original article

Phenotypic and genotypic characterization of extended-spectrum β-lactamases producing *Escherichia coli* and *Klebsiella pneumoniae* in a tertiary care hospital in Riyadh, Saudi Arabia

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BACKGROUND AND OBJECTIVES: Extended-spectrum beta-lactamase (ESBL)-producing pathogens remain a public health concern, with limited data on the molecular characterization of isolates. We aimed to determine the molecular characterization of ESBL-producers circulating in our setting and correlate the molecular types with the minimal inhibitory concentration (MIC) to third-generation cephalosporins.

DESIGN AND SETTING: Retrospective study conducted during the period from January to June 2013 at King Khalid University hospital, a tertiary-care hospital in Riyadh, Saudi Arabia.

MATERIALS AND METHODS: All *Escherichia coli* and *Klebsiella pneumoniae* confirmed to be ESBL producers were included. The MICs of ceftriaxone and ceftazidime were determined by the E-test. Molecular characterization of ESBL-genes was performed using the Check-MDR-CT102 DNA microarray.

RESULT: Of 77 isolates comprising 50 (65%) *E coli* and 27 (35%) *K pneumoniae*, the majority (n=63; 81%) were from urine. Most isolates were bla_{CTX-M} gene positive (n=72/77; 93.5%) comprising bla_{CTX-M1} (n=62), bla_{CTX-M9} (n=9) and $bla_{CTX-M25}$ (n=1). Two or more ESBL genes were present in 45% of isolates with bla_{SHV} predominating in *K pneumoniae* and bla_{TEM} in *E coli*. Two isolates were positive for blaOXA-48 carried in combination with bla_{CTX-M9} and bla_{TEM} in *E coli* and $bla_{CTX-M1}/_{CTX-M9}$ in *K pneumoniae*. Ceftriaxone MIC50 and MIC90 of ≥256 μg/mL were seen in *E coli* and *K pneumoniae* harboring bla_{CTX-M} alone or in combination with bla_{SHV} or bla_{TEM}. For ceftazidime the highest MIC50 and MIC90 was seen in *K pneumoniae* harboring bla_{CTX-M}+bla_{SHV} and *E coli* with bla_{CTX-M}+bla_{TEM} combinations.

CONCLUSION: A preponderance of bla_{CTX-M} suggests dissemination of the gene in our setting. The MIC for ceftriaxone and ceftazidime correlate well with molecular characterization of ESBL-producing *Enterobacteriaceae*.

The main mechanism of acquired resistance to extended-spectrum cephalosporins among the *Enterobacteriaceae* is the production of plasmid-mediated extended-spectrum β-lactamases (ESBLs) and/or AmpC β-lactamases (pAmpCs).[1] The bla_{SHV}-type and bla_{TEM}-type ESBLs were the most prevalent enzymes in the world a decade ago, but now the epidemiology of dominant ESBL types has shifted to the bla_{CTX-M} -type β-lactamases.[2] The bla_{CTX-M} family, first described in 1992,[3] is known to be the most dominant non-bla_{TEM} and non-bla_{SHV} ESBL among *Enterobacteriaceae* and is recognized as a rapidly growing family of ESBLs that selectively prefer to hydrolyze cefotaxime rather than ceftazidime.[4]

Clinical laboratories have to accurately screen isolates suspected of harboring ESBLs. The Clinical and Laboratory Standards Institute (CLSI) recommends screening of *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Proteus mirabilis* isolates for ESBL production by the use of cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone, followed by phenotypic confirmation with clavulanate.[5]

However, multiple resistance mechanisms may obscure the ability to detect ESBLs, as the new classes of non-ESBL enzymes that are emerging may result in resistance patterns that overlap those caused by the ESBLs, leading to the failure of conventional clavulanate-based ESBL detection.

Our aim was to determine the epidemiology as well as genotypic and phenotypic characterization of ESBL-producing isolates including carbapenem-resistant *Enterobacteriaceae* (CRE) and to correlate the molecular types of ESBLs with the minimal inhibitory concentration (MIC) of ceftriaxone and ceftazidime.

