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Prevalence and molecular epidemiology of extended spectrum β-lactamase producing *Escherichia coli* from hospital and community settings in Egypt

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ABSTRACT

The prevalence and molecular epidemiology of *Escherichia coli* that produce extended spectrum β-lactamase (ESBL) in Cairo, Egypt was investigated. Ninety *E. coli* isolates were collected along the period of September to November 2012 from hospital and community settings. Antibiotic susceptibility of the *E. coli* isolates was determined by disk diffusion method. All isolates were screened phenotypically for ESBL production by combination disk method. The presence of ^{bla}CTX-M-I, ^{bla}CTX-M-IV, ^{bla}TEM and ^{bla}SHV genes in ESBL-producing *E. coli* was examined by PCR and sequencing experiments. The results showed high prevalence of ESBL-producing *E. coli*, 52% of the collected isolates were ESBL producers. The ESBL-producing isolates significantly ($P < 0.05$) had increased resistance compared with non–ESBL producers to cefuroxime, cefotaxime, ceftazidime, cefepime, ciprofloxacin, and co-trimoxazole. Imipenem was the most effective drug against ESBL producing isolates. All ESBL producing *E. coli* isolates were multi drug resistant (MDR) to eight antibiotics or more. Detection of ESBL genes in selected MDR-ESBL producing *E. coli* revealed that ^{bla}CTX-M-I was the most prevalent ESBL type. It is clear that the prevalence of ESBL producing *E. coli* in Cairo, Egypt is alarming high. This study is useful for clinician in order to improve the empiric treatment.

INTRODUCTION

In the early 1980s, third-generation cephalosporins were introduced to the clinical practice as β-lactam antibiotics able to overcome resistance caused by the common β-lactamases produced by *Escherichia coli* (Livermore, 2012). However, within few years; *E. coli* produced mutated versions of these β-lactamases called extended spectrum β-lactamases (ESBLs) which enable them to neutralize the activity of expanded-spectrum cephalosporins, and monobactams (Livermore, 2012). ESBLs are enzymes capable of conferring bacteria resistance to penicillins, 1st, 2nd, 3rd, 4th generation cephalosporins, and monobactams by hydrolysis of these antibiotics. ESBLs do not hydrolyze cephamycins (e.g., cefoxitin or cefotetan) or carbapenems (e.g., imipenem and meropenem), and they are inhibited by β-lactamase inhibitors such as clavulanic acid (Paterson and Bonomo, 2005). The majority of ESBLs belongs to class A Ambler classification which includes the ^{bla}SHV, ^{bla}TEM, and ^{bla}CTX-M types (Paterson and Bonomo, 2005). Since 2000, *E. coli* producing ^{bla}CTX-M have emerged worldwide as an important cause of community-onset UTIs (Lahlaoui *et al.*, 2014). ESBLs are mostly encoded by large plasmids (up to 100 kb and even more) that are transferable from strain to strain and between bacterial species (AitMhand *et al.*, 2002). Genes encoding ESBLs are frequently found on the same plasmid as genes encoding resistance for other classes of antibiotics such as aminoglycosides, tetracyclines, and sulfonamides. Taking into the account, many of *E. coli* strains possess chromosomal changes that confer resistance to fluoroquinolones (Livermore, 2012). As a result, ESBL producing *E. coli* is frequently multidrug resistant (MDR), posing particular difficulties in the treatment of infections, especially in critically ill patients. ESBL-producing *E. coli* have recently reported in Egypt (Abdul Rahman and El-Sherif, 2011). A more comprehensive survey of ESBLs from Egypt is urgently needed not only for the hospital setting but also for the community. This study was carried out to determine the prevalence of ESBL-producing *E. coli* in hospital and community settings in Cairo, Egypt and to assess antibiotic susceptibility patterns of these isolates in order to define appropriate antimicrobial therapy. We are also aimed to reveal basic aspects of the molecular epidemiology of these isolates in Cairo, Egypt.

