

Molecular characterization of extended-spectrum beta-lactamase producing *Enterobacteriaceae* in a Saudi Arabian tertiary hospital

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Abstract

Introduction: The aim of this study was to determine the prevalence of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), and *Proteus mirabilis* (*P. mirabilis*). In addition, different methods for detection of these enzymes, including the newly introduced CHROMagar ESBL, were evaluated.

Methodology: A total of 382 *Enterobacteriaceae* clinical isolates were obtained from King Fahad Specialist Hospital – Dammam, during 2011 and screened for production of ESBL using advanced expert system of Vitek 2, CHROMagar and ESBL-E-strips. PCR assay was used to detect *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M} genes. Susceptibility to a panel of antibiotics was determined.

Results: The overall proportion of ESBL-producing enterobacterial isolates was 30.6%, which was higher in *E. coli* (35.8%) than in *K. pneumoniae* (25.7%). ESBL genotypes showed remarkable increase in the CTX-M (97.4%) compared to SHV (23.1%). The predominant ESBL was CTX-M-15 (92.1%). No TEM ESBL was detected in this study. The Vitek2 showed the highest sensitivity (100%), and the CHROMagar had the lowest specificity (97.3%) compared to the molecular method. All isolates were susceptible to imipenem and meropenem.

Conclusions: This study confirms a high level of *bla*_{CTX-M} positive ESBL isolates are circulating in the Eastern Province of Saudi Arabia. The trend of a multidrug-resistant profile associated with the recovery of the *bla*_{CTX-M} gene is alarming.

Key words: ESBL; CTX-M; *Enterobacteriaceae*

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Introduction

Microbial resistance through extended-spectrum beta-lactamase (ESBL) was first reported in the early 1980s in Europe and subsequently in the United States soon after the introduction of third-generation cephalosporins in clinical practice [1]. The dominant enzyme variants until the late 1990s were TEM, SHV, and OXA, while CTX-M ESBLs were rarely recorded [1,2]. The epidemiology of ESBLs has undergone dramatic changes in recent years with the emergence and spread of the CTX-M-type enzyme worldwide [3-6].

The detection of ESBL-mediated resistance in Gram-negative bacilli is of paramount importance because of its clinical implications and the limited therapeutic options [3,7,8]. Conventional methods for resistance detection rely on phenotypic identification based on bacterial growth inhibition in disk diffusion or dilution tests, which are cost-effective but which usually require two days to complete [9]. A faster and more reliable detection method is desirable, as the

determination of antibiotic susceptibility is crucial for positive antimicrobial therapy outcomes and significantly influences subsequent procedures and actions [10].

In recent years, chromogenic media were initially developed for the detection and presumptive identification of urinary tract pathogens. The selective chromogenic agar medium CHROMagar ESBL (CHROMagar, Paris, France) enables the detection and presumptive identification of ESBL-producing *Enterobacteriaceae* directly from clinical specimens with good selectivity [11]. In addition, automated systems are increasingly used for routine species identification and susceptibility testing in clinical laboratories to decrease the in-laboratory turnaround time and to improve cost effectiveness.

Although ESBL-producing members of *Enterobacteriaceae* have been reported in Saudi Arabia, situated in the Arabian Gulf region of Asia [12-15], scant data about the genetic background of the involved organisms is available [16-17]. Therefore,

the aim of the study was to screen for the dominant genes of ESBL in Saudi Arabia and to evaluate the different phenotypic methods in comparison to the molecular method for the detection of ESBL producers.

Methodology

Bacterial isolates

From January to June 2011, a total of 382 consecutive clinical isolates (251 *E. coli*, 107 *K. pneumoniae*, and 24 *P. mirabilis*) were collected at King Fahad Specialist Hospital – Dammam (KFSHD). Copy strains were excluded from the study. The isolates were all from different patients and units. They were isolated from urine (n = 259; 67.8%), wounds (n = 65; 17%), blood and central venous lines (n = 32; 8.4%), sputum (n = 16; 4.2%), sterile body fluids (n = 8; 2.1%), and ear infections (n = 2; 0.5%). No epidemic outbreak was observed. In each assay, *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as the positive and negative control for ESBL production, respectively. The criterion to include a strain in this study was either an alert from the automated system (Vitek 2) about ESBL detection, growth on CHROMagar, or a noticeable pattern of resistance against third-generation cephalosporins in the antimicrobial susceptibility testing using E-test. The molecular method (PCR analysis and sequencing of amplified PCR products) was chosen as the reference method. Isolates were cultured from frozen stock or from lyophilized material onto Columbia agar with 5% sheep red blood cells for 16 to 24 hours at 37°C, subcultured, and grown again for 16 to 24 hours at 37°C just before testing.

