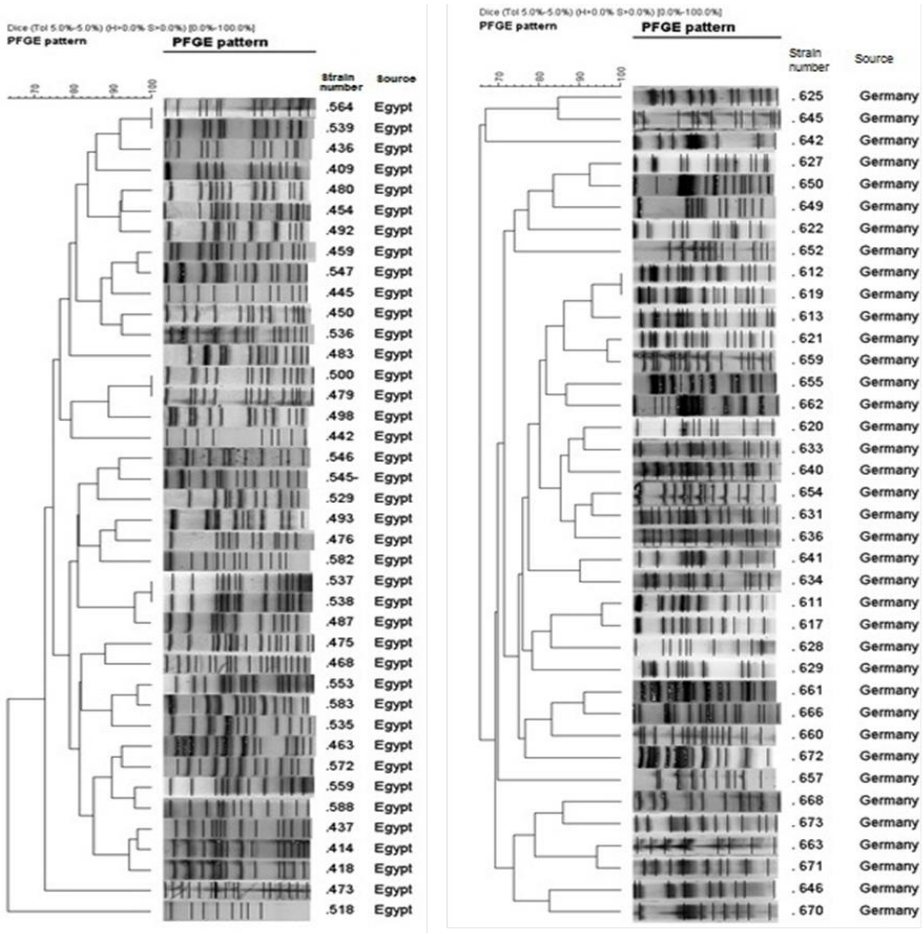


Figure 10: Dendogram illustrating the genetic relatedness of the PFGE-typeable 40 Egyptian and 38 German CTX-M-producing clinical *E. coli* isolates created by using the band based similarity coefficient and unweighted pairs geometric-matched analysis (UPGMA) with 5% position tolerance for band comparison. The major clusters were defined by similarity co-efficient of ≥ 80%.

3.3.5. Phylogenetic group typing

PCR-based phylogenic analysis revealed that 48.9% (n = 20) and 17% (n = 7) of the CTX-M-producing *E. coli* isolates derived from Egyptian University Hospitals were belonging to the virulent extraintestinal phylogenic group D and B2, respectively. Unexpectedly, 31.7% (n = 13) and 2.4% (n = 1) were belonging to commensal and intestinal virulent group A and B1, respectively.

On the other hand, 36.6% (n = 15) and 39% (n = 16) of the CTX-M-producing *E. coli* isolates derived from German University Hospital were belonging to phylogenetic types B2 and A, respectively. The remaining isolates were distributed between the phylogenic type D 22% (n = 9) and B1 (one isolate) 2.4%.

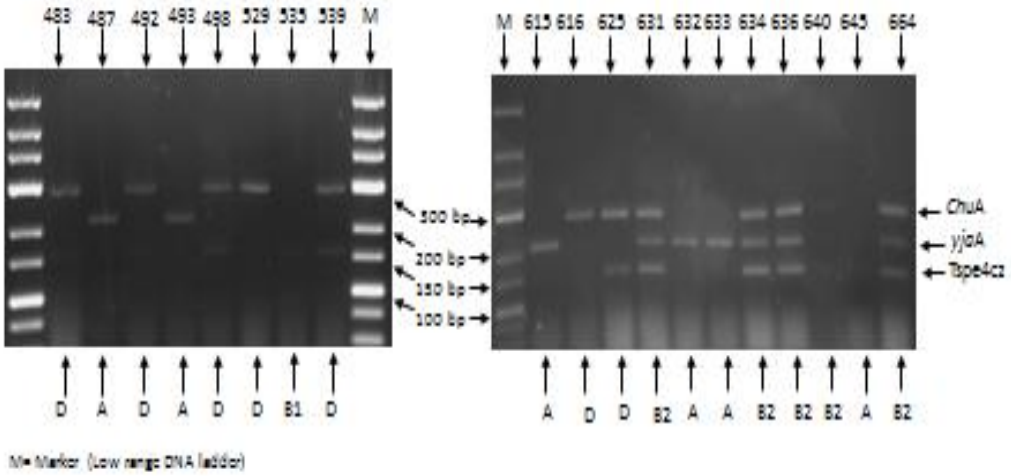


Figure 11: Triplex PCR profiles specific for *E. coli* phylogenetic groups for some Egyptian (left hand side) and German (right hand side) University Hospital-derived isolates. Showing amplification of *chu*A and *yja*A gene and DNA fragment TSPE4.C2 which is interpreted as shown by arrows and according to the previously illustrated dichotomous tree.

Table 17: Frequency of the different phylogenetic types among CTX-M-encoding *E. coli* isolates derived from Egyptian and German University Hospitals.

Source	Phylogenic type			
	A	B1	B2	D
Egypt	13	1	7	20
Germany	16	1	15	9

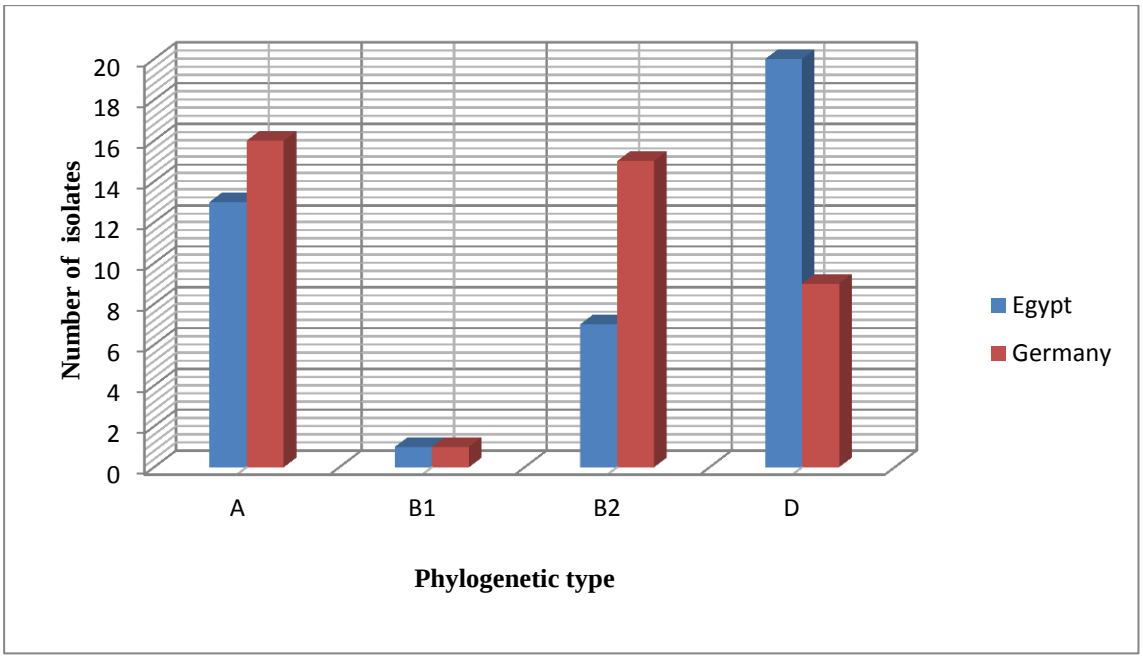


Figure 12: Frequency of the different phylogenetic types among CTX-M-encoding *E. coli* isolates derived from Egyptian and German University Hospitals.

3.3.5.1. Distribution of the different *bla*$_{\text{CTX-M}}$ allele types among *E. coli* phylogenetic types

Since *bla*$_{\text{CTX-M-15}}$ was the only allele type that has been detected in *E. coli* isolates derived from Egyptian University Hospitals and the four *E. coli* phylogenetic types are represented by these isolates, the distribution of this allele on the different phylogenetic types was the same as the distribution of *E. coli* isolates on the four phylogenetic types**.**

On the other hand, *E. coli* isolates harboring *bla*$_{\text{CTX-M-15}}$, which were derived from German University Hospital, were distributed onto three phylogenetic types, 35.3% (12/34) belonged to A, 40% (14 /34) belonged to B2 and 23.5% (8 /34) belonged to D phylogenic type**.** Three *E. coli* isolates of a total of 5 isolates that encoded the *bla*$_{\text{CTX-M-1}}$ belonged to phylogenetic type A, while the remaining two isolates, one belonged to the phylogenetic type B2 and the other belonged to the phylogenetic type B1.

The unique isolate that harbored *bla*$_{\text{CTX-M-3}}$ was belonging to the phylogenetic type B2 while the unique isolate that encoded *bla*$_{\text{CTX-M-61}}$ belonged to the phylogenetic type A.

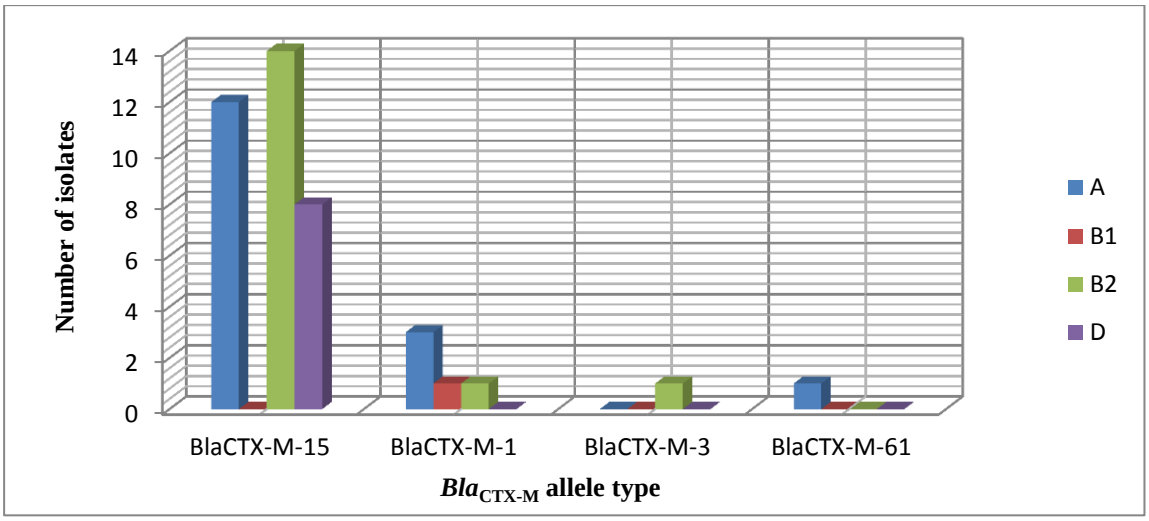


Figure 13: Frequency of the different *bla*$_{CTX-M}$ allele types among clinical *E. coli* isolates of the different phylogenic types.

3.3.6. Multi -Locus Sequence Typing (MLST)

MLST analysis of 13 CTX-M-producing *E. coli* isolates (7 isolates derived from Egyptian University Hospitals and 6 isolates derived from German University Hospital) identified seven different sequence types. Three sequence types (ST648, ST354 and ST1574) identified only among Egyptian isolates.

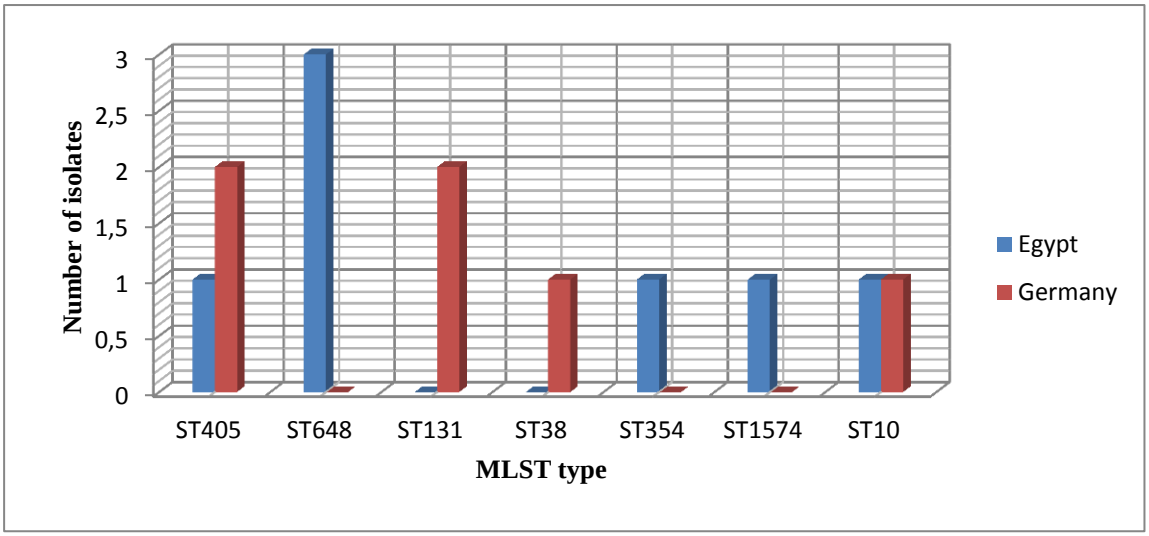


Figure 14: Frequency of the different MLSTs among CTX-M-encoding clinical *E. coli* isolates derived from Egyptian and German University Hospitals.

Two sequence types (ST131, ST38) identified only among German isolates, while the remaining two sequence types (ST405 and ST10) were identified among both of Egyptian and German Hospitals-derived isolates. 42.9% (3/7) of the isolate that are

derived from Egyptian Hospitals belonged to ST648 and the remaining four isolates belonged to the sequence types ST405, ST354, ST1574 and ST10 one each. On the other side, 66.7% (4/6) of the isolates that are derived from German hospital distributed equally onto ST131 and ST405 two each. The remaining two isolates, one belonged to ST38 and the other belonged to ST10.

Table 18: Allelic profiles and sequence types of the tested isolates.

Serial number	Strain number	Source	*adk*	*fum*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	ST
1	442	Egypt	35	37	29	25	4	5	73	405
2	459	Egypt	85	88	78	29	59	58	62	354
3	483	Egypt	92	4	87	96	70	58	2	648
4	493	Egypt	56	4	12	1	20	18	7	1574
5	529	Egypt	92	4	87	96	70	58	2	648
6	546	Egypt	92	4	87	96	70	58	2	648
7	476	Egypt	10	11	4	8	8	8	2	10
8	613	Germany	53	40	47	13	36	28	29	131
9	619	Germany	53	40	47	13	36	28	29	131
10	622	Germany	35	37	29	25	4	5	73	405
11	627	Germany	35	37	29	25	4	5	73	405
12	628	Germany	4	26	2	25	5	5	19	38
13	649	Germany	10	11	4	8	8	8	2	10

3.3.7. PCR-based replicon typing (PBRT) of plasmid

PBRT revealed that, the majority of isolates 68.3% (n = 28) and 63.4% (n = 26) of CTX-M-producing *E. coli* isolates derived from Egyptian and German University Hospitals were positive for both FIA and FIB, while, 12.2% (n = 5) and 4.9% (n = 2) were positive for FIA alone and 12.2% (n = 5) and 17% (n = 7) were positive for FIB alone respectively. Three of the remaining strains derived from German University Hospital were carrying blasmids belonged to Inc N group and one belonged to Inc I1. The replicon types of the plasmids of two strains failed to be detected by this method.

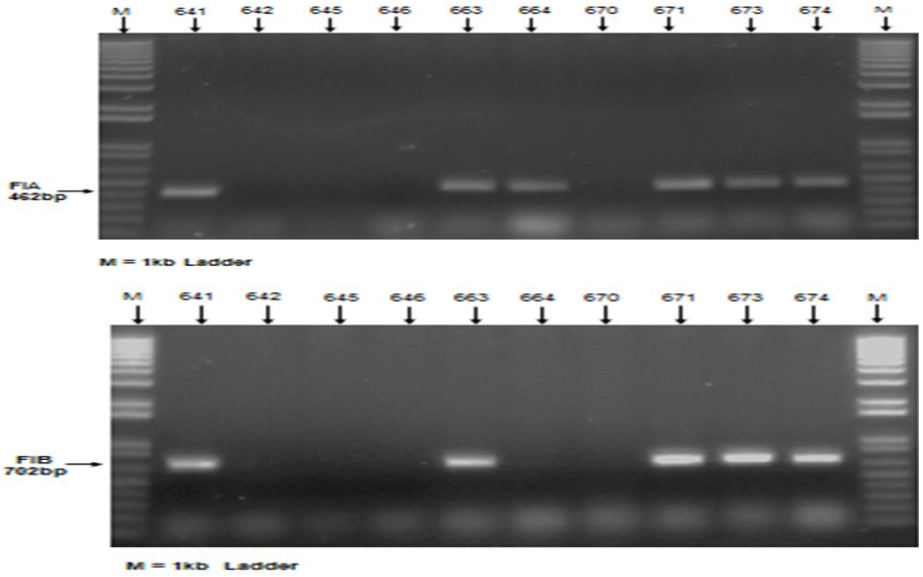


Figure 15: PCR screening of some clinical *E. coli* isolates for FIA and FIB replicons

On the other hand, the plasmids of two of the remaining three strains derived from Egyptian University Hospitals were belonged to A/C and the plasmid of one strain failed to be detected by this method.

Table 19: Results of PCR-based replicon typing (PBRT) of the large plasmids carried by CTX-M-encoding clinical *E. coli* isolates derived from Egyptian and German University Hospitals

Source	Plasmid replicon type						
	FIA+FIB	FIA	FIB	N	A / C	I 1	Non detected
Egypt	28	5	5	0	2	0	1
Germany	26	2	7	3	0	1	2

3.3.7.1. Association of the different plasmid replicon types with the different *bla*$_{\text{CTX-M}}$ alleles in *E. coli* strains derived from German University Hospital

Co-relating the result of sequencing based *bla*$_{\text{CTX-M}}$ allele typing and PCR-based replicon typing for *E. coli* isolates derived from German University Hospital revealed that, the majority 25/34 (74%) of *bla*$_{\text{CTX-M-15}}$ were associated with isolates harbouring FIA-FIB replicon-type plasmids, while 5 (14.7%) and 2 (5.9%) were associated with FIB alone and FIA alone, respectively. Three isolates of a total 5 isolates (60%) those

were harboring the *bla*$_{CTX-M-1}$ were carrying plasmids of Inc N group and one of the remaining two isolates was carrying plasmid of FIB Inc group while the other was carrying FIA-FIB replicon type plasmid. The unique isolate that encode *bla*$_{CTX-M-3}$ was carrying plasmid of I1 replicon type. The unique isolate that harbored *bla*$_{CTX-M-61}$ was carrying plasmid FIB replicon type. Unfortunately, the replicon type of the plasmids of two isolates carrying *bla*$_{CTX-M-15}$ failed to be recognized by the PCR-based replicon typing method that utilized in this study

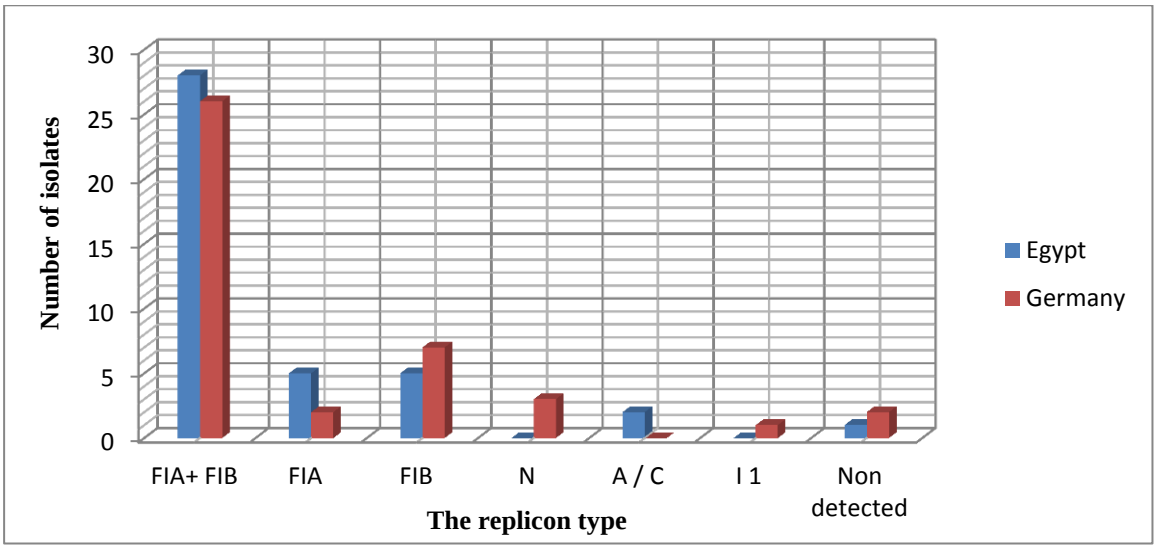


Figure 16: Frequency of the different plasmid replicon types among CTX-M-encoding clinical *E. coli* isolates derived from Egyptian and German University Hospitals.