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MATERIALS AND METHODS

Bacterial isolates

Bacterial isolates were collected along the period of September to November 2012 from three hospitals (University of Ain Shams Hospital, El-Salam International Hospital, National Cancer Institute) and two community clinical labs (Cairo Lab - Helwan branch, Shaker Lab - Misr El-Gedida branch). The isolates were non-consecutive (Only a single positive culture per patient). Isolates were streaked on Tryptic soy agar medium to ensure purity and viability.

Isolates that were gram-negative, lactose-fermenting, non-swarming, indole positive, oxidase negative, producing acid slant/acid butt reaction with or without gas on triple sugar iron medium test, citrate negative and urease negative identified as *E. coli* (Engelkirk and Duben-Engelkirk, 2007). Isolates identified as *E. coli*, were stored on glycerol Tryptic soy broth at -20° C freezer. When a fresh seed-stock vial is required, it was removed and used to inoculate a series of working cultures. Quality control strains; *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were kindly provided by the U.S. naval medical research unit no. 3 (NAMRU-3) in Egypt.

Antibiotic susceptibility testing and phenotypic ESBL detection

Antibiotic susceptibility of the *E. coli* isolates was determined by Kirby-Bauer disk diffusion method according to CLSI recommendations (Bauer *et al.*, 1966; CLSI 2009). Antibiotics to be tested; were selected referring to CLSI document M100-S21 (CLSI, 2011). Antibiotics discs were obtained from Oxoid (UK) and they included cephalothin (30 µg), cefuroxime (30 µg), co-amoxiclav (20/10 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), imipenem (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), and co-trimoxazole (1.25/23.75 µg).

The diameters of the inhibition zones were interpreted by referring to CLSI document M100-S21 (CLSI, 2011), and the examined isolates were reported as susceptible, intermediate, or resistant to the agents under test. Control strain used to validate susceptibility tests was *E. coli* ATCC 25922. Quality control strain was tested daily to ensure the test system is working and gives results within specified limits.

All tested *E. coli* isolates were screened for ESBL production. Combination disk method as recommended by (CLSI, 2011) was used. ESBL Kits were obtained from Himedia (India) and they included cefotaxime (30 µg), cefotaxime/clavulinic acid (30/10 µg), ceftazidime (30 µg), and ceftazidime/clavulinic acid (30/10 µg).

ESBL testing requires use of both cefotaxime and ceftazidime, alone and in combination with clavulinic acid, ≥ 5-mm increase in a zone diameter for either antibiotic (ceftazidime or cefotaxime) tested in combination with clavulinic acid versus its zone when tested alone was determined to be ESBL producer. *E. coli* ATCC 25922 (negative control) and *Klebsiella pneumoniae* ATCC 700603 (positive control) were used as quality control strains.

Statistical analysis

Fisher exact test (Agresti, 1992) was used as a statistical tool to determine the significance of difference between the resistance level to various antibiotics in ESBL and non ESBL producing isolates, $P < 0.05$ was considered significant.

Detection of ESBL genes

PCR amplification

Boiling lyses method was used for extraction of DNA from isolates under test (Moore *et al.*, 2004). *Klebsiella pneumoniae* ATCC 700603 was used as positive ESBL producer (^{bla}SHV-type). Primers (Biosearch Technologies, USA) were purchased in lyophilized form. DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, USA) containing (DreamTaq DNA polymerase, 2X DreamTaq green buffer, dATP, dCTP, dGTP, dTTP, 0.4mM each, and 4mM $MgCl_2$) was used. PCR amplification was used to identify the presence of ^{bla}CTX-M genes using specific primers that targeted ^{bla}CTX-M group I and IV (Pitout *et al.*, 2004). The presence of genes encoding ^{bla}TEM and ^{bla}SHV were also analyzed by PCR (Hanson *et al.*, 1999). The primers sequence, sizes of the expected amplification product and nucleotide positions are listed in Table 1.

