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Characterization of CTX-M ESBLs in *Enterobacter cloacae*, *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates from Cairo, Egypt

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Abstract

Background: A high rate of resistance to 3rd generation cephalosporins among Enterobacteriaceae isolates from Egypt has been previously reported. This study aims to characterize the resistance mechanism (s) to extended spectrum cephalosporins among resistant clinical isolates at a medical institute in Cairo, Egypt.

Methods: Nonconsecutive *Klebsiella pneumoniae* (Kp), *Enterobacter cloacae* (ENT) and *Escherichia coli* (EC) isolates were obtained from the clinical laboratory at the medical institute. Antibiotic susceptibility was tested by CLSI disk diffusion and ESBL confirmatory tests. MICs were determined using broth microdilution. Isoelectric focusing (IEF) was used to determine the pI values, inhibitor profiles, and cefotaxime (CTX) hydrolysis by the β-lactamases. PCR and sequencing were performed using $bla_{\text{CTX-M}}$ and IS*Ecp1*-specific primers, with DNA obtained from the clinical isolates. Conjugation experiments were done to determine the mobility of $bla_{\text{CTX-M}}$.

Results: All five clinical isolates were resistant to CTX, and were positive for ESBL screening. IEF revealed multiple β-lactamases produced by each isolate, including a β-lactamase with a pI of 8.0 in Kp and ENT and a β-lactamase with a pI of 9.0 in EC. Both β-lactamases were inhibited by clavulanic acid and hydrolyzed CTX. PCR and sequence analysis identified $bla_{\text{CTX-M-14}}$ in Kp and ENT and a $bla_{\text{CTX-M-15}}$ in EC. Both $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ were preceded by IS*Ecp1* elements as revealed by partial sequence analysis of the upstream region of the $bla_{\text{CTX-M}}$ genes. $bla_{\text{CTX-M-15}}$ was transferable but not $bla_{\text{CTX-M-14}}$.

Conclusion: This is the first report of CTX-M-14 in Kp and ENT isolates from Egypt, the Middle East and North Africa.

Background

Recent studies on Enterobacteriaceae isolates from Egypt have reported a resistance rate to third generation cephalosporins of 70% [1,2]. A survey, carried out in 2001–2002 and covered medical centers in Northern and Southern European countries, Egypt, Lebanon, Saudi Arabia

and South Africa, reported the highest incidence of extended spectrum β-lactamases (ESBLs)-producing isolates in Egypt [3].

CTX-M ESBLs are the most prevalent ESBLs worldwide [4]. Recently, CTX-M ESBLs have been reported in Egypt [5], with CTX-M-15 being the most common ESBL reported in the Middle East region and North Africa [6,5,7]. However, CTX-M-14 has also been detected in *Escherichia coli* isolates from Egypt and Tunisia [5,8]. But CTX-M-14 has not been reported in *Klebsiella pneumoniae* isolates in this geographical region before.

CTX-Ms are class A ESBLs that are most active against cefotaxime [9]. However, some CTX-Ms can hydrolyze ceftazidime such as CTX-M-15 and CTX-M-19 [10,11]. The nucleotide sequences of $bla_{\text{CTX-M}}$ genes are highly related to the nucleotide sequence of *Kluyvera spp.* [12,13].

Clinical isolates of *K. pneumoniae*, *Enterobacter cloacae* and *E. coli* were sent from the clinical microbiology laboratory in a medical institute in Cairo, Egypt to investigate the mechanism (s) responsible for resistance to extended spectrum cephalosporins.

Methods

Bacterial strains

Five clinical isolates were sent on blood agar plates from the clinical laboratory at the medical institute. The isolates were three nonconsecutive *K. pneumoniae* isolates and one *E. coli* isolate, which were collected from chest wound swabs from patients in an adult surgical ICU ward. In addition, one *E. cloacae* isolate was obtained from central venous line of a patient in the pediatric ICU ward. Informed written consents were obtained from patients. Identification of the isolates was performed using Phoenix® bacterial identification panels (NMIC/ID-107) and API® 20E strips (Biomerieux SA, Marcy-l'Etoile, France).