MATERIALS AND METHODS

This study was carried out at King Khalid University Hospital, Riyadh, Saudi Arabia. The study included 77 non-duplicate, consecutive, phenotypically confirmed *E coli* and *K pneumoniae* ESBL-producing isolates identified between January to June 2013. These isolates were from various specimen types including, blood, wound swabs, endotracheal secretions, sputum, body fluids, and urine, which were routinely cultured in the bacteriology laboratory. Identification of the organisms and susceptibility testing were carried out according to our laboratory policy by Vitek 2 (Biomerieux, Marcy l'Etoile) for blood and sterile body fluids samples and by MicroScan Walkaway 96 plus System (Siemens Healthcare Diagnostic Inc.) for other samples. We used MicroScan as a backup automated identification system in case the isolate was not identified or results were equivocal by Vitek 2. The isolates flagged as ESBL by Microscan or Vitek-2 were confirmed using the E-test method as recommended by CLSI. Only the first representative isolate per patient was included and all other repeat isolates were excluded.

Antimicrobial susceptibility testing

Forty-eight of 77 isolates were tested for AmpC production using the Kirby-Bauer method on Mueller Hinton agar using commercially available discs Mastdiscs ID inhibitor combination disks (MDI) (Mast Diagnostics company, Bootle, Liverpool, UK) in accordance with manufacturer's guidelines. This methodology utilizes three discs: disc A, containing cefpodoxime 10 μg + AmpC inducer; disc B, consisting of cefpodoxime 10 μg + AmpC inducer + ESBL inhibitor; and disc C, consisting of cefpodoxime 10 μg + AmpC inducer + ESBL inhibitor + AmpC inhibitor. Interpretation of the test requires comparison of the zone of inhibition of disc C with the inhibition zones of each disc A and B. If disc C shows a zone difference of ≥5 mm from discs A and B this is indicative of AmpC production.

The minimum inhibitory concentration for ceftriaxone and ceftazidime was determined by E-test (AB Biodisk, Solna, Sweden) following the manufacturer's instructions and results were interpreted as per CLSI guidelines.5 *E coli* ATCC 25922, *K pneumoniae* ATCC 700603 and *P aeruginosa* ATCC 27853 were used for quality control.

Genotypic testing for ESBL and Carbapenemases genes

Testing was carried out using the Check-MDR CT102 DNA microarray (CheckPoints BV, Wageningen, Netherlands) at the Antimicrobial Resistance Research Laboratory, Alfaisal University, Riyadh, Saudi Arabia. At the time of genotypic testing, the research laboratory was blinded to the findings of the phenotypic assays. The Check-MDR CT102 combines ligation-mediated amplification with detection of amplified products on a microarray to detect ESBL genes bla_{CTX-M}, bla_{TEM}, and bla_{SHV} as well as carbapenemase genes including KPC, NDM, VIM, IMP, OXA-48. Whole-cell DNAs were extracted from overnight bacterial cultures using the Mo Bio UltraClean Microbial DNA Isolation Kit. Microarray assays were performed according to manufacturer instructions with provided tubes and reagents. Briefly, 10 μL of purified genomic DNA (5-50 ng/μL) was added to 5 μL of proprietary mix containing ligation probes and thermostable DNA ligase. The sample was run in T100 thermal cycler (Bio-Rad) for 3 min at 95°C, followed by 24 cycles of 0.5 min at 95°C and 5 min at 65°C, and final denaturation at 98°C for 2 min. For the DNA amplification step, 30 μL of manufacturer provided mix containing PCR primers, deoxynucleoside triphosphates, and thermostable polymerase were added and the sample was heated for 10 min at 95°C followed by 30 cycles of 0.5 min at 95°C, 0.5 min at 55°C, 0.5 min at 72°C, and a final denaturation step of 2 min at 98°C using the Veriti thermal cycler (Applied Biosystem). The ligation and DNA amplification steps were carried out in separate rooms in accordance with manufacturer guidelines. DNA hybridization of the amplified ligation products was carried out in customized Array Tubes (3 samples per tube). Hybridization was performed using 10 μL of each amplified reaction product with 300 μL of preheated hybridization buffer for 30 min at 50°C under rotational shaking (400 rpm) in a thermo-mixer (Eppendorf, Hamburg, Germany). Unbound DNA was washed away using two 5-min incubation steps with 300-μL blocking buffer under the same conditions. Then 150-μL freshly prepared conjugate solution was added to each array tube and incubated for 15 min at 30°C at 400 rpm on the thermo-