Amplifications of ESBL genes were carried out on a DNA Thermal Cycler instrument (PTC-100; MJ Resrach Inc, USA). The composition of the reaction mixture was as follows: 12.5 µl of DreamTaq Green PCR Master Mix (2X), 3 µl of bacterial lysate, 1 µl of each primer (10 pM/ µl), and nuclease-free water was added to complete a final PCR reaction volume to 25 µl; the mixture was mixed gently before running the PCR program. The PCR program consisted of an initial denaturation step at 96°C for 4 minutes, followed by 24 cycles of; DNA denaturation at 96°C for 30 seconds, primer annealing at 55°C for 30 seconds, and primer extension at 72°C for 2 min, and final elongation step at 72°C for 10 minutes (Pitout *et al.*, 1998). Aliquots of 10 µl of PCR products were analyzed by gel electrophoresis with 1.6 % agarose gels in 1× TAE buffer for 30 minutes at 90 V. Gels were stained with 2 µl ethidium bromide (10 mg/L), visualized and compared to 100 bp DNA ladder (fisher bioreagents, Canada) by UV transilluminator.

Sequencing of ESBL genes

Randomly selected products from the CTX-M group I and CTX-M group IV PCRs were sequenced on an Applied Biosystems 3100 DNA Analyser to identify the specific ESBL type. The primers used for sequencing were the same as those used for PCR. The PCR products were purified with high pure PCR product purification kits (Roche Molecular Biochemicals, Espoo, Finland). ABI BigDyeTM terminator cycle sequencing kit version 3.1 (Applied Biosystems, Espoo, Finland) was used. The DNA sequences were analysed and translated into amino acid sequences with DNA sequencing analysis software, version 5.3.1 (Applied

Biosystems, Espoo, Finland). Amino acid sequences were compared to the known CTX-M variants by BLAST at website search (http://www.ncbi.nlm.nih.gov/blast).

RESULTS AND DISCUSSION

ESBLs are now commonly found in *E. coli* isolates from patients in nursing homes and long term- care facilities, and even in patients with community-acquired infections (Lahlaoui *et al*., 2014). In this study; during the 3 months study period; a total of 90 *E. coli* isolates were collected from three hospitals included University of Ain Shams Hospital (32 isolates), El Salam International Hospital (32 isolates), National Cancer Institute (6 isolates), and two clinical community labs included Cairo Lab – Helwan branch (10 isolates), Shaker Lab - Misr El Gedida branch (10 isolates). The majority of isolates were from urine samples (67 isolates). There were 7 isolates from stool samples, 5 from sputum samples, 4 from pus samples, 2 from groin samples, 2 from drainage samples, and 3 isolates from miscellaneous sites.

High prevalence level of ESBL producing *E. coli* was recorded in the present study, as of the 90 *E. coli* isolates collected; 47 (52%) were ESBL producers (Table 2). Many studies from Egypt also recorded high percentage of ESBL production among *E. coli* isolates. In a study carried out at Cairo University Hospitals, 400 bacterial isolates from 632 stool samples were found to be ESBL producers. Out of these 400 isolates, 285 (71.25%) were identified as *E. coli* (Abdul Rahman and El-Sherif, 2011). In another study from Egypt, which included only blood stream infections in patients admitted to intensive care units (ICUs), the proportion of ESBL-producing *E. coli* was 39% (Saied *et al.*, 2011). In contrast to our results, low prevalence level of ESBL-producing *E. coli* was reported in many African countries such as Morocco (1.3%) (Barguigua, *et al.*, 2011), Nigeria (12.8%) (Aibinu *et al.*, 2012), and South Africa (7.6%) (Brink *et al.*, 2012). Prevalence of ESBL producing *E. coli* isolates in the United States and Europe was lower than that reported in Egypt. Bhusal, *et al.* (2011) reported that out of 443 *E. coli* isolated from cancer patients at a cancer center in USA, only 41 (9.2%) isolates were ESBL producers.

