Phenotypic and genotypic characterization of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from patient's urine

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Abstract

Introduction: Extended-spectrum β -lactamases (ESBLs)-producing *Enterobacteriaceae* are a clinical threat that may cause nosocomial as well as community-acquired infections. *E.coli* and *Klebsiella pneumoniae* are among the most common Gram-negative bacilli causing urinary tract infections. This study investigated certain molecular characteristics of phenotypically proved ESBL-producing *E. coli* and *K. pneumoniae*.

Materials and Methods: This study includes a total of 64 community and hospital-acquired *Enterobacteriaceae* suspected to produce ESBLs using routine antimicrobial susceptibility test. Identification of species of *Enterobacteriaceae* was done by the API 20E identification system. ESBL production was detected by double disk synergy test (DDST) followed by detection of the encoding genes by PCR using primers for *bla*-TEM, *bla*-CTX-M1, *bla*-CTX-M2, *bla*-SHV and *bla*-PER genes.

Results: The identity of confirmed isolates was 40 *E.coli* and 24 *Klebsiella pneumoniae*. Out of these, 49 isolates were positive for ESBL-production by DDST. Fifty seven isolates were proved to produce ESBLs using PCR. The *bla*-TEM, *bla*-CTX-M1 and *bla*-PER were the most prevalent ESBL genes (45.3%, 45.3% and 34.375%; respectively).

Conclusion: The double disk synergy test showed sensitivity of 82.5% compared to PCR. The study showed high prevalence of ESBLs in *E. coli* and *K. pneumoniae* isolates from urine specimens with *bla*-TEM, *bla*-CTX-M1 and *bla*-PER as the predominant detected genes.

Key words: *E. coli*, *Klebsiella pneumoniae*, ESBLs, DDST, PCR.

Introduction

Infections due to extended-spectrum beta-lactamase-producing *Enterobacteriaceae* are associated with impaired outcome, delay in administration of adequate antimicrobial chemotherapy and can be associated with serious complication [1]. The most evolving mechanism of ESBL production among *Enterobacteriaceae* is the selective pressure imposed by inappropriate use of third generation cephalosporins, most often encountered in ICU settings [2]. Patients at high risk for developing colonization or infection with ESBL-producing organisms are often seriously ill patients with prolonged hospital stay and often are exposed to invasive medical devices (urinary catheters, endotracheal tubes, central venous lines) for a prolonged duration [3]. ESBLs are β -lactamases capable of conferring bacterial resistance to the penicillin and all generation cephalosporins as well as aztreonam; but not the cephamycins such as cefoxitin and cefotetan or carbapenems, although they can hydrolysis these antibiotics. However, ESBLs are inhibited by β -lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam [4]. Most of the genes encoding class A ESBLs are plasmid-borne which may facilitate the intra- or inter-species exchange of the resistance genes, resulting in the acquisition of new resistance phenotypes [5-7]. When a significant proportion of Gram-negative isolates emerged as ESBL-producers in any patient, empirical therapy may change towards the use of imipenem, quinolones, or beta-lactamase inhibitor combinations [8]. Endemic transmission of ESBL-producers can be controlled by appropriate infection control measures and prudent antibiotic management interventions [4]. Reliable detection of ESBL-producers by clinical microbiology laboratory is essential to provide appropriate therapy and to control nosocomial outbreaks [9, 10].

The aim of the present study was to detect common ESBL-producing *Enterobacteriaceae* (*E.coli* and *K. pneumoniae*) by Double Disk synergy test (DDST)

and to compare it by molecular characterization of common genes encoding ESBLs using PCR.

Material and Methods

Bacterial isolates. This study was conducted on 64 non-duplicate community and hospital-acquired isolates of *Enterobacteriaceae* during the last 3-year. The isolates were selected first by routine disk diffusion method due to their reduced susceptibility to any of the 3rd generation cephalosporins (e.g. cefotaxime, ceftazidime, cefoxitin, ceftriaxone instead cefoxitin) and/or aztreonam. All isolates were obtained from urine samples cultured on MacConkey's agar (Oxoid Hampshire, England) and were incubated aerobically at 37°C 24-48 hours. Conventional microbiological standard tests (Gram stain, glucose fermentation test and oxidase test) were used. All isolates found to be Gram-negative, glucose fermenter, glucose and oxidase negative were considered preliminary to be *Enterobacteriaceae* [10].