3.3.8. Transfer of antibiotic resistance genes by conjugation

All of the randomly selected *bla*$_{CTX-M-}$carrying *E. coli* isolates (10 isolates derived from Egyptian University Hospitals and 12 isolates derived from German Hospital) were capable of transferring their plasmids by conjugation to their corresponding transconjugants.

Table 20: Characteristic of *Escherichia coli* strains which were selected as donors.

Strain No.	Clinical specimen	Medical ward	CTX-M allele type	Other ß-lactams	Phylogenic type	replicon type	ST	PFGE type	resistance to Non B- lactam
409	Urine	Oncology	15	----	D	FIA+ FIB	ND	E3	Tetra., Gent., SXT, Tobra. and Quinolones.
414	ETA	Pediatric neonatal	15	----	B2	FIA+FIB	N.D	A 16	Tetra., Gent., SXT, Tobra. and Quinolone
459	Sputum	Oncology	15	TEM	D	FIA+ FIB	354	B7	Tetra, Gent, SXT, Tobra, Quinolones
468	Urine	Pediaric	15	TEM	D	FIB	N.D	D1	Tetra. and SXT
475	Wound swab	Oncology	15	----	B2	FIA+FIB	N.D	A 5	Tetra., Tobra. and Quinolones
476	Ascetic fluid	Tropical Medicine	15	TEM	A	FIA+FIB	10	F3	Tetra ,Gent, SXT, Tobra. and Quinolones
483	Urine	Neurology	15	TEM	D	FIA+FIB	648	(I)	Tetra., SXT, Tobra. and Quinolones
500	T-tube aspirate	GIT	15	TEM	D	FIA+FIB	N.D	H1	Tetra., SXT, Tobra. and Quinolones
529	Urine	Oncology	15	TEM	D	FIA+FIB	648	C3	Tetra., Gent., SXT, Tobra. and Quinolones
559	Urine	Urology	15	TEM	A	FIA+FIB	N.D	C1	Tetra., Gent., SXT, Tobra. and Quinolones
613	Anal swab	Surgery	15	TEM	B2	FIA+FIB	131	B4	Tetra., Gent., SXT, Tobra. and Quinolones.
620	Sputum	Internal surgery	1	----	B2	FIB	N.D	A 8	None
622	Cervical swab	Gynecology	15	TEM	D	FIB	405	E4	Gent. and Tobra.
629	Vaginal swab	Gynecology	15	TEM	B2	FIA+FIB	N.D	(IV)	Tetra., Gent., SXT, Tobra. and Quinolones.
632	Anal swab	Surgery	15	----	A	FIA+FIB	N.D	NT	Tetra., Gent., SXT, Tobra. and Quinolones.
633	Urine	Orthopedics	15	TEM	A	FIA+FIB	N.D	A7	Tetra., Gent., SXT, Tobra. and Quinolones.
642	Urine	Medical clinic	1	----	B1	N	N.D	(V)	SXT and quinolones
649	Abdomenal swab	Medical clinic	15	TEM	A	FIA+FIB	10	F3	Tetra., Gent. SXT, Tobra. and Quinolones
652	Anal swab	Surgery	3	TEM	B2	I1	N.D	(III)	None
662	Urine	Urology	15	TEM	A	FIA+FIB	N.D	D2	Tetra., Gent. SXT, Tobra. and Quinolones
668	Anal swab	Operation Intensive care	15	TEM	A	FIA+FIB	N.D	K1	Tetra., SXT, Tobra. and Quinolones.
670	Oral swab	Gynecology	1	----	A	N	N.D	L 2	None

ETA: Endotracheal aspirate; N.D: not determined; NT: non-typeable Tetra.: Tetracycline; Gent.: Gentamicin; SXT: Sulfamethoxazole /Trimethoprim; Tobra. : Tobramycin.

3.3.9. Transfer of non ß-lactam resistance encoding genes

All the obtained transconjugants were subjected to antimicrobial susceptibility test by disk diffusion to confirm the transfer of *bla*$_{\text{CTX-M}}$ and to detect also any associated transfer of other resistance phenotypes indicating co-transfer of genes encoding resistance to non ß-lactam antimicrobials. In this concern, we have tested the resistance to the following antimicrobial agents: Tetracycline, gentamicin, sulfamethoxazole/ trimethoprim (SXT) combination, tobramycin and quinolones. Egyptian isolates transferred tetracycline, gentamicin, and sulfamethoxazole/trimethoprim and tobramycin resistance respectively to 60 %, 40 %, 30 % and 50 %, of the corresponding transconjugants through conjugation. Similarly, 41.7 %, 58.7 %, 58.7 %, 41.7% of German Hospital-derived transconugants have acquired resistance to tetracycline, gentamicin, sulfamethoxazole/trimethoprim and tobramycin respectively from their corresponding clinical isolates via conjugation. The highest rate of transfer of resistance to non ß-lactam shown by Egyptian isolates was against tetracycline while the lowest one was against sulfamethoxazole/trimethoprim. On the other hand, the highest rate of transfer of resistance to non ß-lactam antimicrobials shown by German isolates against sulfamethoxazole/trimethoprim and gentamicin, while the lowest rate of transfer of resistance was against tobramycin and tetracycline. Moreover, the antimicrobial susceptibility test of the transconjugants towards the non ß-lactam antimicrobial agents revealed nine different resistance profiles among the trasconjugants corresponding to the parent clinical isolates derived from both countries. Only two of resistance profiles were common to the transconjugants of both countries, one exhibited resistance to four non ß-lactam antimicrobial agents namely tetracycline, gentamicin, SXT, tobramycin, while the other reflected sensitivity to all the tested non ß-lactam antimicrobial agents. The remaining resistance profiles demonstrated resistance to a number ranged from one to three non ß-lactam antimicrobials and were distributed among Egyptian and German University Hospital-derived transconjugants (see AppendixIII, Table III-C).

Table 21: Associated transfer of resistance of non ß-lactam antimicrobials along with CTX-M-encoding gene through conjugation.

Source	Non ß-lactam antimicrobial				
	Tetracycline	**Gentamicin**	**SXT**	**Tobramycin**	**Quinolones**
Egypt	6 (60%)	4 (40%)	3 (30%)	5 (50%)	0 (0%)
Germany	5 (41.7%)	7 (58.7%)	7 (58 .7%)	5 (41.7%)	0 (0%)

SXT: Sulfamethoxazole /Trimethoprim.

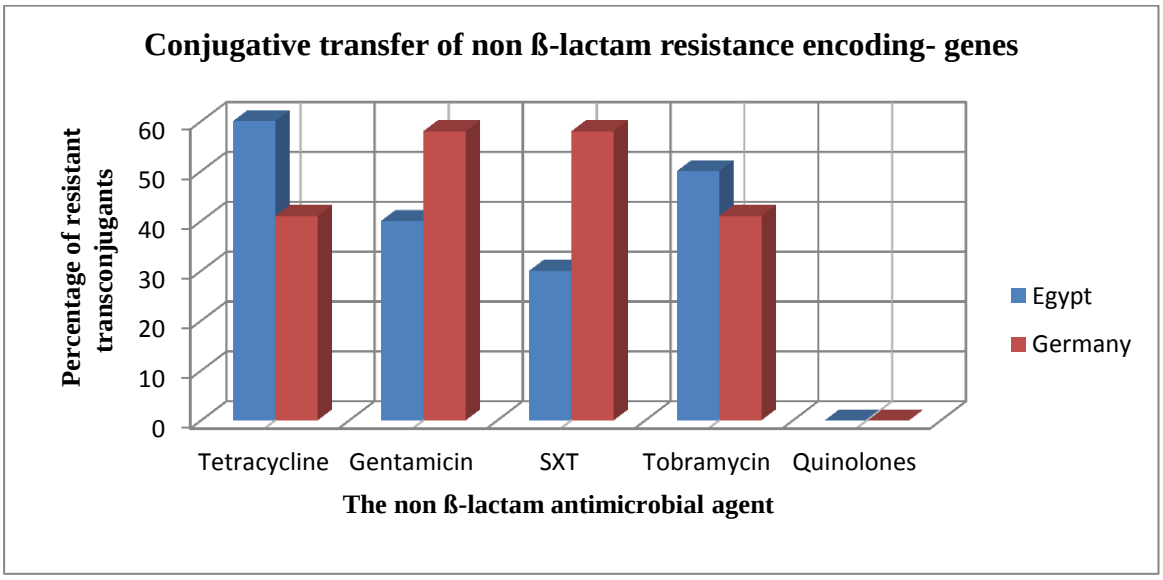


Figure 17: Co-transfer of resistance to non ß-lactam antimicrobials together with CTX-M-encoding gene through conjugation.

3.3.10. Plasmids size and number

S1-nuclease / PFGE - based plasmid profiles were obtained for 33 isolates. The number of plasmids ranged from one to two per isolate and ranged in size from < 48.5 Kb to ~170 kb in German isolates and ranged in number from one to two/strain and in size from ~97 kb to ~ 242.5 kb in Egyptian isolates. A total of 24 (7 from Egypt and 17 from Germany) single plasmid carrying isolates and 9 (6 from Egypt and 3 from Germany) multiple plasmids carring isolates have been detected (see Appendix III, Tables III-D and III-E). It is to be mentioned that, low molecular weight plasmids of approximately 45 kb were not detected by the used electrophoresis conditions.

Table 22: Frequency of the different large-sized plasmids among CTX-M-producing *E. coli* isolates derived from Egyptian and German University Hospitals.

Source	Plasmid size						
	242.5 kb	**194.5 kb**	**170 kb**	**145.5 kb**	**120 kb**	**97 kb**	**<48 kb**
Egypt	1 (7.7%)	1 (7.7%)	4 (30.8%)	4 (30.8%)	1 (7.7%)	9 (69.2%)	0 (0.0%)
Germany	0 (0.0%)	0 (0.0%)	9 (45%)	2 (10%)	1 (5%)	8 (40%)	3 (15%)

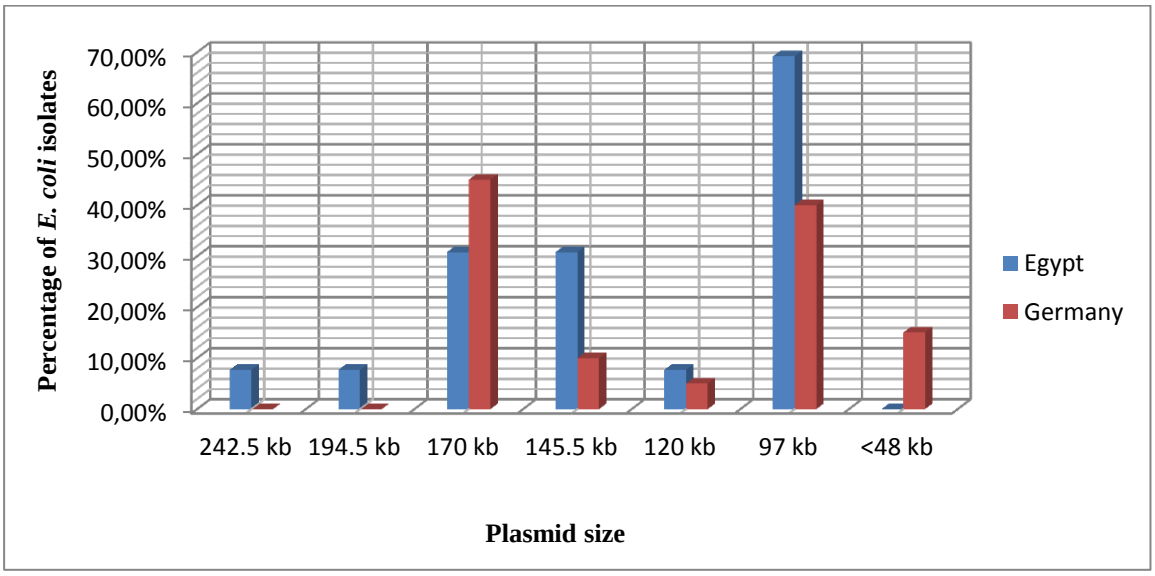


Figure 18: Frequency of different large-sized-plasmids of among CTX-M-producing clinical *E. coli* isolates derived from Egyptian and German University Hospitals.

From the previous Table we could demonstrate that, the majority (69.2% of Egyptian and 40% of German) of isolates were carrying plasmids of approximately 97 kb. 30.8% of Egyptian and 45% of German isolates were carrying plasmids of approximately 170 kb. Thirty-one percent of Egyptian and 10% of German isolates were carrying plasmids of approximately 145 kb. Only one isolate from each of Egyptian and German isolates was carrying ~120 kb plasmid. None of German isolates were found to carry ~194 kb or ~242 kb while each of which was detected in an *E. coli* isolate derived from Egyptian Hospitals. Plasmids of approximate size 48 kb were detected in three (15%) of German isolates.

3.3.11. Southern blot and hybridization

Representative isolates/transconjugants from Egyptian and German University Hospitals were selected and subjected to S1-nuclease restriction followed by pulsed-field gel electrophoresis with Lambda ladder PFGE marker. After that, the plasmid DNA was transferred from the agarose gel onto positively charged nylon membrane (Amersham Hybond™-N^+, GE Healthcare, US) and hybridized with *bla*$_{CTX-M}$ probe (prepared by labeling the purified PCR product with high DIG prime labeling mixture) and the following results were revealed.

3.3.11.1. DNA hybridization results for isolates derived from Egyptian University Hospitals

Bla$_{CTX-M}$ was located on plasmid of approximate size of 97 kb in three trasconjugants corresponding to the following clinical isolates 459, 483 and 529. And it was located on ~170 kb in another three transconjugants corresponding to the clinical isolates 414, 476 and 468. It was also located on ~145kb plasmid in the transconjugant corresponding to the isolate 475. It is noteworthy that, all the three transconjugants that carry ~ 97 kb were susceptible to all non ß-lactam tested unlike their parent clinical isolates. On contrary, the transconjugants which were carrying ~170 kb were resistant to all tested non ß-lactam antimicrobials.

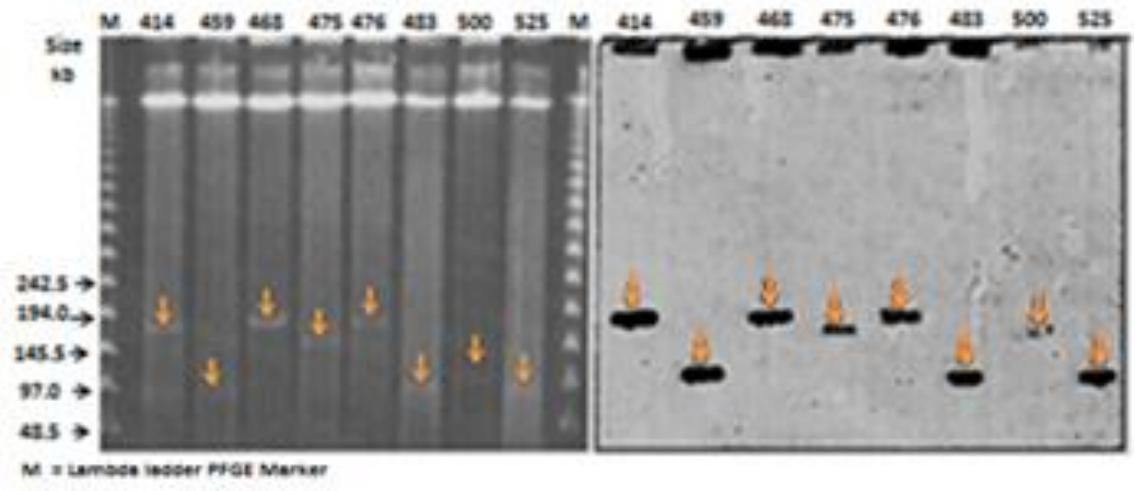


Figure 19: Agarose gel picture of PFGE/S1-nuclease-based sizing of the large plasmids derived from 8 Egyptian clinical isolates (the left hand side) and the consequent DIG-labeled CTX-M probe hybridization with CTX-M-encoding plasmids which are previously southern blotted on positively-charged nylon membrane (the right hand side).

3.3.11.2. DNA hybridization results for isolates derived from German University Hospital

DIG-labeled *bla*$_{CTX-M}$ probe was hybridized with three DNA fragments (that have been blotted to a positively charged nylon membrane by southern transfer) corresponding to ~170 kb plasmid. These fragments were derived from the isolates 649, 633, 636.

It was also hybridized with two DNA fragments corresponding to the plasmid of approximate size 97 kb derived from the isolates 629 and 654 and one fragment derived from the isolate 670 corresponding to < 48 kb size plasmid. It is to be mentioned that, the DIG-labeled *bla*$_{CTX-M}$ probe was hybridized with two fragments originated from the isolates 636 and 671 in the chromosomal fragment location.

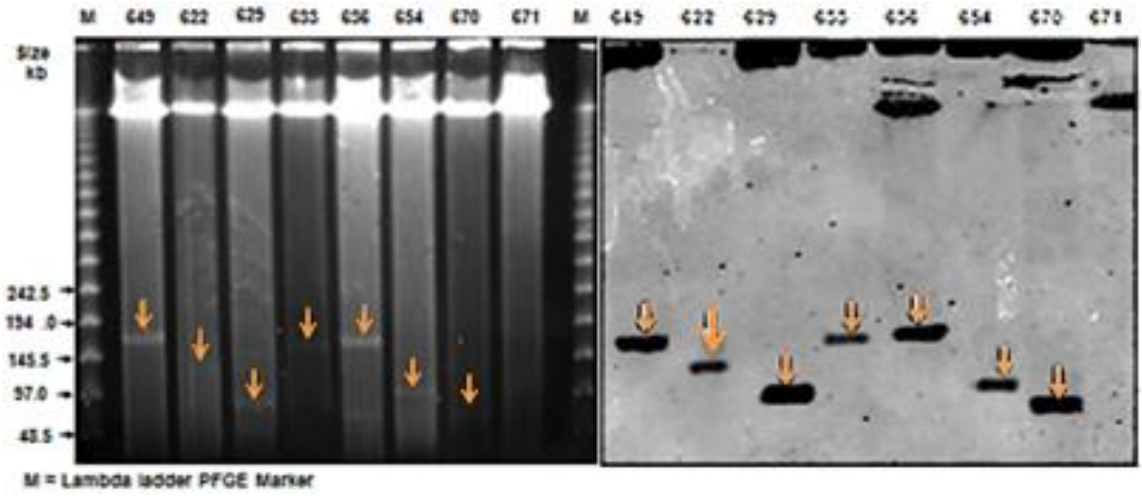


Figure 20: Agarose gel picture of PFGE/S1-nuclease-based sizing of large plasmids derived from 8 German clinical isolates (the left hand side) and the consequent DIG-labeled *bla*$_{CTX-M}$ probe hybridization with CTX-M-encoding plasmids which were previously southern blotted on positively-charged nylon membrane (the right hand side).