Susceptibility test

Antibiotic susceptibility was tested using disk diffusion with the following drugs: cefotaxime, ceftazidime, tetracycline, gentamicin, amikacin, ciprofloxacin, and sulfamethoxazole. ESBL production was investigated using cefotaxime and ceftazidime, alone and in combination with clavulanic acid (BBL, Beckton Dickinson, Sparks, MD., USA) as recommended by the Clinical Laboratory Standard Institute [14]. The minimum inhibitory concentrations (MICs) of cefpodoxime, cefepime, cefoxitin, aztreonam, and imipenem, and the β-lactam/β-lactamase inhibitor combinations: cefpodoxime/clavulanate, and cefepime/clavulanate were determined by broth microdilution according to CLSI guidelines [14] using TREK microbroth dilution panels (Cleveland, Ohio, USA).

β-lactamase characterization

Crude β-lactamase extracts from the clinical isolates and strains producing reference β-lactamases were assessed for β-lactamase pI values, inhibitor and substrate characteristics by isoelectric focusing (IEF) [15].

β-lactamase gene identification and analysis of upstream region

PCR amplification was used to identify the presence of $bla_{\text{CTX-M-15-like}}$ in the *E. coli* clinical isolate, and $bla_{\text{CTX-M-14-like}}$ in *K. pneumoniae* and *E. cloacae* isolates using specific primers that targeted CTX-M group I and IV; respectively [16]. The presence of genes encoding TEM and SHV enzymes was analyzed by PCR [17]. The $MgCl_2$ concentration used was 2 mM for bla_{TEM} and bla_{SHV} PCR and 1.5 mM for $bla_{\text{CTX-M}}$ PCR. Template DNA preparation and PCR amplifications were carried out as previously described [17].

PCR amplification and sequencing of the full-length $bla_{\text{CTX-M-14-like}}$ gene was performed, using primers that flanked the gene (CTXM14 F1 5'-GAG TGT TGC TCT GTG GAT AAC-3', designed using accession number AF252622 and annealing at positions 1857–1876; and CTX14R4 5'-GTT ACA GCC CTT CGG CGA TG-3' designed using accession number AF252622 and annealing at positions 2617-2598). Sequence analysis of the $bla_{\text{CTX-M-15-like}}$ gene was done using CTX3 FLF 5'-CGT CTC TTC CAG AAT AAG G-3', designed using accession number AY995205 and annealing at positions 169–187; and CTX3 FLR 5'-GTT TCC CCA TTC CGT TTC CGC-3' designed using accession number AY995205 and annealing at positions 1092-1072).

Sequence analysis of the 524 bp upstream region of the structural gene for $bla_{\text{CTX-M-15}}$ was performed on an amplified product generated using primers IS*Ecp1* (AGC CAA ATA CGA CAT GGC GGT G, this primer corresponds to nucleotide numbers 1179 to 1200 of the sequence with accession no. DQ658222) and CTX15 (CTT CCT AAC AAC AGC GTG AC, this primer corresponds to nucleotide numbers 261-242 of the sequence with the accession number AY995205). The 184 bases upstream of the $bla_{\text{CTX-M-14}}$ were sequenced using the CTX14 upstream primer (GCA CCT GCG TAT TAT CTG C, this primer corresponds to nucleotide numbers 184-166 of the sequence with the accession number DQ359215).

Five microliters aliquots of PCR products were analyzed by gel electrophoresis with 1% agarose gels (BioRad, Hercules, Calif.) in TAE buffer. Gels were stained with ethidium bromide (10 mg/L) and visualized by UV transilluminator.

The PCR products were purified with Microcon YM-50 columns (Micon bioseparations, Bedford, MS, USA). The amplicons were sequenced using automated PCR cycle sequencing with dye terminator chemistry using ABI PRISM 3100 Genetic Analyzer and Data collection software (version 3.7).

The nucleotide and deduced amino acid sequences were analyzed and compared using BLAST software available online at http://www.ncbi.nlm.nih.gov/BLAST.

Conjugation experiments

To determine whether the cefotaxime resistance was carried on a conjugative plasmid, conjugation experiments were performed with *K. pneumoniae* (only one isolate was tested), *E. coli* and *E. cloacae* as donors and the *E. coli* (Na azide^R) as the recipient. The filter mating technique was carried out as previously described [18]. Transformants were selected on Mueller Hinton agar plates containing sodium azide 200 mg/L and cefotaxime 2 mg/L and were confirmed for bla_{CTX-M} genes using PCR as described above.