Also, Hawser *et al.* (2011) examined 3160 isolates of *E. coli* collected from 44 hospitals in different European countries (i.e. France, Germany, Greece, Romania, Spain, Turkey, Estonia, Italy, Latvia, Lithuania, Portugal, and UK), and found that only 11% of isolates were ESBL producers. Variable percentage of ESBL-producing *E. coli* was reported in Asia by different workers, for example, a study from India reported that 30% of *E. coli* isolates obtained from different clinical samples were found to be ESBL producers (Agrawal *et al.*, 2008). On the other hand; in Taiwan; Hsieh *et al.* (2010) conducted a study on hospitalized patients with *E. coli* bacteremia, and found that the frequency of ESBL producers was 4.7%. As it was expected, all ESBL producing *E. coli* isolates in this study were resistant to all cephalosporins tested (Table 2).

It was also noted that ESBL producing *E. coli* had significantly ($P< 0.05$) diminished susceptibility compared with non ESBL producers for all cephalosporin tested (cefuroxime, cefotaxime, ceftazidime, ceftriaxone, cefepime), except cephalothin. This finding is in accordance with other reports (Koksal *et al.*, 2009; Gorgec *et al.*, 2015). Some studies mentioned that co-amoxiclav may have considerable antimicrobial activity against ESBL producing *E. coli* isolated in the community, and it may constitute an effective therapeutic option for community-acquired urinary tract infections caused by ESBL-producing *E. coli* (Falgas *et al*., 2008).

Table 1: Primers used for amplification of DNA of ESBL genes.

Targets	Primers	Sequence 5' to 3'	Product size	Nucleotide positions
CTX-M group I	(forward)	GAC GAT GTC ACT GGC TGA GC	499	416–435
	(reverse)	AGC CG C CGA CGC TAA TAC A		914–896
CTX-M group IV	(forward)	GCT GGA GAA AAG CAG CGG AG	474	1857–1876
	(reverse)	GTA AGC TGA CGC AAC GTC TG		2330–2311
TEM	(forward)	AGA TCA GTT GGG TGC ACG AG	750	313-332
	(reverse)	TGC TTA ATC AGT GAG GCA CC		1061-1042
SHV	(forward)	GGGAAACGGAACTGAATGAG	149	606-625
	(reverse)	ATCGTCCACCATCCACTGCA		757-738

Group I includes CTX-M-1, -3, -10 to -12, -15 (UOE-1), -22, -23, -28, -29, and -30
Group IV includes CTX-M-9, -13, -14, -16 to -19 and -21, and -27 and Toho-2.

Table 2: The antimicrobial resistance percentages of ESBL-producing and non ESBL-producing *E. coli* isolates.

Antibiotics	*E. coli* isolates (n=90)				*P-value*	Resistant isolates	
	ESBL-producing (n=47: 52%)		non ESBL-producing (n=43: 48%)				
	n	%	n	%		n	%
Cephalothin	47	100	41	95	*0.225*	88	98
Cefuroxime	47	100	30	70	*<0.05*	77	86
Co-amoxiclav	34	72	33	77	*0.809*	67	74
Cefotaxime	47	100	17	39	*<0.05*	64	71
Ceftazidime	47	100	11	26	*<0.05*	58	64
Ceftriaxone	47	100	8	19	*<0.05*	55	61
Cefepime	47	100	11	26	*<0.05*	58	64
Imipenem	0	--	0	--	N/D	0	--
Gentamicin	20	43	15	35	*0.519*	35	39
Ciprofloxacin	40	85	18	42	*<0.05*	58	64
Co-trimoxazole	42	89	29	67	*<0.05*	71	79

The difference in resistance level between ESBL and non ESBL isolates was significant at $p < 0.05$.