3.3.12. Comparison between CTX-M-producing *Escherichia coli* isolates derived from University Hospitals of Egypt and Germany

Table 23: Comparison between CTX-M-producing *Escherichia coli* isolates derived from Egyptian and German University Hospitals

Common features

	University Hospitals of Egypt	University Hospital of Giessen, Germany
Number of isolates	41	41
Collection period	During the period from October 2008 to March 2009	Simultaneously in the same time period

Variables

	University Hospitals of Egypt	University Hospital of Giessen, Germany
Types of clinical specimens	Urine (63.4%), swabs (34.1%) and sputum (2.4%)	Urine (48.8%), swabs (48.8%) and sputum (2.4%)
***Bla*$_{CTX-M}$ variants**	CTX-M-15 (100%)	CTX-M-15 (83%), CTX-M-1 (12.2%), CTX-M-3 (2.4%) and CTX-M-61 (2.4%)
Other ß-lactamases	TEM (68.3%)	TEM (65.9%)
PFGE analysis **PFGE clusters (>80%similarity)** **Single (unique) genotypes** **Clonal isolates (100%similarity)**	 5 4 Three clonal pairs of isolates	 10 6 one clonal pair of isolates
Phylogenetic group	A (31.7%), B1(2.4%), B2 (17%) and D (48.8%)	A (39%), B1(2.4%), B2 (36.3%) and D (22%)
MLST analysis	ST648, ST405, ST10, ST354 and ST1574	ST131, ST405, ST10 and ST38
Co-resistance to non ß-lactam antimicrobials **Tetracycline** **Sulfamethoxazole/trimethoprim** **Gentamicin** **Tobramycin** **Fluoroquinolones** **Fosfomycin** **All tested non ß-lactam antimicrobials**	 95.1% 90.2% 70.7% 92.7% 87.8% 0.0% 53.7%	 61% 73.2% 48.8% 58.5% 85.4% 12.2% 41.5%
Co-transferable resistance to non ß-lactam antimicrobials **Tetracycline** **Sulfamethoxazole/trimethoprim** **Gentamicin** **Tobramycin**	 60% 30% 40% 50%	 41.7% 58.7% 58.7% 41.7%
Plasmid analysis **(I) plasmid replicon type** **(II)The size range of large plasmids** **(III)The most predominant conjugative *bla*$_{CTX-M}$ carrying plasmid**	 FIA (12.2%), FIB (12.2%), FIA+FIB (68.3%) and A/C (4.9%) 97 kb -242 kb 97 kb,170 kb	 FIA (4.9%), FIB (17%), FIA+FIB (63.4%), N (7.3%) and I1 (2.4%) 48 kb -170 kb 170 kb, 97 kb

4.0. Discussion

Extended-spectrum ß-lactamases (ESBLs) are a major global problem in the clinical and community setting. The prevalence of ESBL among *Enterobacteriaceae* is variable from country to country and even among two different institutions in the same country and continuously changing over the time. The available data on ESBL in Egypt till yet do not cope with the expected consequences of the continuing evolution and the tremendously increasing prevalence of the different ESBL types. Since *Klebsiella pneumoniae* and *E. coli* are the most predominant ESBL-producing members among *Enterobacteriaceae* especially in the clinical setting, it is more beneficial to estimate the prevalence of ESBL-producer among them in Egyptian clinical setting. Although it is important to determine the ESBL prevalence, it is not sufficient to understand the reasons of evolution and the driving force behind their dissemination in Egypt. To do so, we have to determine the molecular epidemiological characters of ESBL-encoding *Enterobacteriaceae* at the cellular (host) and the plasmid level. Since ESBL production is a global rather than national or local problem, it is more intriguing to know the reason behind their spread in the different countries that follow variable antibiotic policies and hence different antibiotic selective pressure in addition to the other factors like geographical location which constitutes strong motive to investigate for the presence of an epidemiologic link among these countries. These are the main reasons which motivated us to establish this comparative study among ESBL-producing clinical isolates obtained from Egyptian University Hospitals and one German University Hospital namely, Giessen University Hospital. Since it is not feasible to compare the total enterobacterial isolates obtained from Egypt (which belong to different enterobacterial members and differ in their content of ESBL-encoding genes) with corresponding German isolates, this comparative study is restricted to isolates containing *E. coli* strains harbouring certain ESBL-type (CTX-M either alone or in combination with TEM) which collected simultaneously in both countries (from October 2008 till March 2009).

The main objective of this comparative study is to estimate the presence of epidemiological links such as identical strains and/or epidemic plasmids in common between Egyptian and German CTX-M-producing clinical *E. coli* isolates and if any, to which extent they are implicated in the dissemination CTX-M-mediated resistance in the clinical setting of both countries. Moreover, this study examine if the spread of

bla$_{CTX-M}$ is attributed to an epidemic *E. coli* clone by vertical spread, a particular epidemic plasmid, diverse epidemic plasmids by horizontal spread circulating in the clinical setting in each country or attributed to both mechanisms.

4.1. Prevalence of ESBL-producing isolates among enterobacterial isolates derived from Egyptian University Hospitals

Prevalence of ESBL-producing *Enterobacteriaceae* was 79.9% of the total 184 clinical isolates including 102 *Klebsiella pneumoniae* and 82 *E. coli* derived from two different University Hospitals in Egypt. This finding was higher than that reported earlier at the national, regional and the global levels (Bouchillon *et al*., 2004; Fam *et al.,* 2011**)**, indicating the persistent increase of ESBL-producing isolates among *Klebsiella pneumoniae* and *E. coli* populations and reflects the lack of the prudent usage of ß-lactam antimicrobials. The rate of ESBL production among the enterobacterial isolates derived from Egyptian Hospitals in this study was consistent with that recorded by Ahmed *et al*., (2009) but higher than that reported previously (65.8%, 38%, 16% and 38.5%) (Fam and El–Damarawy, 2008; El-Kholy *et al.,* 2003; Fam *et al.,* 2011; Bouchillon *et al*., 2004). The reported rate in this study was also higher than that reported in the other Middle-Eastern countries namely Lebanon, Saudi Arabia and Bahrain, respectively (18.2%, 18.6% and 22.6%) (Bouchillon *et al*., 2004; Bindayna *et al*., 2009).

In details, the prevalence of ESBL-producing *Klebsiella pneumoniae* in this study was higher than that reported earlier (55.3%, 18%, 37.9%, 55% and 24.3%) (Fam and El-Damarawy, 2008; Fam *et al.,* 2011; Bouchillon *et al*., 2004; Al-Agamy *et al*., 2009; Bindayna *et al*., 2009). On the other hand, the prevalence of ESBL-producing *E. coli* in this study was higher than that reported earlier (57%, 19%, 42% and 52.2%) (Fam and El-Damarawy, 2008; Fam *et al.,* 2011; Bouchillon *et al*., 2004; Bindayna *et al*., 2009).

The difference in the prevalence of ESBL-producer among the enterobacterial isolates from study to study may be attributed to the sensitivity of the method used for detection, the type and source of the clinical specimens (specimen type and hospital ward) from which these strains were isolated, the antibiotic administration policy (consequently the antibiotic selective pressure) in the medical institute the proportion of each enterobacterial member since the ESBL production bias to certain members than the other (Paterson and Bonomo, 2005; Sturenburg and Mack, 2003)

4.1.1. Determination of the types of ß-lactamases among clinical enterobacterial isolates derived from Egyptian University Hospitals

In this study, we have identified *bla*$_{TEM}$ as the most frequent ß-lactamase-encoding gene being detected in 81.6% of the total ESBL-producing isolates, 83.3% of ESBL-producing *K. pneumoniae* and 79.4% of ESBL-producing *E. coli*. *Bla*$_{CTX-M}$ was detected in 63.3% of the total ESBL-producers, 59.5% of ESBL-producing *Klebsiella pneumoniae* and 68.3% of ESBL-producing *E. coli*. *Bla*$_{SHV}$ was detected in 25.2% of the total ESBL-producing isolates, 39.3% of ESBL-producing *K. pneumoniae* and 6.3% of ESBL-producing *E. coli*.

Prevalence of *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ among the total *Enterobacteriaceae* in this study were higher than what recorded earlier for *bla*$_{TEM}$ (61.1%), *bla*$_{SHV}$ (55.6%) and *bla*$_{CTX-M}$ (38.7%) (Ahmed *et al*., 2009).

The prevalence of *bla*$_{TEM}$ among *K. pneumoniae* isolates in our study was concordant with that reported earlier (84.1%), but the prevalence of *bla*$_{CTX-M}$ in our study was higher than that reported earlier (34.1%), while the prevalence of *bla*$_{SHV}$ in our study was lower than that reported previously (97.3%) (Al-Agamy *et al.,* 2009).

Lower prevalence (60.8%) of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ among *E. coli* isolates than that detected in our study was reported earlier (Mohamed Al -Agamy *et al.,* 2006).

The presence of single ß-lactamase-encoding gene was detected in 59 (40%) of ESBL-producing clinical isolates including 28 *K. pneumoniae* and 31 *E. coli* isolates. The presence of two different types of ß-lactamase-encoding genes in the same host was detected in 73 (49.7%) of ESBL-producers including 43 *K. pneumoniae* and 30 *E. coli*.

The association of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ was the dominant combination and it was detected in 52 (35.4%) of the total ESBL-producers with a slight bias towards *E. coli* 28 (19%) strains versus 24 (16.2%) *K. pneumoniae*, while *bla*$_{SHV}$ and *bla*$_{TEM}$ association was in the second order and was detected in 14 (9.9%) of the total ESBL-producers, the majority of them (12) were *K. pneumoniae*. The association of the *bla*$_{CTX-M}$ and *bla*$_{SHV}$ was the least prevalent combination, being detected only in seven *K. pneumoniae* isolates. The presence of three ß-lactamase encoding genes in association was detected in 15 (10.2%) of ESBL-producing isolates including 13 *K. pneumoniae* and two *E. coli* isolates. The rate of co-existence of two different ß-lactamase encoding genes among *Enterobacteriaceae* in our study was higher than that reported by Ahmed *et al*. (2009), while the rate co-existence of *bla*$_{TEM}$ + *bla*$_{SHV}$ + *bla*$_{CTX-M}$ that reported in this study was

higher than our finding (Ahmed *et al*., 2009). The Occurrence of *bla*$_{SHV}$ gene either alone, in association with *bla*$_{TEM}$ or together with *bla*$_{CTX-M}$ and *bla*$_{TEM}$ was detected in 6.8%, 56.8% and 25% , respectively among ESBL-producing *K. pneumoniae* in a previous study (Al-Agamy *et al.,* 2009). The first finding was lower, while the last two findings were higher than that detected in our study.

The dominance of *bla*$_{CTX-M}$ gene either alone or in association with *bla*$_{TEM}$ among *E. coli* isolates was reported previously in Egyptian clinical setting **(**Fam and El-Damarawy, 2008; Mohamed Al-Agamy *et al.,* 2006). The previous findings demonstrate the increasing tendency of *K. pneumoniae* and *E. coli* to accumulate ß-lactamase resistance determinants which is higher among *K. pneumoniae* isolates than *E. coli* isolates. The previous finding ascertain also the predilection of *E. coli* to aggregate *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes together as well as the predilection of *K. pneumoniae* to associate either *bla*$_{CTX-M}$ together with *bla*$_{TEM}$ or *bla*$_{TEM}$ together with *bla*$_{SHV}$. Existence of *bla*$_{SHV}$ among *E. coli* isolates is seldom encountered.

Presence of a single ß-lactamase-encoding gene in an ESBL-producing isolate suggests that the relevant ß-lactamase is an extended one. Therefore, it is responsible for the resistance of their host to the third generation cephalosporins, but if two ß-lactamase co-exist in the same host either both or at least one of them are/is an ESBL. In case of presence of multiple ß-lactamase genes in the same bacterial host, sequencing of their PCR amplicons is necessary to identify the allele types of these ß-lactamase-encoding genes to classify each of them as an extended or a narrow spectrum-ß-lactamase (Al-Agamy *et al.,* 2009).

4.2. Screening for *bla*$_{CTX-M}$ and determination of the associated ß-lactamase types among clinical *E. coli* isolates derived from German University Hospital

PCR assay of 64 enterobacterial (entirely *E. coli*) isolates derived from Giessen University Hospital in the period from October 2008 to March 2009 revealed that 41 (64%) of the isolates were positive for *bla*$_{CTX-M}$ and 27(42.2%) were positive for *bla*$_{TEM}$ but none of them were positive for *bla*$_{SHV}$. Analysis of the multiplicity of ß-lactamase-encoding genes among the isolates revealed that, two thirds of CTX-M-encoding isolates were also encoding TEM, while the rest (34%) were encoding CTX-M alone. The prevalence rate of *bla*$_{CTX-M}$ in this study was slightly lower than that was recorded

previously in the same medical institution (77.7%) (Mshana *et al.*, 2009), but it still the dominant ESBL-encoding gene in this medical institution. Higher *bla*$_{CTX-M}$ prevalence rates 80%, 95.5% and 92% among ESBL-producing *E. coli* have previously been reported in Dresden, Germany and Ulm, Germany as well as in Stockholm, Sweden (Schmitt *et al*., 2007; Cullik *et al*., 2010; Fang *et al*., 2008).

The prevalence rate of *bla*$_{TEM}$ in our study was lower than those have previously been reported in other parts of Germany and Sweden (50%, 63.6% and 63%) (Schmitt *et al*., 2007; Cullik *et al*., 2010; Fang *et al*., 2008). *Bla*$_{SHV}$ has not been detected among CTX-M-producing isolates. However it has been detected at rates of 20%, 4.5% and 6% among the other ESBL-producers (Schmitt *et al*., 2007; Cullik *et al*., 2010; Fang *et al*., 2008). The rate of co-existence of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ among CTX-M-producing *E. coli* was twice as much as the rate of occurrence of *bla*$_{CTX-M}$ alone in this study and it was higher than the rates of co-existence of *bla*$_{CTX-M}$ and *bla*$_{TEM}$ among CTX-M-producing *E. coli* that was reported earlier (50% and 59%) (Schmitt *et al*., 2007; Cullik *et al*., 2010).

4.3. Characterization of CTX-M-producing *E. coli* isolates derived from Egyptian and German University Hospitals

4.3.1. Sequence based allele typing for *bla*$_{CTX-M}$

The sequence-based allele typing of *bla*$_{CTX-M}$ revealed that *bla*$_{CTX-M-15}$ was the only detected *bla*$_{CTX-M}$ allele type among clinical *E. coli* isolates obtained from Egypt. This finding is identical to that reported earlier (Fam and El –Damarawy, 2008; Fam *et al.*, 2011). The detection of other *bla*$_{CTX-M}$ alleles like *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-27}$ (both are members of *bla*$_{CTX-M-9}$ like group) in addition to predominant of *bla*$_{CTX-M-15}$ among Egyptian clinical *E. coli* isolates has also been reported (Mohamed Al-Agamy *et al.*, 2006; Khalaf *et al.*, 2009).

Bla$_{CTX-M-15}$ was also detected in 34 (82.9%) among the clinical *E. coli* isolates obtained from Germany representing the dominant *bla*$_{CTX-M}$ allele.

Bla$_{CTX-M-1}$ was detected in five isolates while only one isolate encoding *bla*$_{CTX-M-3}$ and another isolate encoding *bla*$_{CTX-M-61}$ have been detected among *E. coli* isolates obtained from German University Hospital. The dominance of *bla*$_{CTX-M-15}$ among ESBL-producing *E. coli* has been reported earlier in the same medical institute in Giessen,

Germany (Mshana *et al*., 2009) who detected *bla*$_{\text{CTX-M-15}}$ allele in 57% followed by *bla*$_{\text{CTX-M-1}}$ (11.1%) and *bla*$_{\text{CTX-M-3}}$ (4.6%) but did not detect *bla*$_{\text{CTX-M-61}}$ (*bla*$_{\text{CTX-M-1}}$ like group member) which was detected in one isolate in our study. *Bla*$_{\text{CTX-M-61}}$ has not been reported in Germany before, but it was previously reported in France (Brasme *et al.*, 2007). The data of sequence analysis indicated that, this allele is a single point mutant of *bla*$_{\text{CTX-M-1}}$. This mutation is the most probable explanation for its occurrence among the clinical CTX-M-producing *E. coli* isolates in this study. Also, presence an epidemiological link with the previously detected French isolate should be taken in consideration. The lower prevalence of *bla*$_{\text{CTX-M-1}}$ as well as the rare occurrence of *bla*$_{\text{CTX-M-3}}$ in our study is concordant with that reported earlier (Mshana *et al*., 2009). It is noteworthy that all the detected *bla*$_{\text{CTX-M}}$ alleles in the previous study (Mshana *et al*., 2009) as well as our study belong to only one CTX-M cluster namely *bla*$_{\text{CTX-M-1}}$ like group. Previous studies carried out by other groups in Germany reported the detection of *bla*$_{\text{CTX-M-9}}$ like group members mainly *bla*$_{\text{CTX-M-14}}$ and to lesser extent *bla*$_{\text{CTX-M-9}}$ in addition to the predominant *bla*$_{\text{CTX-M-1}}$ like group members mainly *bla*$_{\text{CTX-M-15}}$ (Cullik *et al.*, 2010; Schmitt *et al*., 2007).

4.3.2. Antibiotic susceptibility

All clinical CTX-M-producing isolates derived from Egyptian Hospitals were resistant to ampicillin, cefazolin, cefpodoxime, cefuroxime, cephalexin, cefotaxime, ceftriaxone, cefepime and ceftazidime (some isolates showed only intermediate resistance against the last two antimicrobials) using disk susceptibility test. This finding has been observed also in an earlier study (Khalaf *et al*., 2009).

All isolates remained susceptible to imipenem, meropenem and fosfomycin. The most effective ß-lactam/ß-lactamase inhibitor combination was piperacillin/tazobactam (68.8% of the isolates were susceptible) followed by amoxicillin/clavulanic acid combination (only 34.1% of the isolates were susceptible) while ampicillin/sulbactam combination was non-effective (92.7% of isolates were resistant). This reflects the greater activity of piperacillin/ tazobactam combination which is due to the fact that CTX-M enzymes are better inhibited by tazobactam than by clavulanate (Edelstein *et al*., 2003). Unfortunately, nearly one third of the isolates was resistant to piperacillin/tazobactam and consequently will limit the use of this combination in the treatment of the infections that are caused by CTX-M-producing *E. coli*. The high rate

of resistance to amoxicillin/clavulanate is not surprising and has commonly been detected among CTX-M-producers than among the producers of other ESBL-types as reported previously (Edelstein *et al*., 2003). The rate of resistance to ß-lactam/ß-lactamase inhibitor in this study was nearly similar to that recorded earlier (Edelstein *et al.*, 2003), but lower than that recorded by Diaz *et al.* (2010), who reported 88.6% of the isolate were susceptible to piperacillin/tazobactam, while 69.4% were susceptible to amoxicillin/clavulanates. Nearly the same rate of susceptibility (70% of the tested isolates) to piperacillin/tazobactam combination and complete failure of clavulanate to retain the activity of amoxicillin against ESBL-producers derived from Egyptian medical institute has been reported earlier (Fam and El-Damarawy, 2008). Lower resistance rates (87%) to ampicillin/sulbactam, (77%) to amoxicillin/clavulanic acid and higher level of resistance (62%) to piperacillin/ tazobactam combination than our finding showed by hospitals and community derived *E. coli* isolates in medical institute in Cairo, Egypt (Fam *et al.*, 2011).

The rates of resistance that have been demonstrated by clinical *E. coli* isolates derived from Egyptian Hospitals against the non-ß-lactam antimicrobials were 95% against tetracycline, 87.8% against quinolones 92.7% against tobramycin and 70.7% against gentamicin reflecting the high resistance rates against the different classes of antimicrobials and the limited therapeutic option for the treatment of the infections that are caused by these CTX-M-producing *E. coli*. The rate of resistance against quinolones in this study was similar to that reported previously (Fam and El-Damarawy, 2008), but greatly higher than that recorded by Mohamed Al-Agamy *et al*. (41.3%) (2006). Strong relation between resistance to quinolones and CTX-M-15 production has been documented. The selective association of $bla_{\text{CTX–M}}$ and plasmid mediated quinolone resistance determinants like the association of *qnr*B with $bla_{\text{CTX-M-15}}$ and *qnr*A with $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-9}}$. Moreover, higher association of $bla_{\text{CTX–M}}$ with *aac(6')-Ib-cr* than *qnr* has been observed (Naseer and Sundsfjord, 2011). The rate of resistance against tetracycline in this study was consistent to that recorded by Mohamed Al-Agamy *et al*. (2006). The recorded rate of resistance to sulfamethoxazole/ trimethoprim in our study was slightly lower than 97.8% that had been reported by Mohamed Al-Agamy *et al*. (2006) and higher than that had been reported in the other studies (Nazik *et al*., 2011; Fam and El-Damarawy, 2008). The rates of resistance against aminoglycosides, in general, in our study were higher than the previously reported rates

(Mohamed Al-Agamy *et al*., 2006; Nazik *et al* 2011). On the other hand, the clinical *E. coli* isolates derived from German University Hospital were resistant to the first, second and third generation cephalosporins and the great majority of them (CTX-M-15-producers) were resistant to ceftazidime and cefepime. All strains were still highly susceptible to imipenem and meropenem. The most striking finding was the detection of resistance to fosfomycin in five isolates (12.2%). The emergence of resistance to fosfomycin in CTX-M-15-producing uropathogenic *E. coli* O_{25}: H_4 / ST131 phylogroup B2 has been described in a study carried out in Spain (Oteo *et al*., 2009 B) who recorded an increase in fosomycin resistance from 2.2% in 2004 to 21.7% in 2008 as a consequence for the extensive use of this antimicrobial in the treatment of urinary tract infections that were caused by ESBL-producing bacteria. The residence of fosfomycin resistance-encoding genes named *fos*A2 and *fos*A3 on a transferable plasmid hosted by CTX-M-producing clinical *E. coli* isolates has been reported (Wachino *et al*., 2010). Therefore, it is only a matter of time then we may lose a valuable therapeutic option for the treatment of urinary tract infections that are caused by ESBL-producing organisms. Another important finding among German clinical isolates was the high rate of resistance to piperacillin/tazobactam combination which is the most effective penicillin/ ß-lactamase combination. The rates of resistance to non ß-lactam antimicrobials were 85.4% against quinolones, 73.2% against sulfamethoxazole/trimethoprim, 60.9% against tetracycline, 58.5% against tobramycin and 48.8% against gentamicin. These finding was in general agreement with those reported earlier (Mshana *et al.,* 2009) but lower than that reported by Coque *et al*. who reported the rate of resistance to tetracycline (84%), gentamicin (82%), tobramycin (89%) and higher than that reported for sulfonamide (61%), trimethoprim (58%) and (61%) for quinolones (Coque *et al*., 2008 B).