Identification and antimicrobial susceptibility testing using Vitek 2 and E-test

All isolates were identified and tested for susceptibility by the Vitek 2 system (bioMérieux, Marcy l’Etoile, France) using the card for Gram-negative strains (GN cards) and AST-N026. The following antimicrobial agents were tested in the study: amikacin, gentamicin, ciprofloxacin, ceftazidime, cefotaxime, piperacillin/tazobactam, and trimethoprim/sulfamethoxazole. The cards were inoculated and incubated in the system according to the manufacturer’s instructions. All results were interpreted using the Advanced Expert System (AES) (software version VT2-R04.03). The isolates were initially screened positive if minimum inhibitory concentration (MICs) of ceftazidime and cefotaxime for these organisms were ≥ 2 mg/L using the Vitek 2

system AST-N026 card. Phenotypic confirmation of ESBLs was done using E-test (epsilometer assay; bioMérieux, Marcy l’Etoile, France) on Mueller-Hinton agar. A decrease of at least three twofold concentrations in MIC of either ceftazidime or cefotaxime tested in combination with clavulanic acid versus its MIC when tested alone was indicative of phenotypic confirmation of ESBL production [18].

Evaluation of CHROMagar ESBL

The CHROMagar ESBL provided by CHROMagar (Paris, France) was obtained from the supplier as a prepared plate medium. Each bacterial strain was cultured on CHROMagar ESBL agar, and was incubated aerobically at 37°C for 18 to 24 hours. Colonies of ESBL producers develop species-specific colors (dark pink to reddish coloration for *E. coli*; metallic blue coloration for *Klebsiella* spp; and a brown halo for *P. mirabilis*). Non-ESBL producers grow with colorless colonies or not at all on CHROMagar ESBL agar [19].

PCR analysis and sequencing of amplified PCR products for bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes

Genotypic characterization of resistance mechanisms was determined by PCR assays targeting *bla_{TEM}*, *bla_{SHV}*, and *bla_{CTX-M}* genes. Briefly, one to three colonies from freshly prepared blood agar plate were suspended in 100 μ L of molecular grade water and boiled at 95°C for 15 minutes. Cellular debris was removed by centrifugation at 15,000g for 5 minutes, and 2 μ L of DNA-supernatant were used for PCR [20]. For the detection of *bla_{TEM}* and *bla_{SHV}*, a multiplex PCR using TEM and SHV universal primers (Table 1) was designed according to the method of Randall *et al.* [21]. For the detection of *bla_{CTX-M}*, the PCR conditions used were according to the method of Paterson *et al.* [22]. The primers used for PCR amplification of CTX-types from groups CTX-M group 1, CTX-M group 2, CTX-M group 9, CTX-M group 8, and CTX-M group 25 are listed in Table 1. Fragments of alleles encoding enzymes of groups 8 and 25 were amplified with two specific forward primers and a shared reverse primer according to the method of Woodford *et al.* [23].

Amplified PCR products for the full length *bla_{TEM}*, *bla_{SHV}*, and *bla_{CTX-M}* genes were sent to be analyzed by an automated DNA sequencing system in Eurofins MWG Operon using primers listed in Table 1 [24-25]. The method used was cycle sequencing, which is based on Sanger sequencing (as recommended by the manufacturer).