mixer. Excess conjugate was washed away by two 5 min incubation steps using 300-µL detection buffer under the same conditions. Finally, 150 µL of the staining solution was added and incubated for 15 min at room $_{TEM}$perature. After 15 min, tubes were inserted in the single-channel ATR03 array tube reader, the images were acquired and interpreted with the manufacturer provided software controls for assessing the success of each critical step in the procedure, including ligation specificity and efficiency, PCR amplification, hybridization efficiency, label detection and label quality, which are inbuilt in the array analysis.

RESULTS

The study included 77 isolates from various body sites, comprising 50 (65%) *E coli* and 27 (35%) *K pneumoniae*, which were phenotypically confirmed to be ESBLs producers. The majority of the isolates (n=60; 77.9%) were isolated from urine samples. Other specimen sources were wound swabs (n=8; 10.4%), blood (n=3; 3.9%), sputum (n=3; 3.9%) and sterile body fluids (n=3; 3.9%). The isolates were mostly from inpatients (n=40; 52%) with a predominance of adult patients (n/N: 62/77; 81%). Thirty-four isolates were from males and 43 from females. **Figure 1** shows the demographic characteristics of the patient population.

The bla$_{CTX-M}$ gene was harbored by majority of the isolates (n=72/77; 93.5%) and comprised of three bla$_{CTX-M}$ groups namely bla$_{CTX-M1}$ (n=62), bla$_{CTX-M9}$ (n=9) and bla$_{CTX-M25}$ (n=1). The bla$_{CTX-M}$ gene was of comparable predominance in both *E coli* (n=48/50; 96%) and *K pneumoniae* (n=23/27; 85%), However, almost half of the isolates harbored at least two or more ESBL genes (n= 35/77; 45%) (**Table 1**) with bla$_{SHV}$ occurring most commonly in *K pneumoniae* (n=16/27; 59%). Although bla$_{TEM}$ was detected in 26% (n= 13/50) of *E coli* only a single *K pneumoniae* isolate was positive for this gene. Two isolates were positive for bla$_{OXA-48}$ which was carried in combination with bla$_{CTX-M}$ (Group 9) and bla$_{TEM}$ in one *E coli* isolate and blaCTX_M (Groups 1 and 9) in a *K pneumoniae* isolate. No AmpC producer was identified. There was 100% concordance between phenotypic detection of ESBL and molecular characterization using the Checkpoint MDR system.

The MIC50 and MIC90 for ceftriaxone in *E coli* harboring bla$_{CTX-M}$ genes alone or in combination with bla$_{SHV}$ or bla$_{TEM}$ were ≥256 µg/mL. In contrast, ceftriaxone MIC90 ≥256 µg/mL was only observed for *K pneumoniae* isolates harboring bla$_{CTX-M}$ alone or bla$_{CTX-M}$ + bla$_{TEM}$ genes (**Table 2**). For ceftazidime, in *K pneumoniae* harboring bla$_{CTX-M}$ + bla$_{SHV}$ only the MIC50 and MIC90 was much higher compared to isolates carrying other gene combinations, although the small sample size precluded a statistical analysis (**Table 2**). In *E coli*, the highest ceftazidime MIC50 and MIC90 was seen in isolates harboring a combination of bla$_{CTX-M}$ + bla$_{TEM}$ genes (**Table 2**). The *E coli* isolate harboring bla$_{OXA-48}$ showed an MIC of >256 for ceftriaxone and 1.5 for ceftazidime while the *K pneumoniae* isolate with bla$_{OXA-48}$ showed MIC >256 for both ceftriaxone and ceftazidime. Both bla$_{OXA-48}$ positive isolates were sensitive to imipenem and meropenem using CLSI breakpoints. They were resistant to amoxicillin-clavulanic acid, ceftazidime, ceftriaxone, gentamicin and piperacillin-tazobactam.