The rates of resistance among CTX-M-producing *E. coli* in German University Hospital to the different non ß-lactam antimicrobials was markedly lower than the corresponding rates in Egyptian University Hospitals, but still considerably high and reflects the predominance of multiple drug resistant clinical isolates and hence extremely limits the therapeutic options.

4.3.3. Pulsed-field gel electrophoresis (PFGE)-based genotyping

Analysis of the total typable 78 isolates derived from Egyptian and German University Hospitals revealed 73 different genotypes (pulsotypes) distributed onto 12 clusters of related isolates which have ≥80% similarity and 8 single unique PFGE genotypes. The distribution of the isolates on multiple clusters, the presence of multiple single unique types, presence the different phylogenetic types, the non-bias of any of the different *bla*$_{\text{CTX-M}}$ alleles to certain cluster or clusters reflect the heterogeneity and the genetic diversity among isolates and strongly support the role of horizontal transfer and mobile genetic elements as the major driving forces behind the spread of *bla*$_{\text{CTX-M}}$ in the clinical setting of both countries. On the other hand, very limited degree of clonality has been detected in five sporadic cases reflected by the presence of five pairs of PFGE-genotypes each pair composed of two indistinguishable PFGE-patterns (100% similarity), two pairs of these patterns represents two pairs of clinical isolates namely 539, 564 in the cluster E and 537, 538 in cluster C all were isolated from urine samples in Urology Department Assiut University Hospital, Egypt. The third pair (in cluster H) represent the isolates 479, 500 both were isolated from T-tube aspirates in GIT ward in El-Mansoura University Hospital, Egypt. These three pairs represents sporadic cases of nosocomial infection in which intra-hospital spread of a clone between two patients admitted to the same ward most likely acquired from medical ward environment as reported earlier (Ahmed *et al*., 2009), but fortunately it did not reach the epidemic level. The fourth pair represents the strains 612 and 619 but in this case, the strains were isolated from different wards, 612 was isolated from nasal swab in the internal surgery ward while 619 was isolated from groin swab recovered from the Medical Clinic. Both wards belong to the Giessen University Hospital, Germany. The two wards are distant to each other excluding inter-wards spread. Therefore, the most probable explanation for such case is the introduction of this clone from the community or transfer via common medical staff personnel. The lack of predominant epidemic clone, despite the occurrence of few sporadic clonal pairs, circulating in the clinical setting of both countries excludes or greatly minimizes the role of clonal expansion in the dissemination of *bla*$_{\text{CTX-M}}$ in the clinical setting in both countries.

The most surprising finding in this concern was the presence of two identical PFGE-patterns in the cluster F representing the isolates 476 and 649, the first was isolated from ascitic fluid specimen recovered from Tropical Medicine Ward at El-Mansoura

University Hospital, Egypt, while the other 649 was isolated from abdominal swab specimen obtained from Medical Clinic, Giessen University Hospital, Germany. Further epidemiological investigation of these isolates revealed that they belonged to phylogenetic type A, the sequence type ST10, and both of them were harboring identical plasmids (FIA, FIB multi-replicon conjugative plasmid of ~170 kb plasmid). The two isolates showed the same antibiotic resistance pattern and their corresponding transconjugants have identical resistance profiles. Unfortunately, the sources of importation these clonal isolates between both countries could not be back-traced in a retrospective manner in addition to insufficiency of the available clinical data to completely unravel the epidemiological link between them. Therefore, it is difficult to ascertain the mode of transfer of this clone or even the direction of transfer (Egypt to Germany or vice versa). The sources and the routes of transportation of such antibiotic resistance bacteria are rarely identified and the points of their introduction almost often pass undetected. But we can postulate, in the light of the previous and the current studies, putative routs for the inter-continental transfer of this clone which include: foreign travel, trans-continental birds' migration and imported food products with special concern for meat and agricultural products.

Foreign travel to area with a higher prevalence of ESBL-producing strains like Indian subcontinent, Middle East and Africa has been suggested to be the major risk factor for acquisition of ESBL-producing *Enterobacteriaceae* (Tängdén *et al.,* 2010). In our situation, colonization of the returning travelers is the most plausible explanation for transfer of this clone. Taking in consideration the high number of travelers between Egypt and Germany for various purpose including leisure tourism; medical tourism; study and business, foreign travel is considered as the main gate for such trans-continental dissemination of antibiotic resistant bacteria clone. Medical tourists are the highly suspected class of travelers for international transfer of such resistant clone (Rogers *et al.,* 2011) supported in our case by the detection of this clone in the clinical setting and by keeping in mind that Germany is one of the most favorable destinations for medical tourists. Another putative route for inter-country transfer of such antibiotic resistant clone is the trans-continental wild birds' migration. Many bird species, including those have been identified as sources for CTX-M-producing *E. coli*, have migratory behavior and demonstrate vast mobility crossing the continents and represent potential risk to create an epidemic foci over remote distances along their migration routes (Bonnedahl, 2011). Therefore, wild birds' migration contributes to the

dissemination of resistance over the globe probably due to the omnipresent nature of their feces. Lack of sewage system, in contrast to human populations, and the direct dropping of bird feces expose the environment as well as human and animal population to substantial public health hazard (Guenther *et al.,* 2011). The detection of ST648 among CTX-M-producing *E. coli* derived from Egyptian University Hospitals in addition to detection of this sequence type among CTX-M-producing *E. coli* recovered from wild birds in Germany as well as Miami Beach, Florida (Guenther *et al.,* 2010; Poirel *et al.,* 2012) is another finding in our study that support the epidemiological role of birds migration in the trans-continental dissemination of antibiotic resistant bacterial host. Food-borne transmission of such antibiotic resistant clone is not unlikely due to the fact that considerable proportion of fruits and vegetables that are retailed in the developed countries are imported from developing ones. The imported food products from the developing countries often contaminated with antibiotic resistant bacteria and frequently consumed uncooked. Therefore, it represents a source for infection or colonization with antibiotic resistant bacteria (Okeke and Adelman, 2001). CTX-M-producing *E. coli* are likely to be globally present in chickens (Randall *et al*., 2010). It has been recovered from other food producing animals and has been detected in high prevalence in the retailed meat (Simões *et al.,* 2010).

4.3.4. Phylogenetic typing

PCR-based phylogenetic typing of CTX-M-producing *E. coli* isolates derived from Egyptian University Hospitals revealed that nearly half of the isolates was belonging to the extraintestinal D phylogenetic type, while only 17% were belonging to the extraintestinal pathogenic phylogenetic type B2 and a surprisingly high percentage (31.7%) of these isolates were belonging to the human commensal phylogenetic type A. The animal commensal phylogenetic type B1 represented by only one isolate. This finding contradicts the previously reported dominance of B2 phylogenetic type among CTX-M-producers (Karisik *et al*., 2008; Carattoli *et al.,* 2008) and reflects the increase in the prevalence of the phylogenetic type D and to lesser extent the phylogenetic type A among CTX-M-producing *E. coli* isolates on the expense of the phylogenetic type B2 in the clinical setting in Egypt. The phylogenetic diversity and the increasing prevalence of D and A phylogenetic types among CTX-M-producing *E. coli* isolates in the clinical setting in Egypt suggests horizontal transfer of $bla_{\text{CTX-M-15}}$ encoding plasmids from B2

to either D or A and mutually between D and A and also indicates the maintenance and stability of these conjugative plasmids regardless the genetic background of the host (Sidjabat *et al.,* 2009). However, the role of the host in the acquisition and dissemination of *bla*$_{\text{CTX-M}}$ genes could never be neglected or even underestimated. Deschamps *et al.* detected strong epidemiological relation between the phylogenetic type D and the presence of *bla*$_{\text{CTX-M}}$ genes (Deschamps *et al.,* 2009). Moreover, the preference of a certain *bla*$_{\text{CTX-M}}$ allele type for a specific phylogenetic type or types like the preference of *bla*$_{\text{CTX-M-15}}$ for B2 and D phylogenetic types as well as the preference of *bla*$_{\text{CTX-M-9}}$ for D and A phylogenetic types has been reported (Naseer and sundsfjord, 2011) which may explain the dominance of the phylogenetic type D among CTX-M-producing isolates in the clinical setting in Egypt.

Occurrence of one isolate (2.4%) belonging to phylogenetic type B1 indicates the sporadic occurrence of this phylogenetic type among human clinical isolates. High rate (40%) of the phylogenetic type A among CTX-M-producing *E. coli* derived from a medical institute in Cairo, Egypt relative to B2 (26%) and D (32%) has also been reported (Fam *et al*., 2011). Higher prevalence (43.5%) of B2 phylogenetic type than that has been found in our study and lower prevalence (39%) and (17.4%) of the phylogenetic type D and A respectively, while lack of the animal commensal B1 phylogenetic type among CTX-M-producing isolates have been reported (Naseer *et al.,* 2009). In contrast, the majority of isolates derived from German University Hospital were distributed on B2 (36.6%) and A (39%) phylogenetic type while D phylogenetic type represent only 22% of the isolates in addition to one isolate belonged to B1 phylogenetic type. Comparison of these findings with that recorded previously in the same medical institution by Mshana *et al.* who detected the occurrence of B2, A, D and B1 phylogenetic types at prevalence rates 44.9%, 31.7%, 15.8% and 7.9%, respectively highlights the current increase of A phylogenetic type on the expense of B2, while D and B1 phylogenetic types still retaining their previous relative orders among CTX-M-producing isolates (Mshana *et al.,* 2009). Replacement of B2 by A phylogenetic type has occurred but at a higher degree in a medical center in Western Pennsylvania (Sidjabat *et al.,* 2009). Higher prevalence rates of B2 (50%) and D (31.8%) phylogenetic types among CTX-M-producing isolates, derived from another University Hospital in Germany, and lower prevalence of phylogenetic types A (13.6%) than that recorded in our study has been reported (Cullik *et al*., 2010). Therefore, the relative

contributions of the phylogenetic type populations in CTX-M-producing *E. coli* in the clinical setting differ significantly from country to country, from city to another in the same country and most likely from a medical institution to another within the same city as well as from year to year in the same medical institution. The most plausible explanation for such geographic and temporal variations is the horizontal transfer of $bla_{\text{CTX-M}}$-encoding plasmids among the different phylogenetic types which in turn depends on the differential interaction between the conjugative plasmid and the host background of each phylogenetic type.

4.3.5. Multilocus sequence typing (MLST)

In this study, five different sequence types have been identified among seven randomly selected CTX-M-producing clinical *E. coli* isolates derived from Egyptian University Hospitals namely ST648, ST405, ST354, ST10 and ST1574. On the other hand, four sequence types have been identified among six CTX-M-producing *E. coli* isolates derived from German University Hospital including ST131, ST405, ST38 and ST10. All the detected sequence types in this study have been detected in association with $bla_{\text{CTX-M}}$ in other parts of the world (Coque *et al*., 2008 B). ST648 that has previously been identified in the clinical setting in Germany and Tanzania (Cullik *et al.,* 2010; Mshana *et al.,* 2011), also it has recently been isolated from wild birds in Germany and Miami Beach, Florida, US (Guenther *et al.,* 2010; Poirel *et al.*, 2012), highlighting emergence and global dissemination of this epidemic clone and providing a circumstantial evidence for the epidemiological role of the wild bird in the global dissemination of this sequence type. ST405 has previously been detected among CTX-M-producing clinical *E. coli* isolates in Egypt and Germany (Fam *et al*., 2011; Cullik *et al.*, 2010). Also, it has been identified among isolates derived from Switzerland, Kuwait and Spain (Coque *et al*., 2008 B). STC405 uropathogenic *E. coli* was one of the major clones that were implicated in the world wide dissemination of $bla_{\text{CTX-M -15}}$ **(**Naseer *et al*., 2009; Coque *et al*., 2008 B). ST10 was represented in this study in two clonal isolates (demonstrated identical PFGE-patterns) derived from Egyptian and German University Hospitals suggesting emergence of this clone in one of them followed by importation of it to the other. STC10 has not been reported in Germany before but it has been previously reported in Spain (Oteo *et al.,* 2009A) and recently in Egypt (Fam *et al*., 2011). It has also been identified among CTX-M-producing *E. coli* isolates that was

recovered from wild birds in Miami Beach, Florida (Poirel *et al.,* 2012). ST354 has previously been reported among CTX-M-producing isolates recovered from Portugal, Spain and France (Coque *et al*., 2008 B). ST1574 was detected in this study among the isolates derived from Egyptian University Hospitals. This ST is not of a common occurrence among CTX-M-producing *E. coli* isolates. However, it has been previously reported in the clinical setting in Germany (Cullik *et al.,* 2010) but it was associated with $bla_{\text{CTX-M-3}}$ and belonging to the phylogenetic type A in contrast to our finding in which ST1574 was belonging to the phylogenetic type D and was encoding $bla_{\text{CTX-M-15}}$ highlighting the diversification of the genetic background and the variation of $bla_{\text{CTX-M}}$ allele types among the populations of this ST and suggests the local emergence and evolution of these clones.

There are many previous studies reported the implication of ST131 phylogenetic type B2 clone in the global dissemination of $bla_{\text{CTX-M-15}}$ (Coque *et al*., 2008 B; Naseer *et al*., 2009). ST38 has been reported previously (Naseer *et al*., 2009) also it has recently been reported in Egypt and Tanzania (Fam *et al*., 2011; Mshana *et al.,* 2011). Moreover, ST648, ST10, ST38 and ST405 have been identified among CTX-M-producing *E. coli* isolates recovered from wild birds suggesting the epidemiological role of wild birds in the global dissemination of these clones **(**Guenther *et al.,* 2010; Poirel *et al.,* 2012).

Presence of multiple sequence types in Egyptian and German University Hospitals indicate the genetic diversity and the heterogeneity of CTX-M-producing *E. coli* in the clinical setting in both countries and highlights the implication of multiple, diverse, epidemic clones rather than of single epidemic clone in dissemination of $bla_{\text{CTX-M}}$. Detection of the same conjugative $bla_{\text{CTX-M}}$-encoding plasmid (~97 kb FIA-FIB) in diverse isolates which were belonging to different sequence types (ST354 and ST648 derived from Egyptian Hospitals as well as ST405 and ST131 derived from German Hospital) as well as the presence of the same plasmid in non-identical strains (demonstrating non identical PFGE patterns) of the same sequence type (ST648) in this study represents an un-equivocal molecular evidence for the horizontal spread of this plasmid as the main mechanism for the dissemination of $bla_{\text{CTX-M}}$ even within the previously reported epidemic clones in the clinical setting of both countries.

4.3.6. Conjugation and co-transfer of non ß-lactam resistance concomitant with ß-lactam resistance

Results of conjugation experiment for the randomly selected CTX-M-producing *E. coli* isolates in this study demonstrated their ability to transfer their bla_{CTX-M}-encoding plasmids by conjugation proving the conjugative nature of these plasmids. Therefore, ascertains the role of conjugation in the dissemination of bla_{CTX-M} in the clinical setting in Egypt and Germany. In addition to the resistance to ß-lactam, most (68.2%) of the transconjugants demonstrated additional resistance to one or more of the tested non ß-lactam antimicrobials including tetracycline, sulfamethoxazole/trimethoprim (SXT), gentamicin and tobramycin, while none of the transconjugants showed phenotypic resistance to any of the tested quinolones members. On the other hand, 31.8% of the transconjugants did not show phenotypic resistance to any of the tested non ß-lactam antimicrobial agents, while they presented the ESBL resistance pattern to ß-lactam antimicrobials as their parent clinical isolates and were PCR positive for bla_{CTX-M}. This finding was expected from three (13.2%) transconjugants because their parent clinical isolates were susceptible to all tested non ß-lactam antimicrobials, while those of the remaining four (18.2%) transconjugants demonstrated resistance to all tested non ß-lactam antimicrobials.

The lack of co-transfer of resistance to non ß-lactam concomitantly with bla_{CTX-M} has been observed in the previous studies (Hopkins *et al*., 2006; Mendonca *et al.*, 2007; Dierikx *et al*., 2010). Suggesting that, the transferred bla_{CTX-M}-encoding conjugative plasmids either did not encode non ß-lactam resistance determinant at all or was encoding non ß-lactam resistance determinants which confer a resistance level too low to be detected phenotypically by the disc diffusion test. Co-transfer of tetracycline resistance observed in 60%, gentamicin 40%, sulfamethoxazole/ trimethoprim 30% and tobramycin 50% of isolates derived from Egyptian University Hospitals, while 41.7%, 58.7%, 58.7% and 41.7% of isolates derived from German University Hospital transferred resistance to tetracycline, gentamicin, sulfamethoxazole/trimethoprim and tobramycin respectively. Therefore, German Hospital-derived isolates demonstrated higher potential to transfer sulfamethoxazole/trimethoprim and gentamicin resistance than Egyptian isolates, while the reverse is true in the case of tetracycline and tobramycin. The rates of non ß-lactam resistance transfer in this study were higher than that reported earlier **(**Cullik *et al*., 2010). The comparison between our finding with that

recorded by Mshana *et al.* revealed that the rate of co-transfer of resistance trait to tetracycline and sulfamethoxazole/trimethoprim among German University Hospital-derived isolates in our study was lower than that of the Mshana study, while the rate of co-transfer of resistance to gentamicin was higher in our study than that has been recorded (Mshana *et al*., 2009).

Transfer of resistance to tetracycline, sulfamethoxazole/trimethoprim, tobramycin and gentamicin has been reported previously in many studies (Hopkins *et al.*, 2006; Karisik *et al.*, 2006 and Dierikx *et al.*, 2010) confirming the prevalent residence of the determinants of resistance to non ß-lactam antimicrobials together with $bla_{\text{CTX-M}}$ on the same conjugative plasmid and subsequent co-transfer en bloc from a strain to another via trans-conjugation (Ahmed *et al*., 2009; Mabilat and Courvalin, 1990). The lack of transfer of resistance to quinolones in all transconjugants in our study is concordant with that reported earlier (Mshana *et al.*, 2009).

Although the resistance to quinolones was observed in most of CTX-M-producing clinical *E. coli* isolates, none of the transconjugants expressed phenotypic resistance to any of the tested quinolones members. Suggesting that, the resistance in these clinical isolates is due to chromosomally-encoded quinolones resistance determinants most probably mutation in quinolone resistance determining region of the genes *gyr*A, *gyr*B and *par*C-encoding gyraseA, gyraseB and topoisomerase enzymes. However, this did not exclude presence of quinolone resistance determinants in the transferred $bla_{\text{CTX-M}}$-encoding conjugative plasmid since some of the plasmid mediated quinolone resistance determinants, those confer low level resistance to fluoroquinolones, namely *aac(6')-Ib-cr* and *qnrs* are commonly encoded with $bla_{\text{CTX-M}}$ on the same plasmid (Harajly *et al*., 2010; Naseer and Sudsfjord, 2011). Harajly *et al.* observed also an intermediate level of resistance to ciprofloxacin in a transconjugant, the PCR amplification of the plasmid extract of which and its parent isolate confirmed the presence of both *aac(6')-Ib-cr* and *qnr* on plasmid of both strains (Harajly *et al*., 2010). Demonstration of low level (4-fold increase in MIC) of resistance rather than frank resistance to flouroquinolone in transconjugants that have accepted ß-lactam resistance as a result of transfer of plasmid encoding $bla_{\text{CTX-M}}$ together with plasmid mediated quinolones resistance determinant has previously been reported **(**Karisik *et al*., 2006).