Identification of bacterial isolates. Identification species of *Enterobacteriaceae* after culture on MacConkey's agar was done by the API 20E identification system (bioMérieux, France) in accordance to manufacture recommendation. This study included 40 *E. coli* and 24 *K. pneumoniae* isolates.

Primary Screening for ESBLs. Antimicrobial susceptibility was determined by Kirby-Bauer's disk diffusion method on Mueller-Hinton agar according to the specification of Clinical and Laboratory Standards Institute (CLSI) [11]. All Isolates showing reduced susceptibility to one or more of the used antibiotics ceftazidime (CAZ) (30 μ g), cefotaxime (CTX) (30 μ g), cefpodoxime (CPD) (10 μ g) and aztreonam (ATM) (30 μ g) (Biorad, France) are considered to be potential ESBL- producers and additional phenotypic tests are mandatory to confirm ESBL-production [11, 12].

Phenotypic detection of ESBLS using the Double-disk Synergy-test (DDST)

Mueller-Hinton agar was inoculated with a suspension of the test strain equivalent to 0.5 McFarland turbidity-standard. DDST was employed by placing cefotaxime (30µg), ceftazidime (30µg), cefpodoxime (10µg) and aztreonam (30µg) disks (Biorad) around amoxicillin/clavulanic acid disk (20 µg and 10 µg, respectively) at a distance of 30 mm center to center. Following an overnight aerobic incubation at 37°C, clearly visible extension of the edge of the inhibition zone of any disk towards the amoxicillin/clavulanic acid disk was interpreted as phenotypic evidence of ESBLS-production [11].

Genotypic detection of ESBLS genes using PCR

DNA Extraction of bacterial isolates was done using Biospin Bacteria Genomic DNA Extraction Kit (BioFlux corporation, Tokyo, Japan). Amplification of the following resistance encoding genes; *bla*TEM, *bla*SHV, *bla*CTX-M1, *bla*CTX-M2 and *bla*PER; was performed by extracted DNA of each isolate. Five sets of primers specific for each resistance gene were designed according to the GenBank database and their sequences are listed in **Table 1** [13-17]. Dream Taq™ Green PCR Master Mix (2X) 200 reactions of 50µl (Thermo- Fermentas, California, USA)

(Dream Taq™ DNA polymerase, optimized Dream Taq™ Green buffer, 4mM MgCl₂, density reagents, 2 dyes for monitoring electrophoresis process and 0.4 mM from each of dATPs, dCTP, dGTP and dTTP). Amplification was achieved using the PCR Biometra T-Personal thermal cycler (48 wells, Ver 09/10, Germany). Five µl of the extracted DNA was added to 25µl of PCR master mix, 1µ of each primer, 10µl nuclease free water to reach a final volume of 50µl. The amplification process was done according to the manufacturer's instructions as follows: an initial denaturation cycle at 95°C for 5 minutes; 35 cycles consisted of denaturation at 95°C for 30 seconds, annealing at temperature ranged from 56 to 82°C for 1 minute and primer extension at 72°C for 2 minutes; with an additional 10 minutes incubation at 72°C after completion of the last cycle. At the end of amplification process, the maintenance temperature was 4°C.

Detection of the amplified DNA products

The amplified DNA products sizes were detected using 2% agarose gel electrophoresis and molecular size marker (100-1000 base pair), and the bands were visualized by UV transilluminator (Biometra, TI). Product sizes of the amplified genes are shown in **Table 1**.

Table 1. Specific primer sequences used in the detection of ESBLS genes and the amplified product sizes.