DISCUSSION

In recent years, data from the Arabian Peninsula has shown a high occurrence of ESBL-producing isolates with rates as high as 31.7% in Kuwait,[6] 41% in United Arab Emirates[7] and 55% in Saudi Arabia.8 A study from Bahrain reported that 22.6% of *Enterobacteriaceae* at a major tertiary center were ESBL producers.[9] Although current data indicate that ESBL-producing pathogens are an emerging public health concern in the region, there are limited data on the molecular characterization of local isolates. The findings in our study indicate that most of the ESBL isolates harbored bla$_{CTX-M}$

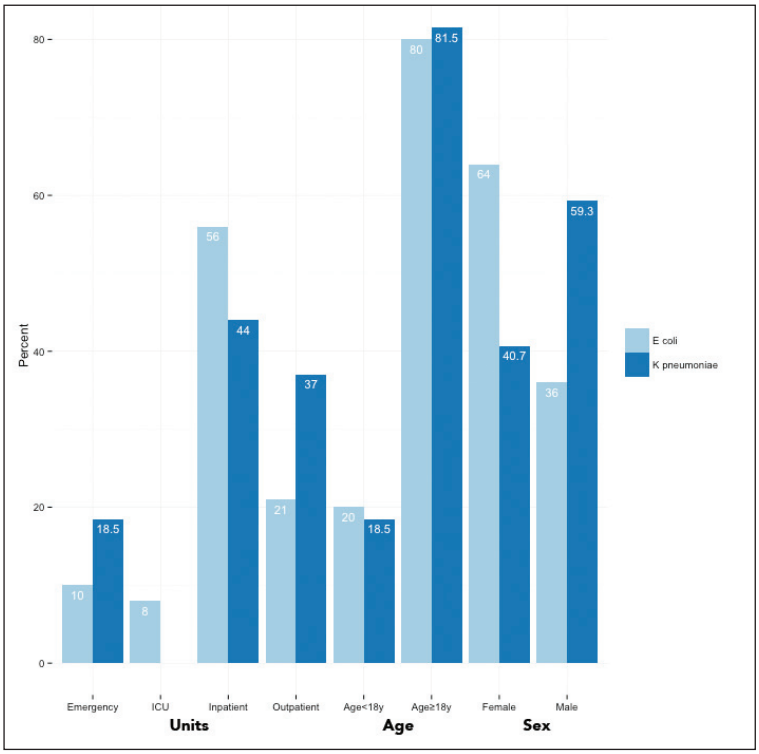

Figure 1. Demographic distribution of patient population.

Table 1. Distribution of ESBL genotypes (number of isolates).

	E coli (n=50)	*K pneumoniae* n=27)	Total (n=77)
Distribution of the single ESBL gene			
bla_{CTX-M} only	34	2	36 (46.75%)
bla_{SHV} only	0	4	4 (5.2%)
bla_{TEM} only	1	0	1 (1.3%)
Total # of isolates with single ESBL gene	35	6	41 (53.2%)
Distribution of the ESBL gene combinations			
bla_{TEM} + bla_{CTX-M}	12	1	13 (16.9%)
bla_{SHV} + bla_{CTX-M}	1	12	13 (16.9%)
bla_{TEM} + bla_{SHV} + bla_{CTX-M}	0	7	7 (9.1%)
bla_{CTX-M} + bla_{TEM} + bla_{OXA-48}	1	0	1 (1.3%)
bla_{CTX-M} + bla_{OXA-48}	0	1	1 (1.3%)
Total # of isolates with ≥2 genes	14	21	35 (45.45%)

Table 2. Comparative analysis of ceftriazone and ceftazidme MIC in isolates habouring different combinations of resistance genes.