Table 1. Primer sets used in PCR runs for tested isolates

Primers, gene sought, or ESBL family	Primer sequence (5'-3')	Gene product length (bp)	Reference
TEMFU	TCGTGTCGCCCTTATCCCTTTT	426	21
TEMRU	GCGGTTAGCTCCTCCGGTCCTC		
SHVFU	GTGGATGCCGGTGACGAACAGC		
SHVRU	TGGCGCAAAAAGGCAGTCAATCCT		
CTX-MuF	CGCTTTGCGATGTGCAG	551	
CTX-MuR	ACCGCGATATCGTTGGT		
CTX-M grp1F	AAAAATCACTGCGCCAGTTC	415	
CTX-M grp1R	AGCTTATTCATCGCCACGTT		
CTX-M grp2F	CGACGCTACCCCTGCTAT T	552	22, 23
CTX-M grp2R	CCAGCGTCAGATTTTTCAGG		
CTX-M grp9F	CAAAGAGAGTGCAACGGATG	205	
CTX-M grp9R	ATTGAAAAGCGTTCATCACC		
CTX-M grp8F	TCGCGTTAAGCGGATGATGC	666	
CTX-M grp8/25R	AACCCACGATGTGGGTAGC		
CTX-M grp25F	GCACGATGACATTCGGG	327	
CTXMSeqF	GTTCGTCTCTCCAGAATAAGG	968	24
CTXMSeqR	CAGCACTTTTGCCGTCTAAG		
TEMFLF	GAAGACGAAAGGGCCTCGTG	1074	
TEMFLR	GGTCTGACAGTTACCAATGC		
SHVFLF	CGCCGGTTATTCTTATTTGTCGC	1016	25
SHVFLR	TCTTCCGATGCCGCCAGTCA		

Table 2. Molecular typing results for PCR ESBL-positive strains

ESBL genotype	Number of isolates (%)		
	<i>E. coli</i>	<i>K. pneumoniae</i>	Total
<i>bla</i> _{CTX-M}	81 (69.2)	9 (7.6)	90
<i>bla</i> _{SHV}		3 (2.5)	3
<i>bla</i> _{CTX-M} + <i>bla</i> _{SHV}	9 (7.6)	15 (12.8)	24
	90	27	117

Table 3. Sensitivity, specificity, PPV and NPV results of AES of Vitek 2, CHROMagar ESBL medium, and phenotypic confirmatory test for ESBLs by E-test. The molecular method was used as a reference method.

	Sensitivity	Specificity	Positive predictive value (PPV)	Negative predictive value (NPV)
AES of Vitek 2	100%	98.9%	97%	100%
CHROMagar	97.4%	97.3%	94%	98%
E-test	97%	100%	100%	98%

Table 4. Co-resistance of ESBLs producing isolates to non-beta-lactam antibiotics

Antibiotics	ESBLs (117) R%	Non ESBLs (265) R%	P value
Gentamicin	69 (59)	33 (12.5)	< 0.05
Amikacin	3 (2.6)	0	< 0.05
Ciprofloxacin	101 (86.3)	97 (36.6)	< 0.05
Piperacillin / tazobactam	74 (63.2)	77 (29)	< 0.05
Trimethoprim / sulfamethoxazole	102 (87.1)	121 (45.7)	< 0.05

R: resistance

The sequencing reaction was run on an automated ABI 3730xl DNA Analyzer. The resulting DNA sequences were compared with genes from GenBank using BLAST (<http://www.ncbi.nlm.nih.gov/blast>). Primers used in this study as described in the literature [21-25] are listed in Table 1.

Analysis of results

The sensitivities, specificities, and positive and negative predictive values (PPV and NPV) respectively of AES of Vitek 2, the CHROMagar ESBL medium, and phenotypic confirmatory test for ESBLs by E-test for all groups were calculated. The molecular method results were used as previously described [26].

Statistical analysis

Categorical variables were compared using Fisher's exact test. A p value of < 0.05 was considered statistically significant [26].

Results

Based on the criteria of selection, 145 isolates were suspected to harbor ESBL out of 382 strains and were screened for the presence of the beta-lactamase genes *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M} by PCR. All *bla*_{TEM} genes detected in the isolates had 100% identity to TEM-1 sequences in GenBank (accession number AF427133). Therefore, strains harboring only *bla*_{TEM} gene (18 strains) as well as 10 isolates screened negative by PCR were excluded from the study. As a result, a total of 117 strains were further characterized in this project.

The PCR data revealed that 114 and 27 of isolates harbored *bla*_{CTX-M} and *bla*_{SHV} genes, respectively. Using CTX-M group specific primers, 105 (92.1%) isolates were positive for the CTX-M-1 group and 9 (7.9%) were positive for the CTX-M-9 group. The full length amplified products of the *bla*_{SHV} and *bla*_{CTX-M} genes were sequenced and compared with ESBL sequences in GenBank to determine the ESBL types. The BLAST results revealed that the *bla*_{SHV} amplification product showed 100% identity to the SHV-12 enzyme (accession number AJ920369). Furthermore, DNA sequence analysis showed that all amplicons obtained for the CTX-M-1 group were characterized as CTX-M-15 (accession number AY044436), and all amplicons obtained for the CTX-M-9 group were characterized as CTX-M-14 (accession number AF252622AAC). Based on these data, 90 strains of *E. coli* (90/251; 35.8%) and 27 (27/107; 25.2%) of the *K. pneumoniae* isolates were