Genes	Primer sequences	Product size	Ref.
TEM	5'-ATGAGTATTCAACATTTCG-3' 5'-CCAATGCTTAATCAGTGAGG-3'	858 bp	[13]
SHV	5'-CTTTACTCGCTTTATCG-3' 5'-TCCCGCAGATAAATCACCA-3'	827 bp	[14]
CTX-M1	5'-AGTTCACGCTGATGGCGACG-3' 5'-AACCCAGGAAGCAGGCAGTCC-3'	676 bp	[15]
CTX-M2	5'-CGGAATTCATGATGACTCAGAGCATG3' 5'-GCTCTAGATTATTGCATCAGAAACCG3'	896 bp	[16]
PER	5'-AATTTGGGCTTAGGGCAGAA-3' 5'-ATGAATGTCATTATAAAAGC-3'	925 bp	[17]

Table 2. Antibiotic susceptibility testing used for screening of *E. coli* and *K. pneumoniae*

Antibiotic	Number (%) of resistance		
	Disk content	<i>E. coli</i> N = 40	<i>K. pneumoniae</i> N = 24
CTX	30µg	28 (70)	18 (75)
CAZ	30µg	19 (47.5)	20 (83.3)
CPD	10µg	35 (87.5)	15 (62.5)
ATM	30µg	11 (27.5)	10 (41.67)

CTX = Cefotaxime, CAZ = Ceftazidime, CPD = Cefpodoxime, ATM = Aztreonam.

This study was carried out in the department of Medical Microbiology and Immunology and the department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University during the period from March, 2013 to August 2015. A permission was obtained from the Institutional Ethical Review Board (IERB) of Cairo University, Egypt.

Statistical Analysis

Data were statistically described in terms of frequencies (number of isolates) and percentages. Comparisons were done using McNemar test. Agreement between the different diagnostic tests was tested using kappa statistic. *P* values less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

Results

Identification of the *Enterobacteriaceae*

The 64 ESBLs-producing *Enterobacteriaceae* included in this study which were isolated from urine samples were identified to be 40 *E. coli* (62.5%) and 24 *K. pneumoniae* (37.5%).

Primary Screening for ESBLs

All of the 40 *E. coli* and the 24 *K. pneumoniae* isolates were resistant to one or more of the tested β -lactam antibiotics (CTX, CAZ, CPD, ATM) as illustrated in **Table 2**.

Phenotypic detection of ESBLs using the double-disk Synergy-test (DDST) showed that 31(77.5%) of *E. coli* isolates were positive for ESBL by DDST, and 18 (75%) of *K. pneumoniae* isolates were positive for ESBL.

Number and percentage of *E. coli* and *K. pneumoniae* isolates showing positive synergism in relation to the tested antibiotics are demonstrated in **Table 3**.

Table 3: Number and percentage of *E. coli* & *K. pneumoniae* isolates showing positive synergism in relation to the used antibiotics

Antibiotics	Negative synergism No. (%)	Positive synergism No. (%)
CTX	13 (20)	51 (80)
CAZ	7 (11)	57 (89)
CPD	33 (52)	31 (48)
ATM	8 (12.5)	56 (87.5)

Table 4: Distribution of the resistance encoding genes among *E. coli* and *K. pneumoniae*

Presence of ESBL Gene	Number (%) of positive isolates		
	<i>E. coli</i> N = 40	<i>K. pneumoniae</i> N = 24	Total N= 64
<i>bla</i> -CTX-M1	18 (45)	11 (45.8)	29 (45.3)
<i>bla</i> -CTX-M2	3 (7.5)	3 (12.5)	6 (9.4)
<i>bla</i> -SHV	4 (10)	10 (41.7)	14 (21.9)
<i>bla</i> -TEM	22 (55)	7 (29.2)	29 (45.3)
<i>bla</i> -PER	13 (32.5)	9 (37.5)	22 (34.4)

Fifty seven isolates (89.1%) out of the 64 isolates were positive for ESBL and only 7 isolates (10.1%) yielded negative results. Distribution of the resistance encoding genes among *E. coli* and *K. pneumoniae* is illustrated in **Table 4**.

Discussion

The emergence and spread of multidrug resistance among *E. coli* and *K. pneumoniae* particularly ESBL-producers complicate therapy and limit treatment options. The influx of these bacteria into hospitals has major implications for infection-control and empirical treatment strategies [7, 18, 19]. The increased incidence of community-acquired ESBLs poses an additional challenge for clinical microbiological laboratories to be detected among multidrug-resistant pathogens [20].