4.3.7. Plasmid analysis and location of *bla*$_{\text{CTX-M}}$

Determination of the plasmid replicon type and the plasmid size together with the resistance gene variants and linking them greatly help to trace this plasmid and to detect its mobility from a bacterial host to another as well as the mobilization of a resistance-encoding gene among the different plasmids (Hopkins *et al.,* 2006). All CTX-M-producing *E. coli* isolates were typed according to their plasmid incompatibility group (Inc group/replicon type). The great majority (68.3%) and (63.4%) of the plasmids that were carried by the clinical isolates derived from Egyptian and German University Hospitals were positive for FIA and FIB multiple replicon type, 12.2% and 4.9% of the isolates were positive for FIA, while 12.2% and 17% were positive for FIB, respectively. Replicon F plasmids have been identified as one of the most frequent carrier of *bla*$_{\text{CTX-M}}$ and were commonly associated with *E. coli* species. In contrary to most of the previous studies which reported the association of *bla*$_{\text{CTX-M-15}}$ with the epidemic FII plasmid either alone or in combination with FIA, FIB in multi-replicon plasmid (Carattoli *et al,*. 2008 ; Coque *et al.,* 2008B), none of isolates in this study was positive for FII replicon type. Our finding was similar to that reported earlier (Mshana *et al.,* 2009) who reported the detection of FIA and FIB either as single replicon or together in multi-replicon plasmid carrying *bla*$_{\text{CTX-M-15}}$ in the same medical institute. Therefore, our finding ascertain the stand-still circulation of FIA-FIB multi-replicon plasmids and to lesser extent FIA or FIB single replicon plasmids among the clinical isolates derived from Giessen University Hospital, Germany. The first detection of *bla*$_{\text{CTX-M}}$ on FI replicon was reported by Gonullu *et al.* (2008**)**. High proportion (47%) of *bla*$_{\text{CTX-M-15}}$ carrying plasmids that were carried by *E. coli* isolates derived from a tertiary hospital in Tanzania was belonging to FI replicon (Mshana *et al*., 2011). These finding together with the predominance of Inc FIA-FIB plasmids among the isolates derived from Egyptian University Hospital suggest a partial shift from epidemic FII to FI multi-replicon plasmids as the major contributor in *bla*$_{\text{CTX-M-15}}$ dissemination. All FI (narrow-host-range) plasmids in this study were associated with *bla*$_{\text{CTX-M-15}}$ except one FIA-FIB multi-replicon plasmid which was associated with *bla*$_{\text{CTX-M-1}}$ and two FIB plasmids; one was associated with *bla*$_{\text{CTX-M-1}}$ and the other associated with *bla*$_{\text{CTX-M-61}}$.
In this study, the wide host range replicon type A/C plasmids were detected in two (4.9%) of Egyptian Hospital-derived isolates both of them were encoding *bla*$_{\text{CTX-M-15}}$.

The wide host range A/C plasmid is of a rare occurrence among *E. coli* isolates. Therefore, the detection of *bla*$_{\text{CTX-M-15}}$-encoding plasmid of this replicon type suggests the acquisition of this plasmid from other bacterial species and predicts further inter-species as well as intra-species transfer. However, the association of *bla*$_{\text{CTX-M-15}}$ with the wide host ranges A/C replicon type plasmid among *E. coli* isolates has been reported earlier (Marcade *et al.,* 2009; Hrabak *et al.,* 2009). Association of A/C plasmids with other *bla*$_{\text{CTX-M}}$ variants, most commonly with *bla*$_{\text{CTX-M-2}}$, has also been reported (Hopkins *et al*., 2008). Three (7.3%) and one (2.4%) of the isolates derived from German Hospital were carrying IncN and IncI1 plasmids, respectively. In our study, most *bla*$_{\text{CTX-M-1}}$ was associated with IncN plasmids. This finding was consistent with that reported previously (Marcade *et al.,* 2009; Cullik *et al*., 2010). Association of *bla*$_{\text{CTX-M-1}}$ with FI replicon type plasmid has been detected in two isolates derived from German University Hospital in this study. Also, it has previously been detected in the same medical institute (Mshana *et al.,* 2009), while *bla*$_{\text{CTX-M-3}}$ was most commonly associated with IncI1 (Naseer and Sundsfjord, 2011).

Bla$_{\text{CTX-M-15}}$ has been located in this study on plasmids of a size ~ 97 kb and ~ 170 kb in most of the clinical isolates derived from both Egyptian and German University Hospitals. Location of *bla*$_{\text{CTX-M-15}}$ on ~95 kb plasmid has been reported in CTX-M-15-producing multi-drug resistant enteroaggregative *E. coli* in the United Arab Emirates (Sonnevend *et al*., 2006). *Bla*$_{\text{CTX-M-15}}$ has been described on plasmid ranging in size from 7 to 430 kb which in the great majority of cases is a conjugative plasmid of a large size and encodes resistance to other antimicrobial classes (Naseer and Sundsfjord, 2011). Plasmid size ranges from 95 kb to 120 kb (Velasco *et al.,* 2007); from 85 kb to 160 kb **(**Coque *et al.* 2008 B); on ~70 kb and ~ 150 kb (Karisik *et al.,* 2006) ~70 kb (Abbassi *et al.,* 2008) from ~ 145 kb to ~194 kb (Mshana *et al.,* 2009) and from 50 kb to 291 kb (Mshana *et al*., 2011). *Bla*$_{\text{CTX-M-1}}$was located on ~ 48 kb replicon N plasmid in an isolate derived from German University Hospital, while *bla*$_{\text{CTX-M-15}}$ was located on ~ 145 kb replicon FIA-FIB plasmid in an isolate derived from Egyptian University Hospital as reported earlier (Naseer *et al.,* 2009). Although the presence of larger plasmids of a size ~ 242 kb and ~ 194 kb in our study, they did not hybridized with *bla*$_{\text{CTX-M}}$ specific probes in contrary to that detected previously (Mshana *et al.,* 2009).

The predominant occurrence of the conjugative FIA-FIB multi-replicon plasmids of a size ~97 kb and ~ 170 kb encoding *bla*$_{\text{CTX-M-15}}$ among unrelated clinical isolates in

Egypt and Germany is an indicator for the emergence of new probable trans-continental epidemic plasmids and strongly supports their role in the horizontal spread of *bla*$_{\text{CTX-M-15}}$ in the clinical setting in both countries.

It is noteworthy here that, the previously mentioned clone that has been shared between the clinical setting in Egypt and Germany was harboring one of these putative trans-continental conjugative plasmids (~170 kb FIA-FIB multi-replicon plasmid). Therefore, it is most likely to be implicated in the horizontal spread through conjugation and inevitably, additional vertical spread through its proliferation. Such two-dimensional pattern of spread of the CTX-M-mediated resistance resembles to great extent a growing snow ball and illustrates the impact of importation of a single resistant strain carrying an epidemic plasmid from one country to another in the creation of a new epidemic focus.

4.4. Concluding remarks

Bla$_{\text{CTX-M-15}}$ is the most common *bla*$_{\text{CTX-M}}$ allele among CTX-M-producing *E. coli* isolates derived from German University Hospital (Giessen University Hospital) and the only detected *bla*$_{\text{CTX-M}}$ allele among those derived from Egyptian Hospitals. The other detected *bla*$_{\text{CTX-M}}$ alleles among German isolates were *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-3}}$ and *bla*$_{\text{CTX-M-61}}$. The last *bla*$_{\text{CTX-M}}$ allele was detected for the first time in Germany. All the detected *bla*$_{\text{CTX-M}}$ alleles in this study belonged to the same evolutionary group i.e. *bla*$_{\text{CTX-M-1}}$ group.

Investigation of the genetic background of the CTX-M-producing *E. coli* has demonstrated a wide diversity among the isolates with an extremely limited degree of clonality. The most striking finding was the detection of a pair of clonal isolates one of them was derived from an Egyptian University Hospital while the other was derived from the German University Hospital. This clone may be imported via infection/colonization of a returned traveler, colonization of a migrating wild bird or contamination of an imported food product.

Phylogenetic typing and MLST confirmed the wide diversity among the isolates reflected by their distribution on the different phylogenetic types with the prevalence of D phylogenetic type among Egyptian isolates and the prevalence of A and B2 phylogenetic types among German ones as well as detection of many diverse sequence types namely ST131, ST405, ST38 and ST10 among German isolates and ST648, ST453, ST405, ST10 and ST1574 among Egyptian isolates.

Bla$_{CTX-M}$ genes were located on many diverse plasmids with different replicon types, different replicon combination and different plasmid size even within the same Inc group. Occurrence of *bla*$_{CTX-M-15}$ in genetically diverse *E. coli* isolates implied their link to mobile genetic elements i.e. conjugative plasmid that facilitates its dissemination among genetically diverse bacterial hosts. Similarly, occurrence of *bla*$_{CTX-M-15}$ on diverse plasmids indicated its successful transfer among the different plasmids implying its association with an insertion element. FIA-FIB was the dominant replicon type plasmids among the isolates derived from both countries and were associated mainly with *bla*$_{CTX-M-15}$. Most of them are large conjugative plasmids of different size. The plasmid of a size about 97 kb is the most common plasmid among Egyptian *E. coli* isolates while ~170 kb and ~97 kb sized plasmids are the most prevalent plasmids among German *E. coli* isolates.

Multiple occurrence of conjugative FIA-FIB multi-replicon plasmids of a size ~97 kb ~170 kb suggest the emergence of new putative trans-continental epidemic plasmids and strongly support their role in the horizontal spread of *bla*$_{CTX-M-15}$ in the clinical setting of both countries.

Spread of *bla*$_{CTX-M}$ is driven mainly by the horizontal transfer of IncFI group of plasmids among the clinical isolates in both countries which is inferred from the predominance of these plasmids among Egyptian and German isolates, the conjugative nature of these plasmids and their occurrence in genetically diverse hosts.

Strong epidemiological link between Egypt and Germany can be concluded from multiple molecular evidences including the presence of identical isolates, sequence types, probable epidemic plasmids in common in both countries. Surprisingly, the shared clone between Egypt and Germany was harboring one of the suggested trans-continental epidemic plasmids. Therefore, it is inevitable that it has been implicated in the horizontal as well as the vertical spread of *bla*$_{CTX-M-15}$-mediated resistance. Such two-dimensional pattern of spread of resistance highlights the impact of importation of a resistant clone harboring an epidemic plasmid from one country to another in the creation of a new epidemic focus.

The great majority of CTX-M-producing isolates showed multiple resistances to one or more classes of non ß-lactam antimicrobials like tetracycline, sulfamethoxazole/ trimethoprim, aminoglycosides and fluoroquinolones beside their resistance to ß- lactam antimicrobials. Resistance to one or more of these non ß-lactam antimicrobial, except

quinolones, has been observed to be co-transferred by conjugation together with *bla*$_{CTX-M}$ indicating the presence of their genetic determinants on the same plasmid that encodes *bla*$_{CTX-M}$ complicating the resistance problem and limiting the treatment options of infections that are caused by such isolates.

Prevalence of the most widely spread ß-lactamase families i.e. CTX-M, SHV and TEM family among Egyptian *K. pneumoniae* and *E. coli* clinical isolates are considerably high. TEM ß-lactamase was the most prevalent. This study ascertains also the previously reported worldwide distribution of CTX-M-encoding genes among *E. coli* and *K. pneumoniae*. Further follow-up studies are still needed to update the detection of prevalence, source, mechanism of spread, mode of transfer of ESBL determinants of resistance and/or ESBL-producing organisms for better insight that facilitates the establishment of strict public health measures and modified antimicrobial therapeutic strategies to eradicate or at least to hamper the spread of ESBL resistance determinants and ESBL-producers.

4.5. Limitations of this study and recommendations for further studies in this field

- This study is limited to only one medical institute in Germany and to only two medical institutes in Egypt. The need for a nation-wide surveillance in both countries especially in Egypt is strongly required.
- This study is confined to ESBL-producing isolates in the clinical setting. Therefore, further studies to investigate ESBL production in the community setting and ambient environment are also needed.
- The number of investigated isolates was comparatively low. So for more representative result, investigation of a larger number of isolates should be taken in the consideration.
- Characterization of ESBL-producing isolates was restricted to those producing CTX-M of *E. coli* species, so the future studies should be directed to the other ESBL-producing organisms as well as other ESBL-types.

Future studies are recommended to be devoted on the following issues

- Detailed plasmids characterization (i.e. sequencing) which could ascertain the identity of the postulated epidemic trans-continental plasmids.
- Investigation of the probable sources for importation of ESBL-producers among distant countries over the globe including
    - Investigation of volunteer travelers before travel and immediately as well as six months after the arrival for detection of acquisition and persistent colonization with an ESBL-producer.
    - Investigation of the wild birds, especially the migrating species as well as imported animals for infection or colonization with ESBL-producing strains.
    - Investigation of the imported foods specially meat and agricultural products for ESBL-producing organisms.

Summary

Extended-spectrum ß-lactamases (ESBLs) production is the major clinical and public health problem in Egyptian Hospitals. However, little data are available on the prevalence and the types of ESBLs in Egypt. This study aimed to determine the prevalence and the probable types of ESBL among *E. coli* and *K. pneumoniae* clinical isolates obtained from Egyptian Hospitals. It was carried out on a total of 248 clinical enterobacterial isolates collected during six months period from October 2008 to March 2009 from different clinical specimens obtained from different medical wards of the University Hospitals in Egypt and Germany including: 184 isolates (102 *K. pneumoniae* and 82 *Escherichia coli*) derived from Egyptian University Hospitals and 64 *E. coli* isolates derived from a German University Hospital (Giessen University Hospital). Phenotypic and molecular characterization was performed, results were compared.

The prevalence of ESBL-producing isolates among *E. coli* and *K. pneumoniae* derived from Egyptian University Hospitals was as high as 78.8% and 82.4% respectively. CTX-M + TEM was the most prevalent ß lactamase profile that has been detected among the ESBL-producing *E. coli* and *K. pneumoniae*. $Bla_{\text{CTX-M-15}}$ was the only detected $bla_{\text{CTX-M}}$ allele among Egyptian isolates and the most predominant allele among German isolates.

The molecular characterization of the bacterial host background revealed the prevalence of D phylogenetic group type among Egyptian isolates and the competitive prevalence of both A and B2 phylogenetic type among German isolates. PFGE analysis revealed a high degree of genetic diversity of the isolates derived from both countries. Presence of a clonal pair composed of an Egyptian isolate and a German isolate which could represent a case of clonal importation from one country to another was also detected.

In addition to ß-lactam resistance, the great majority of the isolates derived from both countries were multiple resistant to one or more of non ß-lactam antimicrobial classes including tetracycline, sulfamethoxazole/ trimethoprim, aminoglycosides and quinolones. CTX-M production was associated with IncFI plasmids most commonly with FIA-FIB multi replicon plasmids. Plasmids sizing and subsequent DNA hybridization revealed the location of $bla_{\text{CTX-M}}$ on ~ 97 kb FIA-FIB and ~ 170 kb FIA-FIB conjugative plasmids in common among the isolates derived from both countries suggesting emergence of a suspected trans-continental epidemic plasmid.

This study highlights the high prevalence of ESBL production among *E. coli* and *K. pneumoniae* in the clinical setting in Egypt as well as the presence an epidemiological relation between Egypt and Germany evidenced by the presence common epidemic plasmids, sharing of an identical clone which in turn harbor one of the epidemic plasmids. The dominance of plasmids of an identical replicon type and molecular size in both countries as well as the presence of other diverse large CTX-M-encoding plasmids in genetically heterogeneous strains proves that, horizontal transfer of multiple epidemic plasmids is the major driving force behind the spread of $bla_{\text{CTX-M}}$ in the clinical setting of both countries.

Zusammenfassung

Die Produktion von β-Lactamasen mit erweitertem Spektrum (ESBL) stellt das größte klinische und öffentliche Gesundheitsproblem in ägyptischen Krankenhäusern dar. Allerdings stehen nur wenige Daten über ESBL-Prävalenz und -Typen in Ägypten zur Verfügung. Die vorliegende Studie hat zum Ziel, die ESBL-Prävalenz und die möglichen ESBL-Typen unter den in ägyptischen Krankenhäusern gewonnenen klinischen Isolaten *E. coli* und *K. pneumoniae* zu bestimmen. Dies wurde an insgesamt 248 klinischen Enterobakterien-Isolaten durchgeführt, die in einem Zeitraum von sechs Monaten zwischen Oktober 2008 und März 2009 aus verschiedenen klinischen Proben unterschiedlicher Krankenstationen in Universitätskrankenhäusern in Ägypten und Deutschland gewonnen wurden, einschließlich: 184 aus den ägyptischen Universitätskrankenhäusern stammende Isolate (102 *K. pneumoniae* und 82 *Escherichia coli*) und 64 aus einem deutschen Universitätskrankenhaus (Universitätsklinikum Gießen) stammende Isolate *E. coli*. Eine phänotypische und molekulare Charakterisierung wurde durchgeführt und die Ergebnisse wurden verglichen.

Die Höhe der Prävalenz der aus ägyptischen Universitätskrankenhäusern stammenden ESBL-produzierenden Isolate unter *E. coli* und *K. pneumoniae* betrug 78,8% bzw. 84,2%. CTX-M + TEM war das am weitesten verbreitete ß-Lactamase-Profil, das unter den ESBL-produzierenden *E. coli* und *K. pneumoniae* nachgewiesen wurde. $Bla_{\text{CTX-M-15}}$ war das einzige nachgewiesene $Bla_{\text{CTX-M}}$-Allel unter den ägyptischen Isolaten und das vorherrschende Allel unter den deutschen Isolaten.

Die molekulare Charakterisierung des Hintergrunds des bakteriellen Wirts ließ die Prävalenz des D-phylogenischen Gruppentyps unter den ägyptischen Isolaten und die kompetitive Prävalenz sowohl des phylogenetischen Typs A als auch B2 unter den deutschen Isolaten erkennen. Die PFGE-Analyse zeigte einen hohen Grad an genetischer Vielfalt der Isolate aus beiden Ländern. Die Präsenz eines aus einem ägyptischen Isolat und eines deutschen Isolats zusammengesetzten Klonpaares, das einen Fall von Klonimport aus einem Land in das andere darstellen könnte, wurde ebenfalls nachgewiesen.

Zusätzlich zur ß-Lactam-Resistenz war die große Mehrheit der aus beiden Ländern stammenden Isolate vielfach resistent gegen eine oder mehrere antimikrobielle non-ß-Lactam-Klassen einschließlich Tetracyclin, Sulfamethoxazol/Trimethoprim,

Aminoglycoside und Quinolone. Die CTX-M-Produktion war verbunden mit IncFI-Plasmiden, am häufigsten mit mehrfach replizierenden Plasmiden FIA-FIB. Die Größenbestimmung der Plasmide sowie die anschließende DNA-Hybridisierung zeigte die Position von *bla*$_{CTX-M}$ auf ~ 97 kb FIA-FIB und ~ 170 kb FIA-FIB konjugativer Plasmide gemeinsam mit aus beidenLändern stammenden Isolaten, was auf ein Aufkommen befürchteter kontinentübergreifender epidemischer Plasmide hindeutet.

Die vorliegende Studie hebt die hohe Prävalenz der ESBL-Produktion unter *E. coli* und *K. pneumoniae* im klinischen Umfeld in Ägypten sowie die Präsenz einer durch das Vorfinden gemeinsamer epidemischer Plasmide nachgewiesenen epidemiologischen Beziehung zwischen Ägypten und Deutschland hervor, die identische Klone teilen, welche wiederum eines der epidemischen Plasmide beherbergen. Die Dominanz von Plasmiden mit identischem Replikationstyp und Molekülgröße in beiden Ländern sowie die Präsenz weiterer unterschiedlich großer CTX-M-codierter Plasmide in genetisch heterogenen Stämmen beweist, dass die horizontale Übertragung mehrfach epidemischer Plasmide die wesentliche Antriebskraft hinter der Ausbreitung von *bla*$_{CTX-M}$ im klinischen Umfeld beider Länder ist.