found to be ESBL producers. The majority of ESBL-producing isolates (n = 83; 71%) were recovered from urine, 20 from wounds (17%), 8 from blood (6.8%), 4 from sputum (3.4%) and 2 from fluids (1.8%). PCR showed that 97.4% (n = 114) and 23.1% (n = 27) of isolates contained *bla*_{CTX-M} and *bla*_{SHV} genes, respectively. Coexistence of the *bla*_{CTX-M} and *bla*_{SHV} was detected in 24 isolates (20.5%) (9 *E. coli* and 15 *K. pneumoniae*). Ninety strains (76.9%) (81 *E. coli* and 9 *K. pneumoniae*) had the *bla*_{CTX-M} gene only, while three *K. pneumoniae* isolates (2.5%) harbored the *bla*_{SHV} gene (Table 2).

ESBL-producing organisms were detected on CHROMagar in 114 isolates (sensitivity 97.4%). The specificity assessed on 265 specimens that were found negative for ESBL-producing isolates by PCR was 97.3%. A total of seven specimens yielding the growth of non-ESBL producing organisms presenting the typical chromogenic character of *Enterobacteriaceae* species were found on CHROMagar. For these specimens, high-level cephalosporinase (five specimens) and impermeability (two specimens) were the resistance mechanisms reported by the advanced expert system (AES) of the Vitek 2.

With the exception of three strains that gave false positive results, the AES of the Vitek 2 was able to detect ESBL-producing isolates. These strains gave negative results with the E-test and CHROMagar ESBL. These isolates produced amplicons corresponding to ESBL beta-lactamase gene in addition to an AmpC beta-lactamases. The sensitivity, specificity, PPV and NPV results of AES of Vitek 2, CHROMagar ESBL medium, and phenotypic confirmatory test for ESBLs by E-test are shown in Table 3.

The lowest rates of resistance in ESBL-producing isolates were observed for amikacin (2.6%), gentamicin (59%), and piperacillin/tazobactam (63.2%). The highest rate of resistance belonged to trimethoprim/sulfamethoxazole (87.1%) and ciprofloxacin (86.3%). Remarkably, as shown in Table 4, ESBL-positive isolates were significantly (p < 0.05) more frequently resistant to gentamicin, amikacin, ciprofloxacin, piperacillin-tazobactam, and trimethoprim/sulfamethoxazole when compared to non-ESBL isolates over the time frame.

Discussion

In the current study, the overall proportion of ESBL-producing enterobacterial isolates was 30.6%. The proportion of ESBL producers was higher in *E. coli* (35.8%) than in *K. pneumoniae* (25.2%). In

neighbouring Asian countries, the ESBL frequency varied from 66.7% in India [27], 54.7%-61% in Turkey [28,29], 41% in United Arab Emirates [30], 31.7% in Kuwait [31], and 72.1% in Iran [32], but was lower in America and Europe [29-33].

The overall prevalence of ESBL producers was found to vary greatly in different geographical areas and in different institutes in Saudi Arabia. In our study, the rate of ESBLs-producer isolates was higher than that reported from Abha and Khobar (27.5% and 12.2%, respectively) in 2001 [34] and 2005, but still lower than the rate reported in a study from Riyadh in 2007 (55%) [17]. This may be due to differences in the type and volume of consumption of antibiotics and differences in the time of collection of isolates. However, it reflects the chronological increase in the prevalence of ESBL producers in the Eastern Province of Saudi Arabia. As in other studies, the majority of ESBL-producing isolates were recovered from urine [35-37].

In this study, ESBL genotypes showed remarkable increase in the CTX-M genotype (97.4%), while SHV was detected in 23%. A study done in a tertiary care hospital in the Eastern Province of Saudi Arabia in 2006 showed that 71% of the strains harbored the *bla*_{CTX-M} gene [38]. This reflects remarkable increase in the *bla*_{CTX-M} genotype in this area. Similarly, in most European countries, Latin America, and East Asia, CTX-M variants have displaced TEM and SHV enzymes as the predominant beta-lactamases produced by Gram-negative bacteria such as *K. pneumoniae* [3,27,32]. In Arab countries, the first description of CTX-M was in Egypt and then in the United Arab Emirates and Kuwait [39-41]. In these countries, the predominant ESBL was CTX-M-15. This data is consistent with our data and with previous reports from Saudi Arabia. CTX-M14 was detected among 7.9% of our strains, correlating with previous studies done in Saudi Arabia that detected CTX-M-14 in a range from 5% to 40% [16,17].