In the present study, we found frequently ESBL-producers *E. coli* (62.5%) and *K. pneumoniae* (37.5%) in urine samples of patients. This finding is in agreement with Poupet *et al.* 2008, who reported that *E. coli* and *Klebsiella* species were the most frequently recovered isolates among ESBL-positive *Enterobacteriaceae* in clinical samples [21]. A study performed by Katteregga *et al.* 2015, also reported that *E. coli* was the most common ESBL-producers (53.9 %) isolated from urine samples, followed by

K. pneumoniae (28.7 %) [22]. The predominance of *E. coli* isolates from urine samples was in concordance with a study done in Lebanon by Daoud *et al.*, 2015, which has compared between two periods of 2005 and 2012, and stated that ESBL-producing *E. coli* was isolated in 60.53% and 73.98%, while *K. pneumoniae* was isolated in 5.32% and 8.33 %, respectively [23].

This study found that all 64 isolates were resistant to one or more of the tested antibiotics as shown in **Table 3**.

A study conducted by Maina *et al* 2011, has reported that β -lactamase producing *Enterobacteriaceae* in the community accounted for 65.4 % resistance to CTX and 21.2 % resistance to CAZ [20]. Another study done by Katteregga *et al.* 2015 showed a higher resistance to CAZ (73%) compared to CTX (57.5%) in ESBL-producing *Enterobacteriaceae* collected from patients in various wards of Mulago Hospital, Uganda [22]. The different rates seen in these studies could be due to regional differences and the type of samples collected [11, 12]. In addition, phenotypic methods for detection of ESBLs are not constantly accurate. In contrast, the genotypic method using specific PCR amplification of resistance genes seems to have 100% specificity and sensitivity [24]. The distribution of ESBL among *E. coli* and *K. pneumoniae* isolates by DDST and PCR

Table 5. Distribution of ESBL among *E. coli* and *K. pneumoniae* isolates

Isolates	No.	No. (%) ESBLs –positive	
		DDST	PCR
<i>E. coli</i>	40	31 (63.3)	35 (61.4)
<i>K. pneumoniae</i>	24	18 (36.7)	22 (38.6)
Total	64	49	57

is shown in **Table 5**. DDST showed a sensitivity of 82.5% for ESBL detection in relation to PCR. This finding was lower than a study done by Nandagopal *et al.* 2015 who reported 96.9% sensitivity of DDST to detect ESBL-producing *Enterobacteriaceae* [25]. All Clinical isolates of *Enterobacteriaceae* found to produce ESBLs should be assumed to be resistant to all extended spectrum cephalosporins irrespective the results in vitro susceptibility testing [12]. In our study the percentage of the detected ESBLs genes by PCR were 45.3% for TEM, 45.3% for CTX-M1, 34.4% for PER, 21.9% for SHV and 9.4% for CTX-M2. A recent study of Daoud *et al.* 2015 in Lebanon, has reported that the detected ESBLs genes from *E. coli* and *K. pneumoniae* were SHV (30.69%), TEM (68.19%) and CTX-M (100%) [23]. While Nandagopal *et al.* 2015 in India, has reported less incidence rates of genes of CTX-M2 (28.5%) , TEM (23.4%) and 4.7% for SHV [25]. In an older study conducted by Welsh *et al.* 2007 in India hospital, CTX-M was also the most common genotype isolated [26]. Similar results have been documented in Europe, Latin America and other countries [27]. A study conducted by Al-Algamy *et al.* 2015 in Saudi Arabia, has found that ESBL-producing *K. pneumoniae* using PCR for detection of SHV, TEM, CTX-M β -lactamase genes have prevalence rates of 97.3%,

84.1%, and 34.1%, respectively [28]. It is well documented that the CTX-M genotypes which are chromosomally encoded has been recently sharply increased in *Escherichia coli* and *K. pneumoniae* in different geographical regions of the world including Middle East Arab countries [28,29].

Conclusion

Routine detection of ESBL-producing *E. coli* and *K. pneumoniae* in the laboratories plays a critical role for their control. Molecular identification of ESBL encoding genes is essential for a reliable investigation of their antimicrobial resistance. The need for new treatment strategies and prudent use of antimicrobials at each segment of healthcare services, continuous monitoring, effective infection control measures are essential to minimize the emergence and spread of multidrug resistant pathogens.

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