List of Abbreviations

AB	Anti-body
Act	Plasmid-encoded AmpC-type ß-lactamase
Adk	Adenylate kinase
Amp C	Class C ß- lactamase
ASP	Aspartic acid, an amino acid
attl	Attachment site
Bla	ß- lactamase-encoding gene.
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
CA	Clavulanic acid
CARB-3	Carbenicillinase-3 ß-lactamase
Cep A	Cephalosporinase
*Chu*A	Gene required for heme transport
CLSI	Clinical Laboratory Standard Institute
CMY	Cephamycin-hydrolyzing ß- lactamase
Co	Centigrade/Celsius degree
CphA	Carbapenem-hydrolyzingclass B metallo-ß-lactamases
CSPD	Chemiluminescent substrate for alkaline phosphatase
CT	Cefotaxime
CTX-M	Cefotaximase
CVP	Catheter for measurement of Central Venous Pressure
DIG	Digoxygenin
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *E. coli*
EDTA	Ethylene diamine tetra acetic acid
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ENT	Ear Nose and Throat ward
EPEC	Enteropathogenec *E. coli*
ESBL	Extended-spectrum ß-lactamase.
ETA	Endotracheal tube aspirate
ETEC	Enterotoxigenic *E. coli*
EUCAST	European Committee of Antimicrobial susceptibility testing
ExPEC	Extraintestinal pathogenic *E. coli*
F	Forward
FOX	Plasmid mediated AmpC-type ß-lactamase resistant to cephamycin
fum C	Fumarate hydratase
G + C	Guanine and cytosine bases content
Gent.	Gentamicin
GES	Guiana extended-spectrum ß- lactamase
GIT	Gastroenterology
Gly	Glycine
gyr B	DNA gyrase encoding gene
h / hr.	Hours
HCl	Hydrochloric acid
icd	Isocitrate /isopropyl malate dehydrogenase encoding gene

ICU	Intensive Care Unit
IMI-1	Class a carbapenem-hydrolyzing ß-lactamase enzyme
IMP-1	Metallo-ß-lactamase
Inc.	Incobatibility group
Int. Medicine	Internal Medicine
Int. Surgery	Internal Surgery
Intl	Integrase gene
Kb	Kilo base
KPC-2	*K. pneumoniae* carbapenemase, Plasmid-mediated, carbapenem-Hydrolyzing ß-lactamase
LB	Lauria-Bertani
mA	Milli Ampere
mdh	Malate dehydrogenase encoding gene
mg	Milligram
MIC	Minimum inhibitory concentration
Min.	Minute
MIR	Plasmid-mediated ß-lactamase (MIR-1) conferring resistance to oxyimino-and alpha-methoxy ß-lactams
MLST	Mutilocus sequence typing
Mm	Millimeter
MUH	El-Mansoura university hospital
Multi	Multi- clinics
ND	Not detected
NDM-1	New Delhi metallo-β-lactamase
OB/GY	Obstetrics and Gynecology ward
Oper. Intensive	Operation Intensive
OXA	Oxacillinase
PBS	Phosphate –buffered saline
PCR	Polymerase chain reaction
PCR-RFLP	PCR-restriction fragment length polymorphism
PCR-SSCP	PCR-single stranded conformation polymorphism
PEARLS	Pan European Antimicrobial resistance Local surveillance
Ped.	Pediatrics
PER	ß-lactamase enzyme named after *Pseudomonas* Extended Resistance
PFGE	Pulsed-Field gel electrophoresis
pH	Negative log hydrogen ion concentration
PM	Cefepime
*pur*A	Adenylosuccinate dehydrogenase encoding gene
R	Reverse
*rec*A	ATP/GTP binding motif encoding gene
*rep*A	Essential replication gene
RNA	Ribonucleic acid
S/ Sec.	Second
SDS	Sodium dodecyl sulphate
SFO	ß-lactamase enzyme named after *Serratia fonticola*
SHV	ß-lactamase enzyme named after Sulfhydryl variable
spp.	Species
SSC	Saline-sodium citrate buffer
SSPE	Saline-Sodium Phosphate-EDTA buffer

ST	Sequence type
SXT	Sulfamethoxazole /trimethoprim
TAE	Tris / Acetate/ EDTA
TBE	Tris/Borate/ EDTA
TE	Tris/ EDTA
TEM	ß-lactamase enzyme named after a Greek patient Temoniera.
Tetra.	Tetracycline
TLA	Plasmid mediated class A ß-lactamase enzyme named after TLAhuicus (Indian tribe)
Tobra.	Tobramycin
Tris	Tris (hydroxymethyl) aminoethan
TspE4C2	Anonymous DNA fragment
TZ	Ceftazidime
TZB	Tazobactam
U	Unit
UK	United Kingdom
USA	United States
UV	Ultraviolet
V	Volt
VEB	Vietnam Extended-spectrum Beta lactamase
VIM	Verona integeron-encoded metalo-ß- lactamase
*Yja*A	Gene has been identified after complete genome sequencing of *E. col* K-12 and its function stills unknown.
µg	Microgram
µl	Microliter

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Appendix

Appendix I

Table I-A_1: Clinical isolates derived from Egyptian University Hospital (El-Mansoura)

Ser No.	Ref. No.	Lab. No	Species	Clinical specimen	Ward	ß-lactamase profile
1	133	406	*Klebsiella pneumoniae*	ETA	Ped.Neonatal	TEM
2	134	407	*Klebsiella pneumoniae*	ETA	Oncology	TEM
3	135	408	*Klebsiella pneumoniae*	Sputum	Chest Medicine	SHV+TEM
4	137	409	*Eschrichia coli*	Urine	Oncology	CTX-M
5	140	410	*Klebsiella pneumoniae*	Urine	Oncology	Negative
6	141	411	*Klebsiella pneumoniae*	Urine	Oncology	Negative
7	142	412	*Klebsiella pneumoniae*	Sputum	Chest Medicine	CTX-M+SHV
8	143	413	*Klebsiella pneumoniae*	Drain	Emergency	CTX-M+TEM
9	144	414	*Escherichia coli*	ETA	Ped.Neonatal	CTX-M
10	145	415	*Klebsiella pneumoniae*	ETA	Ped.Neonatal	CTXM+SHV+TM
11	146	416	*Escherichia coli*	Sputum	Chest Medicine	TEM
12	147	417	*Klebsiella pneumoniae*	Sputum	Oncology	Negative
13	149	418	*Escherichia coli*	Urine	Oncology	CTX-M
14	150	419	*Klebsiella pneumoniae*	Throat swab	Oncology	CTX-M +TEM
15	151	420	*Klebsiella pneumoniae*	Blood	Ped.Neonatal	SHV+TEM
16	153	421	*Klebsiella pneumoniae*	Blood	Ped.Neonatal	SHV+TEM
17	154	422	*Escherichia coli*	Wound swab	Surgery	Negative
18	156	423	*Escherichia coli*	Sputum	Oncology	TEM
19	158	424	*Klebsiella pneumoniae*	Blood	Ped.Neonatal	SHV+TEM
20	160	425	*Klebsiella pneumoniae*	Urine	Oncology	Negative
21	165	426	*Escherichia coli*	Blood	Neuro-surgery	Negative
22	166	427	*Klebsiella pneumoniae*	Sputum	GIT	TEM
23	167	428	*Klebsiella pneumoniae*	Urine	Neurology	CTX-M+TEM
24	168	429	*Klebsiella pneumoniae*	Blood	Ped.Neonatal	SHV+TEM
25	170	430	*Escherichia coli*	Throat swab	Oncology	Negative
26	171	431	*Klebsiella pneumoniae*	Sputum	GIT	TEM
27	172	432	*Klebsiella pneumoniae*	Sputum	Oncology	CTX-M +TEM
28	173	433	*Klebsiella pneumoniae*	Sputum	Oncology	Negative
29	174	434	*Klebsiella pneumoniae*	Drain	GIT	CTXM+SHV+TM
30	176	435	*Escherichia coli*	ETA	Ped.Neonatal	SHV+TEM
31	177	436	*Escherichia coli*	Urine	Oncology	CTX-M
32	178	437	*Escherichia coli*	Urine	Oncology	CTX-M
33	180	438	*Escherichia coli*	Blood	Oncology	Negative
34	181	439	*Klebsiella pneumoniae*	Blood	Ped.ICU	TEM
35	184	440	*Klebsiella pneumoniae*	Wound swab	MUH	CTX-M +TEM
36	186	441	*Escherichia coli*	Wound swab	MUH	CTXM+SHV+TM
37	188	442	*Escherichia coli*	Wound swab	Surgery	CTX-M+TEM
38	189	443	*Klebsiella pneumoniae*	Wound swab	MUH	Negative
39	190	444	*Klebsiella pneumoniae*	Urine	Chest Medicine	SHV+TEM
40	191	445	*Escherichia coli*	Wound swab	Oncology	CTX-M+TEM

41	192	446	*Klebsiella pneumoniae*	T-tube aspirate	Ped-ICU	CTXM+SHV+TEM
42	198	447	*Klebsiella pneumoniae*	T-tube aspirate	Chest Medicine	Negative
43	200	448	*Klebsiella pneumoniae*	ETA	Pediatric	TEM
44	201	449	*Klebsiella pneumoniae*	Stool	Sp. medical Hos.	CTX-M
45	203	450	*Escherichia coli*	Urine	Chest Medicine	CTX-M+TEM
46	204	451	*Klebsiella pneumoniae*	Blood	Ped.Neonatal	SHV+TEM
47	205	452	*Klebsiella pneumoniae*	Blood	Ped.Neonatal	CTXM+SHV+TEM
48	209	453	*Klebsiella pneumoniae*	ETA	Ped.Neonatal	SHV+TEM
49	210	454	*Escherichia coli*	Urine	Sp.medical Hos	CTX-M
50	211	455	*Escherichia coli*	T-tube aspirate	GEC	CTX-M
51	212	456	*Klebsiella pneumoniae*	U-tube aspirate	GEC	TEM
52	213	457	*Klebsiella pneumoniae*	Unknown	Unknown	TEM
53	221	458	*Escherichia coli*	Skin swab	Pediatric	SHV+TEM
54	223	459	*Escherichia coli*	Sputum	Oncology	CTX-M +TEM
55	226	460	*Klebsiella pneumoniae*	Sputum	Chest Medicine	Negative
56	227	461	*Escherichia coli*	Sputum	Chest Medicine	Negative
57	228	462	*Klebsiella pneumoniae*	Urine	Pediatric	SHV+TEM
58	231	463	*Escherichia coli*	Urine	Dermatology	CTX-M
59	232	464	*Klebsiella pneumoniae*	Blood	Pediatric	CTX-M+TEM
60	233	465	*Klebsiella pneumoniae*	Throat swab	Oncology	CTX-M + SHV
61	234	466	*Escherichia coli*	Sputum	Oncology	TEM
62	235	467	*Klebsiella pneumoniae*	Wound swab	Surgery	CTX-M+TEM
63	240	468	*Escherichia coli*	Urine	Pediatric	CTX-M+TEM
64	241	469	*Klebsiella pneumoniae*	Sputum	Chest Medicine	Negative
65	252	470	*Klebsiella pneumoniae*	Ascitic fluid	Tropical med.	TEM
66	229	471	*Klebsiella pneumoniae*	Blood	Oncology	CTX-M+TEM
67	12	472	*Klebsiella pneumoniae*	Blood	Ped.Neonatal	CTXM+SHV+TEM
68	57	473	*Escherichia coli*	Throat swab	Oncology	CTX-M+TEM
69	101	474	*Escherichia coli*	Suction apparatus	Ped.Neonatal	Negative
70	95	475	*Escherichia coli*	Wound swab	Oncology	CTX-M
71	65	476	*Escherichia coli*	Ascitic fluid	Tropical med.	CTX-M+TEM
72	123	477	*Klebsiella pneumoniae*	Wound swab	Multi-clinics	Negative
73	60	478	*Klebsiella pneumoniae*	T-tube aspirate	Neuro-surgery	CTX-M
74	22	479	*Escherichia coli*	T-tube aspirate	GIT	CTX-M+TEM
75	88	480	*Escherichia coli*	Urine	Oncology	CTX-M
76	94	481	*Escherichia coli*	Throat swab	Neuro-surgery	Negative
77	127	482	*Klebsiella pneumoniae*	Blood	Ped.Neonatal	SHV
78	83	483	*Escherichia coli*	Urine	Neurology	CTX-M+TEM
79	32	484	*Klebsiella pneumoniae*	Blood	Ped.Neonatal	CTXM+SHV+TEM
80	98	485	*Klebsiella pneumoniae*	ETA	Ped.Neonatal	CTX-M+TEM
81	129	486	*Escherichia coli*	Blood	Pediatric	TEM
82	59	487	*Escherichia coli*	Wound swab	Surgery	CTX-M+TEM

83	48	488	*Klebsiella pneumoniae*	Urine	Ped-ICU	Negative
84	80	489	*Klebsiella pneumoniae*	Blood	Ped.Neonatal	Negative
85	128	490	*Escherichia coli*	Ventilator	Oncology	TEM
86	45	491	*Escherichia coli*	Sputum	Oncology	TEM
87	117	492	*Escherichia coli*	Urine	Neurology	CTX-M
88	107	493	*Escherichia coli*	Urine	Oncology	CTX-M+TEM
89	26	494	*Klebsiella pneumoniae*	T-tube aspirate	GIT	CTX-M+TEM
90	46	495	*Klebsiella pneumoniae*	T-tube aspirate	Neuro-surgery	Negative
91	69	496	*Klebsiella pneumoniae*	Urine	GIT	CTX-M+TEM
92	100	497	*Escherichia coli*	Urine	Pediatric	Negative
93	96	498	*Escherichia coli*	Wound swab	Oncology	CTX-M+TEM
94	130	499	*Klebsiella pneumoniae*	Sputum	Neurology	CTX-M+TEM
95	20	500	*Escherichia coli*	T-tube aspirate	GIT	CTX-M+TEM
96	50	501	*Klebsiella pneumoniae*	Wound swab	Oncology	CTX-M
97	44	502	*Klebsiella pneumoniae*	Blood	Ped.Neonatal	Negative
98	97	503	*Escherichia coli*	Urine	Chest Medicine	Negative
99	86	504	*Klebsiella pneumoniae*	Unknown	Unknown	CTX-M+SHV
100	16	505	*Escherichia coli*	Sputum	Oncology	TEM
101	87	506	*Escherichia coli*	Blood	Ped.Neonatal	CTXM+SHV+TEM
102	62	507	*Klebsiella pneumoniae*	T-tube aspirate	GIT	TEM
103	116	508	*Klebsiella pneumoniae*	Wound swab	ENT	CTX-M+TEM
104	125	509	*Escherichia coli*	Blood	Pediatric	TEM
105	61	510	*Klebsiella pneumoniae*	Throat swab	Neurology	CTX-M+SHV
106	93	511	*Escherichia coli*	Urine	Oncology	TEM
107	75	512	*Escherichia coli*	Throat swab	Oncology	TEM
108	82	513	*Klebsiella pneumoniae*	Throat swab	Neurology	CTX-M+TEM
109	114	514	*Escherichia coli*	Urine	Ped.Neonatal	TEM
110	64	515	*Klebsiella pneumoniae*	T-tube aspirate	Neuro-surgery	CTX-M+SHV
111	37	516	*Escherichia coli*	Urine	Oncology	TEM
112	79	517	*Klebsiella pneumoniae*	T-tube aspirate	Ped.Neonatal	CTX-M+ SHV
113	120	518	*Escherichia coli*	T-tube aspirate	Pediatric ICU	CTX-M+TEM
114	104	519	*Escherichia coli*	Wound swab	Surgery	TEM
115	84	520	*Klebsiella pneumoniae*	Sputum	Neuro-surgery	TEM
116	71	521	*Klebsiella pneumoniae*	ETA	Ped.Neonatal	CTX-M+TEM
117	91	522	*Klebsiella pneumoniae*	T-tube aspirate	Cardio-thoracic	CTXM+SHV+TEM
118	49	523	*Escherichia coli*	Urine	Outpatient	Negative
119	85	524	*Klebsiella pneumoniae*	CVP	Neuro-surgery	CTX-M
120	29	525	*Klebsiella pneumoniae*	Ascitic fluid	Tropical med.	TEM
121	63	526	*Klebsiella pneumoniae*	Urine	Pediatric	CTX-M+ SHV
122	70	527	*Klebsiella pneumoniae*	Blood	Ped.Neonatal	CTXM+SHV+TEM
123	122	528	*Klebsiella pneumoniae*	ETA	Emergency	SHV+TEM
124	103	529	*Escherichia coli*	Urine	Oncology	CTX-M+TEM
125	119	530	*Klebsiella pneumoniae*	Wound swab	Multi-clinics	CTX-M
126	38	531	*Klebsiella pneumoniae*	Chest-tube aspirate	Pediatric	TEM

Table I-A_2: Clinical isolates derived from Egyptian University Hospital (Assiut).

Ser No.	Ref. No.	Lab. No	*Species*	Clinical specimen	Medical ward	ß-lactamase profile
127	1010	532	*Klebsiella pneumoniae*	Urine	Urology	CTX-M
128	1022	533	*Klebsiella pneumoniae*	Urine	Urology	CTX-M+TEM
129	1025	534	*Klebsiella pneumoniae*	Urine	Urology	CTXM+SHV+TEM
130	1026	535	*Escherichia coli*	Urine	Urology	CTX-M+TEM
131	1027	536	*Escherichia coli*	Urine	Urology	CTX-M +TEM
132	1028	537	*Escherichia coli*	Urine	Urology	CTX-M
133	1031	538	*Escherichia coli*	Urine	Urology	CTX-M
134	1034	539	*Escherichia coli*	Urine	Urology	CTX-M+TEM
135	1035	540	*Klebsiella pneumoniae*	Urine	Urology	Negative
136	1037	541	*Klebsiella pneumoniae*	Urine	Urology	CTX-M+TEM
137	1038	542	*Klebsiella pneumoniae*	Urine	Urology	CTX-M+TEM
138	1039	543	*Escherichia coli*	Urine	Urology	Negative
139	1040	544	*Escherichia coli*	Urine	Urology	TEM
140	1041	545	*Escherichia coli*	Urine	Urology	CTX-M+TEM
141	1044	546	*Escherichia coli*	Urine	Urology	CTX-M+TEM
142	1047	547	*Escherichia coli*	Urine	Urology	CTX-M +TEM
143	1049	548	*Klebsiella pneumoniae*	Urine	Urology	Negative
144	1051	549	*Klebsiella pneumoniae*	Urine	Urology	CTXM+SHV+TEM
145	1052	550	*Klebsiella pneumoniae*	Urine	Urology	TEM
146	1053	551	*Klebsiella pneumoniae*	Urine	Urology	CTX-M+TEM
147	1054	552	*Klebsiella pneumoniae*	Urine	Urology	TEM
148	1055	553	*Escherichia coli*	Urine	Urology	CTX-M+TEM
149	1056	554	*Klebsiella pneumoniae*	Urine	Urology	CTXM+SHV+TEM
150	1059	555	*Escherichia coli*	Urine	Urology	Negative
151	1060	556	*Escherichia coli*	Urine	Urology	Negative
152	1061	557	*Klebsiella pneumoniae*	Urine	Urology	CTX-M+TEM
153	1063	558	*Escherichia coli*	Urine	Urology	Negative
154	1065	559	*Escherichia coli*	Urine	Urology	CTX-M+TEM
155	1060	560	*Escherichia coli*	Urine	Urology	TEM
156	1067	561	*Klebsiella pneumoniae*	Urine	Urology	CTXM+SHV+TEM
157	1070	563	*Escherichia coli*	Urine	Urology	Negative
158	1071	564	*Escherichia coli*	Urine	Urology	CTX-M+TEM
159	1072	565	*Klebsiella pneumoniae*	Urine	Urology	TEM
160	1073	566	*Escherichia coli*	Urine	Urology	Negative
161	1074	567	*Klebsiella pneumoniae*	Urine	Urology	SHV+TEM
162	1075	568	*Escherichia coli*	Urine	Urology	TEM
163	1083	569	*Klebsiella pneumoniae*	Urine	Urology	CTX-M+TEM
164	1084	570	*Escherichia coli*	Urine	Urology	Negative
165	1086	571	*Escherichia coli*	Urine	Urology	TEM
166	1088	572	*Escherichia coli*	Urine	Urology	CTX-M+TEM
167	1069	573	*Klebsiella pneumoniae*	Urine	Urology	CTX-M+TEM
168	1013	574	*Escherichia coli*	Urine	Urology	TEM
169	1016	575	*Klebsiella pneumoniae*	Urine	Urology	CTXM+SHV+TEM
170	1017	576	*Klebsiella pneumoniae*	Urine	Urology	CTX-M+TEM
171	1018	577	*Klebsiella pneumoniae*	Urine	Urology	TEM
172	1092	578	*Klebsiella pneumoniae*	Urine	Urology	TEM
173	1002	579	*Klebsiella pneumoniae*	Urine	Urology	Negative

Table I-A_3: Clinical isolates derived from Egyptian University Hospital (Assiut +El-Mansoura).