In our study; ESBL *E. coli* showed high resistance percentage against co-amoxiclav (72%) which might suggest that co-amoxiclav is not the suitable drug for treatment of infections caused by ESBL producing *E. coli* in our country.

Co-resistance to non-β-lactam antibiotics is common among ESBL producers, especially for fluoroquinolones, co-trimoxazole and/or aminoglycosides. In this study, the obtained results revealed that; resistance level to ciprofloxacin and co-trimoxazole was highly significant in ESBL producing *E. coli* in comparison with non ESBL producing isolates, however; there was no significant difference in the resistance level to gentamicin between ESBL producing and non ESBL producing *E. coli* (Table 2).

Other studies reported high degree of resistance to fluoroquinolones and co-trimoxazole and less resistance degree to gentamicin among ESBL producing *E. coli* when compared to non-ESBL producing *E. coli* (Chander and Shrestha, 2013, Somily, *et al.*, 2014). In contrast to our results; some studies reported significant difference of resistance level to gentamicin between ESBL producing and non ESBL producing *E. coli* (Cagan Aktas *et al.*, 2014).

All ESBL producing *E. coli* isolates in this study were susceptible to imipenem (Table 2), indicating that it could be the proper drug for treating serious infections caused by ESBL producing *E. coli*. Many studies reported high susceptibility rate to imipenem (100%) among ESBL producing *E. coli* (Hawser *et al*., 2011, El-Bouamri *et al.*, 2014). In this study, *E. coli* isolates expressed multi drug resistance (MDR) phenotype at high level. Eighty six (95%) isolates were found to be resistant to 3 or more antibiotic classes (Table 3).

MDR level was higher among ESBL producers than in non-ESBL producers; all ESBL producing *E. coli* were resistant to 8 antibiotics or more. Other workers also found high proportions of multidrug resistance among ESBL producers (Serefhanoglu *et al.*, 2009; Chander and Shrestha, 2013). High resistance level to all antibiotics used in this study with the exception of imipenem and high prevalence of MDR-ESBL producing *E. coli* detected in our study as well as in the previous reports from Egypt compared to other countries, may be attributed to the uncontrolled consumption of large amount of antibiotics by patients in Egypt.

A patient's previous exposure to an antibiotic, especially to extended spectrum cephalosporins, and fluoroquinolones has been widely reported as a risk factor for infection with ESBL-producing bacteria (Hsieh *et al*., 2010; Goulenok *et al*., 2013). Twelve ESBL-producer *E. coli* isolates; which showed resistance to 10 antibiotics in the test panel (Table 3); were further subjected to PCR assay for the detection of ESBL genes and the results revealed that ^{bla}CTX-M-I was the most prevalent gene type (Table 4).

Co-production of ^{bla}CTX-M (^{bla}CTX-M-I or ^{bla}CTX-M-IV) with ^{bla}TEM enzymes was observed in 8 isolates. Sequencing of one amplicon from ^{bla}CTX-M-I group, was characterized as ^{bla}CTX-M-15 enzyme, the sequence obtained was submitted to GenBank under KP325147.1 accession number. Sequencing of one amplicon from ^{bla}CTX-M-IV group was characterized as ^{bla}CTX-M-14 enzyme, the sequences obtained was submitted to GenBank under NG_041766.1 accession number.

Table 3: Antibiotic resistance pattern of the multi drug resistant (MDR) *E. coli* isolates.