Results and discussion

Antimicrobial susceptibility

Disk diffusion showed that all isolates were resistant to cefotaxime and positive for ESBL production by disk confirmatory test using cefotaxime/clavulanate and ceftazidime/clavulanate (Table 1). The MICs of β-lactams and β-lactam/inhibitor combinations were determined by broth microdilution technique. All clinical isolates were resistant to cefpodoxime, cefepime and resistant or intermediately resistant to aztreonam. The phenotypic ESBL microdilution confirmatory test was positive, showing a decrease by 7 doubling dilutions in the presence of clavulanic acid (Table 2). The *K. pneumoniae* clinical isolates were also resistant to other non-β-lactam antibiotics such as tetracycline, gentamicin and fluoroquinolones (Table 1).

Isolates of the Enterobacteriaceae producing CTX-M ESBLs are resistant to cefotaxime (MICs ≥ 64 mg/L) [9] and cefepime (MICs ≥ 32 mg/L) [19-22], but are susceptible or intermediate to ceftazidime [9]. The phenotypic characteristics of the clinical isolates in this study suggested the presence of CTX-M ESBLs. Screening using ceftazidime alone is not sufficient for organisms producing CTX-M ESBLs [16]. However, CTX-M-15 has been reported to possess some hydrolytic activity against ceftazidime [10]. The *E. coli* isolate producing CTX-M-15 was intermediate to ceftazidime using disk susceptibility test (Table 1).

Characterization of β-lactamases

Isoelectric focusing (IEF) of crude sonicates of the clinical isolates was done by a cefotaxime/β-lactamase inhibitor overlay technique. Two enzymes focused at pI values of 8.0 and 9.0, were inhibited by clavulanic acid, and showed an extended spectrum of activity by hydrolyzing cefotaxime (Table 2). PCR and sequence analysis identified $bla_{CTX-M-14}$ in one isolate of *K. pneumoniae* (KP 4) and the *E. cloacae* isolate, and $bla_{CTX-M-15}$ in the *E. coli* clinical isolate (Table 2). Only one *K. pneumoniae* isolate was evaluated by sequence analysis because all three of the *K. pneumoniae* isolates showed the same enzymes on the IEF gel (Table 2).

All isolates produced multiple β-lactamases that were inhibited by clavulanate: *K. pneumoniae* (pI values 5.4, 6.3, 7.6, and 8.0), *E. cloacae* (pI values 6.3, 7.6, 8.0), and *E. coli* (pI values, 5.4, 6.0, 6.6 and 9.0) (Table 2). The bla_{TEM} gene was detected in all *K. pneumoniae* and *E. coli*

Table 1: Susceptibility data of the clinical isolates and the transconjugant

Clinical Isolate	Zone diameter of inhibition (mm)								
	CTX	CTX/CLA	CAZ	CAZ/CLA	TET	GEN	AMK	CIP	SXT
Kp4	6	18	18	25	6	8	20	6	ND
Kp8	6	16	19	26	6	8	20	6	ND
Kp15	6	16	18	25	6	8	20	6	ND
ENT	10	20	21	25	20	17	21	30	ND
EC	9	22	15	26	19	19	20	32	6
TcEC[a]	11	31	20	32	25	ND	ND	ND	33
EC Na az^R	34	32	29	29	22	23	ND	28	30

Isolates were tested for susceptibility using disk diffusion according to CLSI guidelines [14].
CTX: cefotaxime, CTX/CLA: cefotaxime/clavulanic acid, CAZ: ceftazidime, CAZ/CLA: ceftazidime/clavulanic acid, TET: tetracycline, GEN: gentamicin; AMK: amikacin; CIP: ciprofloxacin, SXT: sulfamethoxazole/trimethoprim,
TcEC: transconjugant carrying plasmid encoding $bla_{CTX-M-15}$.
EC Na az^R: recipient strain resistant to sodium azide
[a] Transconjugant was tested by PCR experiment for $bla_{CTX-M-15-like}$ using specific primers [16].
ND: Not determined

Table 2: MIC data of selected β-lactams and characteristics of β-lactamases produced by clinical isolates of Enterobacteriaceae from Egypt