Ser. No.	Ref. No.	Lab. No	Species	Clinical specimen	Medical Ward	Source	ß-lactamase profile
174	199R	580	*Klebsiella pneumoniae*	Throat swab	Oncology	El-Mansoura	TEM
175	199B	581	*Klebsiella pneumoniae*	Throat swab	Oncology	El-Mansoura	TEM
176	206R	582	*Escherichia coli*	Wound swab	MUH	El-Mansoura	CTX-M+TEM
177	206B	583	*Escherichia coli*	Wound swab	MUH	El-Mansoura	CTX-M+TEM
178	195	584	*Klebsiella pneumoniae*	Wound swab	Neurology	El-Mansoura	CTX-M+TEM
179	207	585	*Escherichia coli*	Throat swab	Oncology	El-Mansoura	Negative
180	99	586	*Klebsiella pneumoniae*	Wound swab	Neurosurgery	El-Mansoura	SHV+TEM
181	1045	588	*Escherichia coli*	Urine	Urology	Assiut	CTX-M+TEM
182	1024	589	*Klebsiella pneumoniae*	Urine	Urology	Assiut	TEM
183	1007	590	*Escherichia coli*	Urine	Urology	Assiut	Negative
184	1020	591	*Klebsiella pneumoniae*	Urine	Urology	Assiut	Negative

Table I-B: Clinical isolates derived from Giessen University Hospital, Germany

Ser. No.	Ref. No.	Lab. No	*Species*	Clinical specimen	Medical ward	ß-lactamase profile of CTX-M (+ve) isolates
185	1	611	*Escherichia coli*	stool	Int. Medicine	CTX-M
186	8	612	*Escherichia coli*	Nasal swab	Int. Surgery	CTX-M + TEM
187	9	613	*Escherichia coli*	Anal swab	Surgery	CTX-M + TEM
188	18	614	*Escherichia coli*	urine	Medical clin.	Non –CTX-M
189	29	615	*Escherichia coli*	Cervical swab	Obs/Gyn	CTX-M
190	33	616	*Escherichia coli*	urine	Urology kl.	CTX-M + TEM
191	43	617	*Escherichia coli*	stool	Medical clin.	CTX-M
192	59	618	*Escherichia coli*	urine	Multi-Clin.	Non – CTX-M
193	77	619	*Escherichia coli*	groin swab	Medical clin.	CTX-M + TEM
194	84	620	*Escherichia coli*	sputum	Int. Surgery	CTX-M
195	102	621	*Escherichia coli*	Anal swab	Int. Surgery	CTX-M + TEM
196	108	622	*Escherichia coli*	Cervical swab	Obs/Gyn	CTX-M+TEM
197	113	623	*Escherichia coli*	unknown	unknown	Non – CTX-M
198	122	624	*Escherichia coli*	Anal swab	Surgery	Non–CTX-M
199	149	625	*Escherichia coli*	unknown	unknown	CTX-M +TEM
200	441	626	*Escherichia coli*	Anal swab	Surgery	Non – CTX-M
201	442	627	*Escherichia coli*	urine	Int. medicine	CTX-M
202	455	628	*Escherichia coli*	Anal swab	Surgery	CTX-M
203	471	629	*Escherichia coli*	vaginal swab	Obs/Gyn	CTX-M + TEM
204	472	630	*Escherichia coli*	Mid-stream urine	Urology	Non–CTX-M
205	482	631	*Escherichia coli*	urine	Psychatric	CTX-M +TEM
206	492	632	*Escherichia coli*	Anal swab	Surgery	CTX-M
207	508	633	*Escherichia coli*	urine	orthopedic	CTX-M +TEM
208	509	634	*Escherichia coli*	urine	Urology	CTX-M +TEM
209	533	635	*Escherichia coli*	Anal swab	Int. Surgery	Non – CTX-M
210	542	636	*Escherichia coli*	Anal swab	Int. Surgery	CTX-M +TEM
211	552	637	*Escherichia coli*	cath. urine	orthopedic	Non–CTX-M
212	562	638	*Escherichia coli*	cult. urine	Urology	Non–CTX-M
213	563	639	*Escherichia coli*	m. d urine	Urology	Non–CTX-M
214	573	640	*Escherichia coli*	unknown	unknown	CTX-M +TEM
215	586	641	*Escherichia coli*	unknown	unknown	CTX-M +TEM
216	593	642	*Escherichia coli*	urine	Medical clin.	CTX-M
217	385	643	*Escherichia coli*	STUHL	Obs/Gyn	Non –CTX-M
218	389	644	*Escherichia coli*	left eye swab	Obs/Gyn	Non –CTX-M
219	390	645	*Escherichia coli*	urine	Int. medicine	CTX-M +TEM
220	392	646	*Escherichia coli*	urine	Urology	CTX-M +TEM
221	399	647	*Escherichia coli*	urine	Pediatric	Non –CTX-M
222	404	648	*Escherichia coli*	left leg swab	Medical clin.	Non –CTX-M
223	406	649	*Escherichia coli*	abdomen swab	Medical clin.	CTX-M +TEM
224	413	650	*Escherichia coli*	urine	Medical clin.	CTX-M
225	418	651	*Escherichia coli*	urine	Multi-Clin.	Non –CTX-M
226	419	652	*Escherichia coli*	Anal swab	Surgery	CTX-M +TEM
227	420	653	*Escherichia coli*	urine	Multi-Clin.	Non –CTX-M

228	421	654	*Escherichia coli*	urine	Medical clin.	CTX-M
229	422	655	*Escherichia coli*	urine	Pediatric	CTX-M
230	425	656	*Escherichia coli*	Anal swab	Int. Surgery	Non –CTX-M
231	427	657	*Escherichia coli*	urine catheter	Pediatric	CTX-M +TEM
232	144	658	*Escherichia coli*	Larynx swab	Medical clin.	Non –CTX-M
233	162	659	*Escherichia coli*	urine	Urology	CTX-M +TEM
234	171	660	*Escherichia coli*	urine	Urology	CTX-M+TEM
235	174	661	*Escherichia coli*	urine	neurology	CTX-M+TEM
236	176	662	*Escherichia coli*	urine	urology	CTX-M+TEM
237	178	663	*Escherichia coli*	urine	Int. Medicine	CTX-M+TEM
238	179	664	*Escherichia coli*	urine	Medical clin.	Non – CTX-M
239	190	665	*Escherichia coli*	urine	Pediatric	Non – CTX-M
240	193	666	*Escherichia coli*	Nasal swab	Medical clin.	CTX-M+TEM
241	194	667	*Escherichia coli*	Anal swab	oper. Intensive	Non – CTX-M
242	195	668	*Escherichia coli*	Anal swab	Oper. Intensive	CTX-M+TEM
243	197	669	*Escherichia coli*	diabetic foot swab	Medical clin.	Non–CTX-M
244	201	670	*Escherichia coli*	oral swab	Obs/Gyn	CTX-M
245	202	671	*Escherichia coli*	urine	Pediatric	CTX-M
246	204	672	*Escherichia coli*	urine	Pediatric	CTX-M
247	213	673	*Escherichia coli*	Anal swab	Pediatric	CTX-M+TEM
248	215	674	*Escherichia coli*	urine	Pediatric	Non – CTX-M

Ser. No: Serial number; Ref. No: Reference (given by source of strain) number; Lab. No: Laboratory number (Given in this study); ETA : Endotracheal aspirate; ENT : Ear, Nose and Throat GIT : Gastroenterology; Gyn : Gynecology; ICU : Intensive care unit; MUH : Mansoura University Hospital; Obs : Obstetrics; Ped.: Pediatric; Sp. Medical Hos : special medical Hospital; Tropical Med: Tropical Medicine; Oper. Intensive: Operation Intensive Care.

Appendix II

Feature of CTX-M-producing *E. coli* isolates derived from Egyptian University Hospitals

Table II-A1: Clinical reference data and ß-lactamase profile of CTX-M-producing *E. coli* isolates derived from Egyptian University Hospitals.

Serial No.	Strain No.*	Source code	Clinical specimen	Medical ward	ß-lactamase profile		
					CTX-M	SHV	TEM
1	409	137	Urine	Oncology	CTXM-15	Negative	Negative
2	414	144	ETA	Ped. neonatal	CTXM-15	Negative	Negative
3	418	149	Urine	Oncology	CTXM-15	Negative	Negative
4	436	177	Urine	Oncology	CTXM-15	Negative	Negative
5	437	178	Urine	Oncology	CTXM-15	Negative	Negative
6	442	188	Wound swab	Surgery	CTXM-15	Negative	Positive
7	445	191	Wound swab	Oncology	CTXM-15	Negative	Positive
8	450	203	Urine	Chest medicine	CTXM-15	Negative	Positive
9	454	210	Urine	Sp. Med. H	CTXM-15	Negative	Negative
10	455	211	U tube aspirate	GEC	CTXM-15	Negative	Negative
11	459	223	sputum	Oncology	CTXM-15	Negative	Positive
12	463	231	Urine	dermatology	CTXM-15	Negative	Negative
13	468	240	Urine	pediatric	CTXM-15	Negative	Positive
14	473	57	Throat swab	Oncology	CTXM-15	Negative	Positive
15	475	95	Wound swab	Oncology	CTXM-15	Negative	Negative
16	476	65	Ascetic fluid	Tropical medicine	CTXM-15	Negative	Positive
17	479	22	T-tube aspirate	GIT	CTXM-15	Negative	Positive
18	480	88	Urine	Neurology	CTXM-15	Negative	Negative
19	483	83	Urine	Neurology	CTXM-15	Negative	Positive
20	487	59	Wound swab	surgery	CTXM-15	Negative	Positive
21	492	117	Urine	Neurology	CTXM-15	Negative	Negative
22	493	107	Urine	Oncology	CTXM-15	Negative	Positive
23	498	96	Wound swab	Oncology	CTXM-15	Negative	Positive
24	500	20	T-tube aspirate	GIT	CTXM-15	Negative	Positive
25	518	120	T-tube aspirate	Ped. ICU	CTXM-15	Negative	Positive
26	529	103	Urine	Oncology	CTXM-15	Negative	Positive
27	535	1026	Urine	Urology	CTXM-15	Negative	Positive
28	536	1027	Urine	Urology	CTXM-15	Negative	Positive
29	537	1028	Urine	Urology	CTXM-15	Negative	Negative
30	538	1031	Urine	Urology	CTXM-15	Negative	Negative
31	539	1034	Urine	Urology	CTXM-15	Negative	Positive
32	545	1041	Urine	Urology	CTXM-15	Negative	Positive
33	546	1044	Urine	Urology	CTXM-15	Negative	Positive
34	547	1047	Urine	Urology	CTXM-15	Negative	Positive
35	553	1055	Urine	Urology	CTXM-15	Negative	Positive
36	559	1065	Urine	Urology	CTXM-15	Negative	Positive
37	564	1071	Urine	Urology	CTXM-15	Negative	Positive
38	572	1088	Urine	Urology	CTXM-15	Negative	Positive
39	582	206 R	Wound swab	MUH	CTXM-15	Negative	Positive
40	583	206 B	Wound swab	MUH	CTXM-15	Negative	Positive
41	588	1045	Urine	Urology	CTXM-15	Negative	Positive

Table II-A_2: Characterization of the CTX-M-producing bacterial hosts derived from Egyptian University Hospitals.

Serial No.	Strain No.*	Phylogenic type	ST	PFGE type	Resistance to Non ß- lactam antimicrobials
1	409	D		E 3	Tetracycline, gentamicin, SXT, tobramycin and quinolones
2	414	B2		A 16	Tetracycline, gentamicin, SXT, tobramycin and quinolones
3	418	B2		A 13	Tetracycline, gentamicin, SXT, tobramycin and quinolones
4	436	B2		E 2	Tetracycline,gentamicin,SXT,tobramycin and quinolones
5	437	B2		A15	Tetracycline ,gentamicin, SXT, tobramycin and quinolones
6	442	D	405	I 3	Tetracycline, SXT, tobramycin and quinolones
7	445	D		B 5	Tetracycline, SXT, tobramycin and quinolones
8	450	D		B 11	Tetracycline, SXT, tobramycin and quinolones
9	454	A		A1	Tetracycline, gentamicin, SXT, tobramycin and quinolones
10	455	D		Non-typeable	Tetracycline, gentamicin, SXT, tobramycin and quinolones
11	459	D	354	B 7	Tetracycline, gentamicin, SXT, tobramycin and quinolones
12	463	B2		A 11	Tetracycline, gentamicin, SXT and tobramycin
13	468	D		D1	Tetracycline and SXT
14	473	A		D6	Tetracycline, gentamicin, SXT and tobramycin
15	475	B2		A5	Tetracycline, tobramycin and quinolones
16	476	A	10	F3	Tetracycline, gentamicin, SXT, tobramycin and quinolones
17	479	D		H 1	Tetracycline,SXT,tobramycin and quinolones
18	480	D		F1	Tetracycline, gentamicin, tobramycin and quinolones
19	483	D	648	(I)	Tetracycline, SXT, tobramycin and quinolones
20	487	A		C10	Tetracycline, gentamicin, tobramycin and quinolones
21	492	D		F2	Tetracycline, gentamicin and SXT
22	493	A	1574	C8	Tetracycline, SXT, tobramycin and quinolones
23	498	D		H 2	Tetracycline, gentamicin, SXT, tobramycin and quinolones
24	500	D		H 1	Tetracycline, SXT, tobramycin and quinolones
25	518	A		(VIII)	Tetracycline, SXT, tobramycin and quinolones
26	529	D	648	C3	Tetracycline, gentamicin, SXT ,tobramycin and quinolones
27	535	B1		A 6	Gentamicin, SXT, tobramycin and quinolones
28	536	D		B 12	Tetracycline, gentamicin, SXT, tobramycin and quinolones
29	537	A		C9	Tetracycline, gentamicin, SXT, tobramycin and quinolones
30	538	A		C9	Tetracycline, gentamicin, SXT, tobramycin and quinolones
31	539	D		E1	Tetracycline, gentamicin, SXT, tobramycin and quinolones
32	545	D		B2	Tetracycline, gentamicin, SXT, tobramycin and quinolones
33	546	D	648	B1	Tetracycline, gentamicin, SXT, tobramycin and quinolones
34	547	D		B 8	Tetracycline, gentamicin, SXT, tobramycin and quinolones
35	553	B2		A3	Tetracycline, gentamicin and SXT
36	559	A		C1	Tetracycline, gentamicin, SXT, tobramycin and quinolones
37	564	D		E1	Tetracycline, gentamicin, SXT, tobramycin and quinolones
38	572	A		A 9	Tetracycline, gentamicin, tobramycin and quinolones
39	582	A		C6	Tetracycline, SXT, tobramycin and quinolones
40	583	A		A 4	Tetracycline, SXT, tobramycin and quinolones
41	588	A		C2	Gentamicin, SXT, tobramycin and quinolones

Table II-A_3 : Characterization of the large plasmids which were carried by CTX-M-producing *E. coli* isolates derived from Egyptian University Hospitals.

Serial No.	Strain * No.	Plasmid replicon type	Plasmid transfer	Co-transferable non ß-lactam resistance	Plasmid (s) size	CTX-M-carrying plasmid
1	409	FIA,FIB	Conjugative	Tetracycline, gentamicin and tobramycin	170kb, 97kb	
2	414	FIA,FIB	Conjugative	Tetracycline, gentamicin and tobramycin	170kb, 97kb	170kb
3	418	FIA,FIB				
4	436	FIA,FIB				
5	437	FIA,FIB				
6	442	FIA,FIB				
7	445	FIA,FIB				
8	450	FIA,FIB				
9	454	FIA,FIB				
10	455	FIA,FIB			120 kb	
11	459	FIA,FIB	Conjugative	None	97kb	97kb
12	463	FIB				
13	468	FIB	Conjugative	Tetracycline and SXT	170kb, > 97kb	170kb
14	473	FIA				
15	475	FIA,FIB	Conjugative	None	145kb, 97kb	145kb
16	476	FIA,FIB	Conjugative	Tetracycline, gentamicin, SXT and tobramycin	170kb	170kb
17	479	FIA,FIB				
18	480	FIA,FIB				
19	483	FIA,FIB	Conjugative	None	145kb, 97kb	97kb
20	487	FIA,FIB				
21	492	Unknown				
22	493	FIA,FIB			145, <145kb	
23	498	FIA,FIB				
24	500	FIA,FIB	Conjugative	Tetracycline, SXT and tobramycin	97kb	
25	518	FIA,FIB			242, 97kb	
26	529	FIA,FIB	Conjugative	None	194kb, 97kb	97kb
27	535	A / C				
28	536	FIA				
29	537	FIA,FIB				
30	538	FIA,FIB				
31	539	FIA				
32	545	FIA,FIB				
33	546	FIB				
34	547	FIB				
35	553	FIB				
36	559	FIA,FIB	Conjugative	Tetracycline, gentamicin and tobramycin	145kb	
37	564	FIA				
38	572	A / C				
39	582	FIA,FIB				
40	583	FIA,FIB				
41	588	FIA				

Feature of CTX-M-producing *E. coli* isolates derived from Giessen University Hospital, Germany.

Table II-B_1 : Clinical reference data and ß-lactamase profile of CTX-M-producing *E. coli* isolates derived from Giessen University Hospital, Germany.

Serial No.	Strain No.*	Source code	Clinical specimen	Medical ward	ß-lactamase profile		
					CTX-M	SHV	TEM
1	611	1	Stool	Internal Medicine	CTX-M-15	Negative	Negative
2	612	8	Nasal swab	Surgery (Internal)	CTX-M-15	Negative	Positive
3	613	9	Anal swab	Surgery	CTX-M-15	Negative	Positive
4	615	29	Cervical swab	Gynecology	CTX-M- 1	Negative	Negative
5	616	33	Urine	Urology	CTX-M-15	Negative	Positive
6	617	43	Stool	Medical clinic	CTX-M-15	Negative	Positive
7	619	77	Groin swab	Medical clinic	CTX-M-15	Negative	Positive
8	620	84	Sputum	Surgery (Internal)	CTX-M-1	Negative	Negative
9	621	102	Anal swab	Surgery (Internal)	CTX-M-15	Negative	Positive
10	622	108	Cervical swab	Gynecology	CTX-M-15	Negative	Positive
11	625	149	Unknown	Unknown	CTX-M-15	Negative	Positive
12	627	442	Urine	Internal Medicine	CTX-M-15	Negative	Negative
13	628	455	Anal swab	Surgery	CTX-M-15	Negative	Negative
14	629	471	Vaginal swab	Gynecology	CTX-M-15	Negative	Positive
15	631	482	Urine	Psychiatric	CTX-M-15	Negative	Positive
16	632	492	Anal swab	Surgery	CTX-M-15	Negative	Negative
17	633	502	Urine	Orthopedics	CTX-M-15	Negative	Positive
18	634	509	Urine	Urology	CTX-M-15	Negative	Positive
19	636	542	Anal swab	Surgery (Internal)	CTX-M-15	Negative	Positive
20	640	573	Unknown	Unknown	CTX-M-15	Negative	Positive
21	641	586	Unknown	Unknown	CTX-M- 1	Negative	Positive
22	642	593	Urine	Medical clinic	CTX-M-1	Negative	Negative
23	645	390	Urine	Internal Medicine	CTX-M-15	Negative	Positive
24	646	392	Urine	Urology	CTX-M-15	Negative	Positive
25	649	406	Abdomen swab	Medical clinic	CTX-M-15	Negative	Positive
26	650	413	Urine	Medical clinic	CTX-M-15	Negative	Negative
27	652	419	Anal swab	Surgery	CTX-M- 3	Negative	Positive
28	654	421	Urine	Medical clinic	CTX-M-15	Negative	Negative
29	655	422	Urine	Pediatric	CTX-M-15	Negative	Negative
30	657	427	Urine catheter swab	Surgery (Internal)	CTX-M-15	Negative	Negative
31	659	162	Urine	Urology	CTX-M-15	Negative	Positive
32	660	171	Urine	Urology	CTX-M-61	Negative	Positive
33	661	174	Urine	Neurology	CTX-M-15	Negative	Positive
34	662	176	Urine	Urology	CTX-M-15	Negative	Positive
35	663	178	Urine	Internal medicine	CTX-M-15	Negative	Positive
36	666	173	Nasal swab	Medical clinic	CTX-M-15	Negative	Positive
37	668	195	Anal swab	Oper. Intensive Care	CTX-M-15	Negative	Positive
38	670	201	Oral swab	Gynecology	CTX-M-1	Negative	Negative
39	671	202	Urine	Pediatric	CTX-M-15	Negative	Negative
40	672	204	Urine	Medical clinic	CTX-M-15	Negative	Negative
41	673	213	Anal swab	Surgery	CTX-M-15	Negative	Positive

Table II-B2: Characterization of the CTX-M-producing bacterial hosts derived from Giessen University Hospital, Germany.