	Antibiotic	MIC values	
		MIC50	MIC90
Isolates with CTX-M gene only			
Ceftriaxone	*K pneumoniae* (n=2)	48	256
	E coli (n=34)	256	256
Ceftazidime	*K pneumoniae* (n=2)	1.5	32
	E coli (n=34)	12	48
Isolates with CTX-M + TEM only			
Ceftriaxone	*K pneumoniae* (n=1)	96	96
	E coli (n=12)	256	256
Ceftazidime	*K pneumoniae* (n=1)	1.5	1.5
	E coli (n=12)	16	64
Isolates with CTX-M + SHV only			
Ceftriaxone	*K pneumoniae* (n=12)	256	256
	E coli (n=1)	256	256
Ceftazidine	*K pneumoniae* (n=12)	24	256
	E coli (n=1)	12	12

gene, mainly $bla_{CTX-M-1}$ followed by bla_{SHV} and bla_{TEM}. Previous studies from Saudi Arabia have reported detection of bla_{CTX-M} in 34% and 71% of ESBL-producing *Enterobacteriaceae* isolates.[8,9] Our finding indicates a higher preponderance of bla_{CTX-M} positive isolates indicating further dissemination of this resistance gene among *Enterobacteriaceae* isolates circulating in our setting. However, as in other studies, we also document a high prevalence of urinary isolates harboring the bla_{CTX-M}, although in contrast to other reports, it appears that we have more isolates among inpatients. This suggests that in addition to further dissemination, these bla_{CTX-M}-positive isolates continue to be important agents of community and nosocomial infections in our setting. Correlation of the presence of resistance genes with the MICs indicate that the bla_{CTX-M} was more effective in driving higher MICs of ceftriaxone and ceftazidime in both *K pneumoniae* and *E coli* isolates.

All ESBL producing *E coli* and *K pneumoniae* isolates had ceftriaxone MIC50 and MIC90 of ≥256 ug/mL. Ceftazidime MIC50 and MIC90 for *E coli* and *K pneumoniae* were 12, 64 and 24, ≥256 ug/mL, respectively. This data shows that both drugs are good for the detection of ESBLs while ceftriaxone is more sensitive than ceftazidime. One isolate (positive for bla_{TEM}) had ceftriaxone MIC 1.0 while ceftazidime MIC ≥256, another isolate (positive for bla_{TEM} and bla_{CTX-M}) had ceftazidime MIC 1.0 while ceftriaxone MIC was 64. This means that use of both ceftriaxone and ceftazidime MICs ensures that all ESBLs would be detected even if only phenotypic methods are used.

There was good correlation between the phenotypic tests for ESBL resistance and the molecular characterization using the Check MDR platform. The molecular methodology has the advantage of providing data on the precise type of resistance gene responsible for the phenotypic characteristic seen. This is of use in the clinical setting as such molecular data provides important baseline information useful for instituting effective control measures. In addition, the use of the molecular methodology is useful for the detection of isolates harboring genes encoding for carbapenemase production in particular bla_{OXA-48}. The low MICs of carbapenems, which bla_{OXA-48}-producing isolates often display, makes their detection difficult, thus facilitating dissemination. Current meropenem susceptibility breakpoints often fail to detect CRE isolates in areas with a high prevalence of bla_{OXA-48} producers.[10] In addition, ertapenem resistance has been shown to have a low positive *P* value for the detection of carbapenemase production, in low-prevalence setting.[11] Our findings reflect these challenges as both bla_{OXA-48} positive isolates were sensitive to meropenem

using current guidelines. Without the use of molecular methodology, these CRE would have been missed and disseminated in the healthcare facility. Our findings indicate the need for specific and sensitive methods for detection of bla_{OXA-48} producers in clinical microbiology laboratories. Both bla_{OXA-48} isolates in this study were resistant to piperacillin/tazobactam. It has been suggested that piperacillin/tazobactam and temocillin can be used as highly sensitive surrogate markers for CRE.[10]

We conclude that MIC methods (E-test) for ceftriaxone and ceftazidime correlate well with molecular classification of ESBLs-producing *Enterobacteriaceae* especially for *E coli*. We recommend performing this test for screening purposes in clinical laboratories.

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