Clinical Isolate	Enzyme characteristics[a]				Gene (s) detected by PCR[b]	MIC (mg/L) of:						
	pI	CTX hydrolysis	Inhibited by Clox	Inhibited by CLA		CPD	CPD/CLA	FEP	FEP/CLA	FOX	ATM	IPM
Kp4	5.4	No	No	Yes	bla_{TEM}	>128	1	>128	0.06	8	128	0.12
Kp8	6.3	No	No	Yes	-[c]	>128	2	>128	0.06	8	128	0.12
Kp15	7.6	No	No	Yes	bla_{SHV}	>128	2	>128	<0.03	8	128	0.12
	8.0	Yes	No	Yes	$bla_{CTX-M-14}$							
ENT	6.3	No	No	Yes	-[c]	>128	2	>128	<0.03	>16	32	0.25
	7.6	No	No	Yes	-[e]							
	8.0	Yes	No	Yes	$bla_{CTX-M-14}$							
	8.9	No	Yes	No	-[d]							
EC	5.4	No	No	Yes	bla_{TEM}	>128	0.25	>128	<0.03	<4	16	0.12
	6.0	No	No	Yes	-[c]							
	6.6	No	No	Yes	-[c]							
	9.0	Yes	No	Yes	$bla_{CTX-M-15}$							

CPD: cefpodoxime, CPD/CLA: cefpodoxime/clavulanic acid, FEP: cefepime, FEP/CLA: cefepime/clavulanic acid, FOX: cefoxitin, ATM: aztreonam, IPM: imipenem.
[a] Enzyme Characteristics: pI: isoelectric point of crude β-lactamase extract preparations; CTX (0.75 mg/L) was used in the substrate-based IEF overlay technique, inhibitors used in the IEF overlay were clavulanic acid (1 mM) and cloxacillin (1 mM).
[b] bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes were detected by PCR experiments using specific primers. Only the bla_{CTX-M} genes were sequenced using primers that flanked the full-length genes (See Methods section).
[c] PCR was not done to detect the gene that corresponds to the β-lactamase band on IEF gel.
[d] β-lactamase band focusing at pI value of 8.9, which is inhibited by cloxacillin, corresponds to the chromosomal *ampC* gene of *E. cloacae* (PCR data not shown).
[e] PCR was negative

isolates, and corresponded to the β-lactamase band that focused at pI value of 5.4 when evaluated by IEF (Table 2). The bla_{SHV} gene was present in the *K. pneumoniae* isolates (β-lactamase band focusing at pI value, 7.6-Table 2). The β-lactamase band that focused at pI value of 7.6 in the *E. cloacae* isolate was most likely not an SHV-enzyme since SHV-specific PCR was negative. Further sequencing experiments for the bla_{TEM} and bla_{SHV} genes were not done.

Analysis of the upstream sequence of $bla_{CTX-M-14}$ and of $bla_{CTX-M-15}$ revealed the presence of the right terminal inverted repeat of the insertion sequence IS*Ecp1* and the putative promoter region (-10 and -35) associated with this element [23].

The results of the conjugation experiment showed that $bla_{CTX-M-15}$ was carried on a conjugative plasmid (Table 1). The movement of $bla_{CTX-M-15}$ was verified in the transconjugant using CTX-M-group 1-specific PCR [16]. The $bla_{CTX-M-14}$ gene was not mobilized by conjugation.

A surveillance report on antibiotic resistance in the South-eastern Mediterranean region screened only *E. coli* isolates from different medical centers in Egypt [2]. Other important nosocomial isolates such as *K. pneumoniae* and *E. cloacae* were not evaluated in that study [2]. A recent outbreak was reported in a neonatal intensive care unit in Cairo, Egypt, in which 80% of the isolates were *K. pneumoniae*, of which 58% were ESBL producers [24]. Therefore, it is important not to limit extended-spectrum cephalosporin susceptibility screening in Egypt to *E. coli* but to include *K. pneumoniae* as well as other Enterobacteriaceae such as *E. cloacae*.

It is important for clinical microbiologists in Egyptian hospitals to screen for CTX-M ESBL producers. In addition, clinical microbiologists and physicians need to be aware that these enzymes are present in many different types of Enterobacteriaceae. This information is essential for determining the most appropriate empirical antibiotic therapy.

Conclusion
This study is the first documentation of CTX-M-14 ESBLs in *K. pneumoniae* and *E. cloacae* isolates in Egypt as well as the Middle East region and North Africa.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
NK participated in the design of the study. NK carried out the susceptibility testing, molecular genetic studies, and sequence alignment; and participated in drafting the man-

uscript. NDH participated in the design of the study and drafting the manuscript, and coordination. Sequencing primers were designed by NDH. Work was carried out at the laboratory of Dr. Hanson, Department of Microbiology and Immunology, Creighton University, Omaha, Nebraska. All authors have analyzed and interpreted the data, and have read and approved the final manuscript.

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