Serial No.	Strain No.*	Phylogenic type	ST	PFGE type	Resistance to Non B- lactam antimicrobials
1	611	A		C4	Tobramycin and quinolones
2	612	B2		B3	SXT and quinolones
3	613	B2	131	B4	Tetracycline, gentamicin, SXT, tobramycin and quinolones
4	615	A		Non-typeable	None
5	616	D		Non-typeable	Tetracycline, gentamicin, SXT, tobramycin, quinolones and fosfomycin
6	617	A		C5	Tetracycline, SXT, tobramycin and quinolones
7	619	B2	131	B3	Tetracycline, SXT ,tobramycin, quinolones and fosfomycin
8	620	B2		A8	None
9	621	B2		D4	Gentamicin, SXT, tobramycin and quinolones
10	622	D	405	E4	Gentamicin and tobramycin
11	625	D		J1	Tetracycline, SXT and quinolones
12	627	D	405	B9	Tetracycline, gentamicin, SX, tobramycin and quinolones
13	628	D	38	(II)	Tetracycline, gentamicin, SXT, tobramycin and quinolones
14	629	B2		(IV)	Tetracycline, gentamicin, SXT, tobramycin and quinolones
15	631	B2		A12	SXT and quinolone
16	632	A		Non-typeable	Tetracycline, gentamicin, SXT, tobramycin quinolones and fosfomycin
17	633	A		A7	Tetracycline, gentamicin, SXT, tobramycin and quinolones
18	634	B2		A14	Tetracycline, gentamicin, SXT, tobramycin and quinolones
19	636	B2		A10	SXT and quinolone
20	640	B2		C7	Quinolones
21	641	A		K2	Tetracycline, gentamicin, SXT, tobramycin and quinolones
22	642	B1		(V)	SXT and quinolones
23	645	A		J2	Tetracycline, gentamicin, SXT, tobramycin and quinolones
24	646	D		L1	Tetracycline, gentamicin, SXT and quinolones
25	649	A	10	F3	Tetracycline, gentamicin, SXT, tobramycin and quinolones
26	650	A		B10	Tetracycline, gentamicin, SXT, tobramycin and quinolones
27	652	B2		(III)	None
28	654	A		A2	Tobramycin and quinolones
29	655	D		D3	Tetracycline, gentamicin, SXT, tobramycin , quinolones and fosfomycin
30	657	B2		(VI)	None
31	659	B2		D5	Tetracycline, gentamicin, SXT, tobramycin , quinolones and fosfomycin
32	660	A		G3	Tetracycline, SXT and quinolones
33	661	B2		G1	SXT and quinolones
34	662	A		D2	Tetracycline, gentamicin, SXT, tobramycin and quinolones
35	663	D		I1	Tetracycline, gentamicin, SXT, tobramycin and quinolones
36	666	A		G2	Tetracycline and quinolones
37	668	A		K1	Tetracycline, SXT, tobramycin and quinolones
38	670	A		L2	None
39	671	D		I2	Quinolones
40	672	B2		(VII)	Tetracycline, SXT and quinolones
41	673	A		B6	Tetracycline, gentamicin, SXT, tobramycin and quinolones

Table II-B3: Characterization of the large plasmids which were carried by CTX-M-producing *E. coli* isolates derived from Giessen University Hospital, Germany.

Serial No.	Strain* No.	Plasmid replicon type	Plasmid transfer	Co-transferable non ß-lactam resistance	Plasmid (s) Size	CTX-M carrying plasmid
1	611	FIA,FIB			97kb	
2	612	FIA,FIB				
3	613	FIA,FIB	Conjugative	Tetracycline, gentamicin	97kb	
4	615	N				
5	616	FIA,FIB				
6	617	FIA,FIB				
7	619	FIA,FIB			145kb	
8	620	FIB	Conjugative	None	97kb	
9	621	FIA			120kb	
10	622	FIB	Conjugative	Gentamicin	97kb	97 kb
11	625	FIA,FIB			97kb	
12	627	FIA,FIB			97kb	
13	628	FIB				
14	629	FIA,FIB	Conjugative	Gentamicin, SXT and tobramycin	170kb, 97kb	97 kb
15	631	FIA,FIB				
16	632	FIA,FIB	Conjugative	Tetracycline, gentamicin, SXT and tobramycin	170kb	
17	633	FIA,FIB	Conjugative	Tetracycline, gentamicin, SXT and tobramycin	170 kb	170 kb
18	634	FIA,FIB				
19	636	FIA,FIB			170 kb, <48kb	170 kb + Chromosomal copy
20	640	FIA				
21	641	FIA,FIB				
22	642	N	Conjugative	SXT.	48kb	
23	645	Unknown				
24	646	Unknown				
25	649	FIA,FIB	Conjugative	Tetracycline, gentamicin, SXT and tobramycin	170kb	170kb
26	650	FIB				
27	652	I1	Conjugative	None	170kb	
28	654	FIA,FIB			145kb, 97kb	97 kb
29	655	FIA,FIB				
30	657	FIB				
31	659	FIA,FIB				
32	660	FIB				
33	661	FIA,FIB			170kb	
34	662	FIA,FIB	Conjugative	Tetracycline, gentamicin, SXT and tobramycin	170kb	
35	663	FIA,FIB				
36	666	FIB				
37	668	FIA,FIB	Conjugative	SXT	170kb	
38	670	N	Conjugative	None	< 48kb	<48 kb
39	671	FIA,FIB				Chromosomal copy
40	672	FIA,FIB				
41	673	FIA,FIB				

Strain No.*: Laboratory number (Given in this study).

Appendix III

Table (III-A): Distribution of the different *bla*$_{CTX-M}$ allele types on the different German University Hospital-derived *E. coli* isolates of the different phylogenic types.

Phylogenic type	*Bla*$_{CTX-M}$ allele type			
	Bla$_{CTX-M-15}$	*Bla*$_{CTX-M-1}$	*Bla*$_{CTX-M-3}$	*Bla*$_{CTX-M-61}$
A	12	3	0	1
B1	0	1	0	0
B2	14	1	1	0
D	8	0	0	0
Total	34	5	1	1

Table III-B: Association of the different plasmid replicon types with the different *bla*$_{CTX-M}$ alleles among the isolates derived from German University Hospital.

Replicon type	Plasmid replicon type					
	FIA-FIB	FIA	FIB	N	I1	unknown
Bla$_{CTXM-15}$	25	2	5	0	0	2
Bla$_{CTX-M-1}$	1	0	1	3	0	0
Bla$_{CTX-M-3}$	0	0	0	0	1	0
Bla$_{CTM-61}$	0	0	1	0	0	0

Table III-C: The different non ß-lactam resistance patterns of transconjugants corresponding to Egyptian and German clinical *E. coli* isolates.

Non ß-lactam resistance profile for transconjugants	The Number of transconjugants	The number of the corresponding parent clinical isolates	Source
None	7	459, 475, 529 and 483	Egypt
		670, 652 and 620	Germany
Sulfamethoxazol / trimethoprim (SXT)	2	642 and 668	Germany
Gentamicin	1	622	Germany
Tetracycline and Gentamicin	1	613	Germany
Tetracycline and SXT	1	468	Egypt
Tetracycline, Gentamicin and Tobramycin	3	409, 414 and 559	Egypt
Tetracycline, SXT and Tobramycin	1	500	Egypt
Gentamicin, SXT and Tobramycin	1	629	Germany
Tetracycline, Gentamicin, SXT and Tobramycin	5	476	Egypt
		632, 633, 649 and 662	Germany

Table III-D: The number and size of large plasmids in clinical *E. coli* isolates derived from German University Hospital.

Isolates Number	Number of plasmids	Size of plasmids
649, 652, 661 662, 668, 633 and 632	1	170 kb
611, 613, 622, 620, 625 and 627	1	97 kb
670	1	48 kb
619	1	145 kb
621	1	120 kb
642	1	<48
654	2	145 kb, 97 kb
636	2	170 kb, <48 kb
629	2	97kb, 170kb

TableIII-E: The number and size of large plasmids in clinical *E. coli* isolates derived from Egyptian University Hospitals.

Isolates Number	Number of plasmids	Size of plasmids
500, 459	1	97 kb
455	1	120 kb
559, 493	1	145 kb
476	1	170
529	2	97 kb, 194kb
475, 483	2	97 kb, 145kb
409, 414, 468	2	97 kb, 170 kb
518	2	97 kb, 242 kb

Appendix IV

Table IV-A: Clinical zone break Points.

Antibiotic	Clinical zone break Points (mm)		
	Resistent	Intermediate	Susceptible
Ampicillin (AM) 10 µg	≤13	14-16	≥17
Ampicillin/Sulbactam (SAM)10/10 µg	≤11	12-14	≥15
Fosfomycin (FF) 200 µg	≤12	13-15	≥16
Tetracycline (TE) 30 µg	≤14	15-18	≥19
Gentamicin (GM) 120 µg	≤12	13-14	≥15
Sulfamethoxazole/Trimethoprim (SXT) 23.75/1.25 µg	≤10	11-15	≥16
Cefazolin (CZ) 30 µg	≤14	15-17	≥18
Cefpodoxime (CPD) 30µg	≤17	18-20	≥21
Cefuroxime (CXM) 30µg	≤14	15-17	≥18
Tobramycin (NN) 10µg	≤12	13-14	≥16
Ofloxacin (OFL) 5µg	≤12	13-15	≥16
Ciprofloxacin (CIP) 5µg	≤15	16 - 20	≥21
Moxifloxacin (MOX) 5µg	≤15	16-18	≥19
Piperacillin/ tazobactam(P/z) 100/10 µg	≤17	18-20	≥21
Cefotaxime (CTX) 30µg	≤14	15-22	≥23
Ceftriaxone (CRO) 30µg	≤13	14-20	≥21
Amoxicillin/ clavulanic acid (AMC) 20/10 µg	≤13	14-17	≥18
Ceftazidime (CAZ) 30µg	≤14	15-17	≥18
Cefepime (FEP) 30µg	≤14	1 5-17	≥18
Imipenem (IMP) 10µg	≤13	14-15	≥16
Meropenem (MEM) 10µg	≤13	14-15	≥16

Reference: Clinical laboratory Standard Institute (CLSI)

Control strain: *E. coli ATCC 25922*

Appendix V

Appendix V-A: Nucleotide sequence of the detected CTX-M Alleles

CTX-M-1

atggttaaaaaatcactgcgtcagttcacgctgatggcgacggcaaccgtcacgctgttgttaggaagtgtgccgctgtatgcgcaaacggcgg
acgtacagcaaaaacttgccgaattagagcggcagtcgggaggaagactgggtgtggcattgattaacacagcagataattcgcaaatacttt
atcgtgctgatgagcgctttgcgatgtgcagcaccagtaaagtgatggccgtggccgcggtgctgaagaaaagtgaaagcgaaccgaatctgt
taaatcagcgagttgagatcaaaaaatctgacttggttaactataatccgattgcggaaaagcacgtcgatgggacgatgtcactggctgagct
tagcgcggccgcgctacagtacagcgataacgtggcgatgaataagctgatttctcacgttggcggcccggctagcgtcaccgcgttcgcccga
cagctgggagacgaaacgttccgtctcgaccgtaccgagccgacgttaaacaccgccattccgggcgatccgcgtgataccacttcacctcggg
caatggcgcaaactctgcgtaatctgacgctgggtaaagcattgggtgacagccaacgggcgcagctggtgacatggatgaaaggcaatacc
accggtgcagcgagcattcaggctggactgcctgcttcctgggttgtgggggataaaaccggcagcggtgactatggcaccaccaacgatatc
gcggtgatctggccaaaagatcgtgcgccgctgattctggtcacttacttcacccagcctcaacctaaggcagaaagccgtcgcgatgtattagc
gtcggcggctaaaatcgtcaccaacggtttgtaatag

CTX-M-61

atggttaaaaaatcactgcgtcagttcacgctgatggcgacggcaaccgtcacgctgttgttaggaagtgtgccgctgtatgcgcaaacggcgg
acgtacagcaaaaacttgccgaattagagcggcagtcgggaggaagactgggtgtggcattgattaacacagcagataattcgcaaatacttt
atcgtgctgatgagcgctttgcgatgtgcagcaccagtaaagtgatggccgtggccgcggtgctgaagaaaagtgaaagcgaaccgaatctgt
taaatcagcgagttgagatcaaaaaatctgacttggttaactataatccgattgcggaaaagcacgtcgatgggacgatgtcactggctgagct
tagcgcggccgcgctacagtacagcgataacgtggcgatgaataagctgatttctcacgttggcggcccggctagcgtcaccgcgttcgcccga
cagctgggagacgaaacgttccgtctcgaccgtaccgagccgacgttaaacaccgccattccgggcgatccgcgtgataccacttcacctcggg
caatggcgcaaactctgcgtaatctgacgctgggtaaagcattgggtgacagccaacgggcgcagctggtgacatggatgaaaggcaatacc
accggtgcagcgagcattcaggctggactgcctgcttcctgggttgtgggggataaaaccggcagcggtgactatggcaccaccaacgatatc
gcggtgatctggccaaaagatcgtgcgccgctgattctggtcacttacttcacccagcctcaacctaaggcagaaagccgtcgcgatgtattagc
gtcggcggctaaaatcgtcaccgacggtttgtaatag

CTX-M-15

atggttaaaaaatcactgcgccagttcacgctgatggcgacggcaaccgtcacgctgttgttaggaagtgtgccgctgtatgcgcaaacggcgg
acgtacagcaaaaacttgccgaattagagcggcagtcgggaggcagactgggtgtggcattgattaacacagcagataattcgcaaatacttt
atcgtgctgatgagcgctttgcgatgtgcagcaccagtaaagtgatggccgcggccgcggtgctgaagaaaagtgaaagcgaaccgaatctgt
taaatcagcgagttgagatcaaaaaatctgaccttgttaactataatccgattgcggaaaagcacgtcaatgggacgatgtcactggctgagctt
agcgcggccgcgctacagtacagcgataacgtggcgatgaataagctgattgctcacgttggcggcccggctagcgtcaccgcgttcgcccga
cagctgggagacgaaacgttccgtctcgaccgtaccgagccgacgttaaacaccgccattccgggcgatccgcgtgataccacttcacctcggg
caatggcgcaaactctgcggaatctgacgctgggtaaagcattgggcgacagccaacgggcgcagctggtgacatggatgaaaggcaatacc
accggtgcagcgagcattcaggctggactgcctgcttcctgggttgtgggggataaaaccggcagcggtggctatggcaccaccaacgatatc
gcggtgatctggccaaaagatcgtgcgccgctgattctggtcacttacttcacccagcctcaacctaaggcagaaagccgtcgcgatgtattagc
gtcggcggctaaaatcgtcaccgacggtttgtaa

CTX-M-3

Atggttaaaaaatcactgcgccagttcacgctgatggcgacggcaaccgtcacgctgttgttaggaagtgtgccgctgtatgcgcaaacggcg
gacgtacagcaaaaacttgccgaattagagcggcagtcgggaggcagactgggtgtggcattgattaacacagcagataattcgcaaatactt
tatcgtgctgatgagcgctttgcgatgtgcagcaccagtaaagtgatggccgcggccgcggtgctgaagaaaagtgaaagcgaaccgaatctg
ttaaatcagcgagttgagatcaaaaaatctgaccttgttaactataatccgattgcggaaaagcacgtcaatgggacgatgtcactggctgagct
tagcgcggccgcgctacagtacagcgataacgtggcgatgaataagctgattgctcacgttggcggcccggctagcgtcaccgcgttcgcccg
acagctgggagacgaaacgttccgtctcgaccgtaccgagccgacgttaaacaccgccattccgggcgatccgcgtgataccacttcacctcgg
gcaatggcgcaaactctgcggaatctgacgctgggtaaagcattgggcgacagccaacgggcgcagctggtgacatggatgaaaggcaatac
caccggtgcagcgagcattcaggctggactgcctgcttcctgggttgtgggggataaaaccggcagcggtgactatggcaccaccaacgatat
cgcggtgatctggccaaaagatcgtgcgccgctgattctggtcacttacttcacccagcctcaacctaaggcagaaagccgtcgcgatgtattag
cgtcggcggctaaaatcgtcaccgacggtttgtaatag

Appendix V-B: Amino acid sequence comparison of the detected CTX-M variants.

```
CTX-M-1   MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQS
CTX-M-3   MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQS
CTX-M-15  MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQS
CTX-M-61  MVKKSLRQFTLMATATVTLLLGSVPLYAQTADVQQKLAELERQS

CTX-M-1   GGRLGVALINTADNSQILYRADERFAMCSTSKVMAVAAVLKKSESEPNLLNQRVEIKK
CTX-M-3   GGRLGVALINTADNSQILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKK
CTX-M-15  GGRLGVALINTADNSQILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKK
CTX-M-61  GGRLGVALINTADNSQILYRADERFAMCSTSKVMAVAAVLKKSESEPNLLNQRVEIKK

CTX-M-1   SDLVNYNPIAEKHVDGTMSLAELSAAALQYSDNVAMNKLISHVGGPASVTAFARQLGD
CTX-M-3   SDLVNYNPIAEKHVNGTMSLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGD
CTX-M-15  SDLVNYNPIAEKHVNGTMSLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGD
CTX-M-61  SDLVNYNPIAEKHVDGTMSLAELSAAALQYSDNVAMNKLISHVGGPASVTAFARQLGD

CTX-M-1   ETFRLDRTEPTLNTAIPGDPRDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNT
CTX-M-3   ETFRLDRTEPTLNTAIPGDPRDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNT
CTX-M-15  ETFRLDRTEPTLNTAIPGDPRDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNT
CTX-M-61  ETFRLDRTEPTLNTAIPGDPRDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNT

CTX-M-1   TGAASIQAGLPASWVVGDKTGSGDYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESR
CTX-M-3   TGAASIQAGLPASWVVGDKTGSGDYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESR
CTX-M-15  TGAASIQAGLPASWVVGDKTGSGGYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESR
CTX-M-61  TGAASIQAGLPASWVVGDKTGSGDYGTTNDIAVIWPKDRAPLILVTYFTQPQPKAESR

CTX-M-1   RDVLASAAKIVTNGL
CTX-M-3   RDVLASAAKIVTDGL
CTX-M-15  RDVLASAAKIVTDGL
CTX-M-61  RDVLASAAKIVTDGL
```

Table V-B$_1$: The twenty amino acids found in proteins, their single-letter data-base codes (SLC), and their corresponding DNA codons

Amino Acid	SLC	DNA codons
Isoleucine	I	ATT, ATC, ATA
Leucine	L	CTT, CTC, CTA, CTG, TTA, TTG
Valine	V	GTT, GTC, GTA, GTG
Phenylalanine	F	TTT, TTC
Methionine	M	ATG
Cysteine	C	TGT, TGC
Alanine	A	GCT, GCC, GCA, GCG
Glycine	G	GGT, GGC, GGA, GGG
Proline	P	CCT, CCC, CCA, CCG
Threonine	T	ACT, ACC, ACA, ACG
Serine	S	TCT, TCC, TCA, TCG, AGT, AGC
Tyrosine	Y	TAT, TAC
Tryptophan	W	TGG
Glutamine	Q	CAA, CAG
Asparagine	N	AAT, AAC
Histidine	H	CAT, CAC
Glutamic acid	E	GAA, GAG
Aspartic acid	D	GAT, GAC
Lysine	K	AAA, AAG
Arginine	R	CGT, CGC, CGA, CGG, AGA, AGG
Stop codons	Stop	TAA, TAG, TGA

http://www.cbs.dtu.dk/courses/27619/codon.html

Table V- C: Summary of Nucleotide and amino acid sequences alignment

Mutation	T / C	A / C	T / C	T / C, G / T	G / A	T / G	T / G	T / C	A / G	A / G
Mutation position	21	138	239	313, 315	349	427	582	609	725	865
Involved codon	cgt/cgc	gga/ggc	gtg/gcg	ttg/ctg	gat/aat	tct/gct	cgt/cgg	ggt/ggc	gac/ggc	aac/gac
Aminoacid position	7	46	80	105	116	143	194	203	242	289
Resulting Aminoacid substitution	Argnine	Glycine	Valine / Alanine	Leucine	Aspartic acid/ Asparagine	Serine/ Alanine	Arginine	Glycine	Aspartic acid / Glycine	Asparagine /Aspartic acid
Effect of mutation	silent	silent	coding	silent	coding	coding	silent	silent	coding	coding
CTX-M-1	T	A	T	T , G	G	T	T	T	A	A
CTX-M-61	T	A	T	T , G	G	T	T	T	A	G
CTX-M-22	C	C	C	C , T	A	G	G	C	A	A
CTX-M-28	T	C	C	C , T	A	G	G	C	G	A
CTX-M-15	C	C	C	C , T	A	G	G	C	G	G
CTX-M-3	C	C	C	C, T	A	G	G	C	A	G

List of publications

M.Ahmed, T.R. El-khamissy., E. Domann., M.B.E. El Hadidy., W. Mohamed., T. Chakraborty., C. Imirzalioglu (2012): Comparative Study for the Molecular and Epidemiological Characteristics of CTX-M producing Escherichia coli Isolates derived from University hospital in Egypt and Germany. In 11th Congress of Infectious Diseases and Tropical Medicine (11.Kongress für Infektionskrankheiten und Tropenmedizin, KIT2012), abstract.

M. Ahmed., T.R. El-khamissy., E. Domann., M.B.E. El Hadidy., W. Mohamed., and T. Chakraborty., C. Imirzalioglu (2012): Comparative Study for the Molecular and Epidemiological Characteristics of CTX-M producing Escherichia coli Isolates derived from University hospital in Egypt and Germany. On preparation.

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Ort, Datum Unterschrift

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