The presence of more than one ESBL in a single isolate is being increasingly detected [17,38,42]. In this study, coexistence of *bla*_{SHV} and *bla*_{CTX-M} genes was detected in 20.5 % of the strains. It is remarkable that this was seen more frequently in *K. pneumoniae*. This highlights the growing complexity of antibacterial resistance problems, and the reasons for this situation require further investigation.

The AES Vitek 2, compared to the molecular method, revealed 100% sensitivity, 98.8% specificity, 97.5% PPV and 100% NPV for detection of ESBL producers. The AES enhances the ability of the Vitek

2 system to identify ESBLs by basing its phenotype determination on the distribution of MICs for various beta-lactam antibiotics rather than simply on neutralization by clavulanic acid [37,39]. Therefore, the AES Vitek 2 can be used as a sole detection method of ESBL in *E. coli*, *K. pneumoniae*, and *P. mirabilis*.

The performance of CHROMagar ESBL for the detection and presumptive identification of extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* directly from clinical specimens was evaluated in a few studies [11,19]. In a study done by Saito *et al.*, the sensitivity, specificity, PPV and NPV were 100%, 93.3%, 51.5%, and 100%, respectively [28]. Better results were obtained in our study; the sensitivity, specificity, positive predictive value and negative predictive value of the CHROMagar were 97.4%, 97.3%, 94.2% and 98.8%, respectively. The CHROMagar failed to detect only 2.3% of the ESBL strains. The discrepancy in the results may be explained by the fact that the previous results showing lower specificity and positive predictive value were obtained by evaluating the media directly from clinical specimens, while our study was the first study to evaluate the media from subculture. Therefore, CHROMagar ESBL media can be used as a sole method for detection of ESBL from subculture in laboratories with limited resources.

In most microbiological laboratories, the ESBL E-test is the most commonly employed confirmatory test, as the integration of PCR assay into the routine diagnostic process is not feasible because of its cost and labor intensiveness. In this study, three isolates were not positive in ESBL E-test but produced amplicons corresponding to *bla*_{TEM}, *bla*_{SHV}, or *bla*_{CTX-M}, which shows that the genotypic determination of the *bla* gene families is the most reliable procedure to identify ESBL-producing *Enterobacteriaceae* [43]. Similar results were also reported by other studies [32,43,44].

The lowest rates of resistance in ESBL-producing isolates were observed for amikacin (2.6%), gentamicin (57.3%), and piperacillin/tazobactam (63.2%). The highest rate of resistance was to ciprofloxacin (84.6%). In Riyadh in 2007, researchers reported that only 11% of their ESBL producers were resistant to ciprofloxacin and 88% and 77% were resistant to gentamicin and amikacin, respectively [19,20]. This may be attributed to misuse of ciprofloxacin and restriction of aminoglycosides in our hospital. Amikacin remained the most effective aminoglycoside against *K. pneumoniae*.

In our study, a detailed analysis of the 90 isolated ESBL-producing *E. coli* showed that co-resistance to non-beta-lactam antibiotics was frequent among these isolates: 78.9% (71/90) were resistant to trimethoprim/sulfamethoxazol and 74.4% (67/90) to ciprofloxacin. Co-resistance to non-beta-lactam antibiotics was less frequent among these isolates: (44.4%; (40/90) to gentamicin and 2.2% (2/90) to amikacin. Moreover, 23 of 27 (85.1%) ESBL-producing *K. pneumoniae* were resistant to ciprofloxacin and 22 (81.5%) were resistant to trimethoprim/sulfamethoxazol. However, no co-resistance to amikacin was detected among these strains.

In conclusion, this study confirms that a high level of *bla*_{CTX-M}-positive ESBL isolates is circulating in the Eastern Province of Saudi Arabia. The trend of multidrug-resistant profiles associated with the recovery of the *bla*_{CTX-M} gene is alarming. This highlights the need to establish an antimicrobial resistance surveillance network for *Enterobacteriaceae* to monitor the trends and new types of resistance mechanisms in clinical isolates in the Gulf area.

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