Resistance patterns	Number of isolates	
	ESBL-producers	Non ESBL-producers
Resistance to (3) antibiotics		
CL, AMC, CN	0	2
CL, CXM, AMC	0	2
Other patterns	0	5
Resistance to (4) antibiotics		
CL, CXM, AMC, SXT	0	3
CL, AMC, CN, SXT	0	2
CL, AMC, CIP, SXT	0	2
Other patterns	0	2
Resistance to (5) antibiotics		
CL, CXM, AMC, CN, SXT	0	2
Other patterns	0	2
Resistance to (6) antibiotics		
CL, CXM, AMC, CTX, CIP, SXT	0	2
Other patterns	0	3
Resistance to (7) antibiotics		
CL, CXM, AMC, CTX, CRO, FEP, SXT	0	1
CL, CXM, CTX, CAZ, CRO, FEP, SXT	0	1
Other patterns	0	4
Resistance to (8) antibiotics		
CL, CXM, CTX, CAZ, CRO, FEP, CIP, SXT	7	1
CL, CXM, CTX, CAZ, CRO, FEP, CN, CIP	3	0
CL, CXM, CTX, CAZ, CRO, FEP, CN, SXT	3	0
CL, CXM, AMC, CTX, CAZ, CRO, FEP, CIP	2	0
CL, CXM, AMC, CTX, CAZ, CRO, FEP, SXT	2	0
Other patterns	0	1
Resistance to (9) antibiotics		
CL, CXM, AMC, CTX, CAZ, CRO, FEP, CIP, SXT	16	3
CL, CXM, AMC, CTX, CAZ, CRO, FEP, CN, SXT	2	0
Resistance to (10) antibiotics		
CL, CXM, AMC, CTX, CAZ, CRO, FEP, CN, CIP, SXT	12	1

CL: Cephalothin; CXM: Cefuroxime; AMC: Co-amoxiclav; CTX: Cefotaxime; CAZ: Ceftazidime; CRO: Ceftriaxone; FEP: Cefepime; IPM: Imipenem; CN: Gentamicin; Cip: Ciprofloxacin; SXT: Co-trimoxazole

Table 4: Occurrence of different ESBL genes among the selected 12 MDR-ESBL *E. coli* isolates.

ESBL genes	Number of *E. coli* isolates
^{bla}CTX-M-I group	11
^{bla}CTX-M-I group only	4
^{bla}CTX-M-I group plus ^{bla}TEM	7
^{bla}CTX-M-IV group	1
^{bla}CTX-M-IV group only	0
^{bla}CTX-M-IV group plus ^{bla}TEM	1
^{bla}TEM only	0
^{bla}SHV	0

Many studies also reported that ^{bla}CTX-M-I was the most prevalent type among ESBL producing *E. coli* in Egypt. For example, Fam *et al.* (2011) collected 47 *E. coli* isolates from all specimens' types at Theodor Bilharz research institute in Egypt, and examined them for presence of ESBL genes. The obtained

results showed that the all 47 isolatess produced ^{bla}CTX-M-15 which belongs to ^{bla}CTX-M-I group. In another study, also carried out at Theodor Bilharz research institute on 44 *E. coli* isolates collected from stool samples; ^{bla}CTX-M-I group was the most prevalent enzyme; detected in 29 isolates, whereas ^{bla}CTX-M-IV group genes were detected in 14 isolates (Fam *et al.*, 2014). Co-production of ^{bla}CTX-M and ^{bla}TEM in ESBL producing *E. coli* was previously reported in other studies from Egypt (Al-Agamy *et al.*, 2006) and Turkey (Gorgec *et al*., 2015).Worldwide; several studies reported that ^{bla}CTX-M was the most prevalent ESBL type among ESBL producing *E. coli* (Hoban *et al.*, 2014; Wang *et al.*, 2014, Gorgec *et al*., 2015).

CONCLUSION

Our data pointed out that the prevalence of ESBL-producing *E. coli* is high in Cairo, Egypt. Carbapenems should be regarded as the drugs of choice for serious infections with ESBL producing *E. coli*. If the causative agent has been found as susceptible to other antibiotics such as aminoglycosides, they can be used in the treatment of non-life-threatening infections caused by ESBL producing *E. coli* to reduce carbapenem utilization. The ^{bla}CTX-M-I group was the most prevalent ESBL type, especially in combination with blaTEM enzymes. Continued surveillance, appropriate use of antibiotics, and implementation of strict infection control measures are recommended to decrease ESBL frequency.

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