Original Article

Detection of extended spectrum beta-lactamases-producing isolates and effect of AmpC overlapping

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Abstract

Introduction: Few reports about the prevalence and genetic basis of extended spectrum beta-lactamases (ESBLs) are available from Saudi Arabia. We sought to determine the prevalence of ESBL-producing Enterobacteriaceae in a university hospital in eastern Saudi Arabia and to characterize the ESBLs produced by these isolates at the molecular level.

Methodology: All clinical isolates of *Escherichia coli, Klebsiella spp*., and *Proteus spp*. collected over two years were evaluated for susceptibility to a panel of antimicrobials and were analyzed for the ESBL phenotype using screening and confirmatory tests. ESBL-positive isolates were then screened for the presence of genes encoding CTX-M, SHV, and TEM beta-lactamases by PCR.

Results and conclusions: The overall prevalence of ESBL-producing isolates was 4.8% (253/5256). Most isolates (80%) were from the inpatient department. The ESBL phenotype was more frequently detected in *K. pneumonia*. CTX-M genes were the most prevalent ESBL genes, detected in 82% of the studied isolates. The ESBL producers demonstrated a high multidrug resistance rate (96.6%). In transconjugation assay, the same ESBL gene pattern was transmitted from 29.7% of *K. pneumoniae* donors to the recipient strain, and the latter exhibited concomitant decreased aminoglycosides and co-trimoxazole susceptibility. We observed the presence of ESBL screen-positive but confirmatory-negative isolates (8.9%). Phenotypic tests for the production of AmpC β-lactamase tested positive in 52% of these isolates. Further studies are needed for appropriate detection of concomitant ESBL and AmpC enzyme production among such isolates. Continued surveillance and judicious antibiotic usage together with the implementation of efficient infection control measures are absolutely required.

Key words: antimicrobials; beta-lactamase; enterobacteriaceae; ESBL; transconjugation

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Introduction

Antibiotic resistance in Gram-negative rods is an increasing problem worldwide. Since the introduction of broad-spectrum cephalosporins into clinical practice in the early 1980s, the selective pressure of the use and overuse of antibiotics has resulted in the emergence and rapid development of resistance to expanded-spectrum beta-lactam antibiotics [1,2]. Numerous outbreaks of infection with organisms producing extended-spectrum beta-lactamases (ESBLs) have been observed in many countries throughout the world [2,3], and these organisms have achieved notoriety for causing nosocomial infections that lead to prolonged hospital stay, increased morbidity and mortality, and consequently increased health-care associated costs [4,5].

Since the description of the first ESBL from Germany in 1983 [6], a steady increase in resistance against cephalosporins has been observed [2,6]. ESBLs evolved via point mutations of key amino acids in parent, broad spectrum beta-lactamases (TEM-1, TEM-2 and SHV-1). They have an extended spectrum profile that permits hydrolysis of oxyimino-cephalosporins and monobactams but not 7-alpha-methoxy-cephalosporins (cephamycins). They are generally inhibited by beta-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam [1,2]. ESBLs undergo continuous mutations, causing the

development of new enzymes showing expanded substrate profiles [7].

During the late 1990s and early 2000s, Enterobacteriaceae (mostly *Escherichia coli*) producing novel ESBLs, the CTX-M enzymes, were identified predominantly from the community as a cause of urinary tract infections [7]. CTX-M-type ESBLs are rapidly expanding, and they share only 40% identity with TEM and SHV enzymes [8]. Other ESBL enzymes are less often encountered and epidemiologic data on these less common ESBLs are very limited [9]. At present, there are more than 300 different ESBL variants, and these have been clustered into nine different structural and evolutionary families based on amino acid sequence [10].

According to the Clinical Laboratory Standards Institute (CLSI) recommendations, routine antimicrobial susceptibility testing should include screening for ESBL production, employing cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone followed by phenotypic confirmation for the positive cases, based on demonstrating the effectiveness of the screening antibiotic in the presence of a beta lactamase inhibitor [11,12,13]. Results are issued with the aim of preventing inappropriate use of cephalosporins or monobactams in the setting of ESBL production [12].

Plasmid-mediated AmpC beta-lactamases were reported in 1988 [14]. In addition to resistance conferred by the ESBLs, they simultaneously confer transferable resistance to the cephamycins, and they are not inhibited by beta-lactamase inhibitors [14]. Isolates that coproduce both an ESBL and a high level of AmpC are becoming more common [15]. The effect of concurrent ESBL and AmpC gene expression may adversely affect the performance of current ESBL screening and confirmatory testing as the two enzyme groups have overlapping hydrolysis spectra [16,12]. The resulting failures to detect ESBLs can endanger patients because of the reported false susceptibility to cephalosporins [16]. Little is known about how often AmpC production creates uncertainty about the accuracy of CLSI ESBL confirmatory tests [17]. Currently, no established guidelines from the CLSI are available for the detection of AmpC beta-lactamases [18].

ESBLs constitute a key antibiotic-resistance mechanism by Gram-negative bacteria. ESBL-producing Enterobacteriaceae are increasing steadily and spreading worldwide [1]. Although *K. pneumoniae* and *E. coli* are the main pathogens producing ESBLs, more recently other Enterobacteriaceae, as well as Pseudomonadaceae, from different parts of the world, have also been reported as ESBLs producers [4].

Reports on the prevalence of ESBL-producing Enterobacteriaceae are few in Saudi Arabia. Apart from two studies in Riyadh and the Al-Qassim area [19,20], no significant information on the genetic basis of ESBLs has been available yet. This study was undertaken to determine the frequency of isolation of ESBL-producing Enterobacteriaceae in a tertiary hospital in eastern Saudi Arabia and to characterize the ESBLs produced by clinical isolates at the molecular level.

Methodology

Bacterial isolates and patients demographics

The study included all consecutive, non-duplicate ESBL-producing *E. coli*, *Klebsiella spp*, and *Proteus spp* that were isolated over the period of two years from various clinical specimens obtained by the clinical microbiology laboratory at a university hospital in eastern Saudi Arabia as part of routine diagnostic activities. Isolates were identified to species level using standard microbiological methods [21]. The isolates were prospectively stored at -70° C in glycerol broth till further investigated. Selection of ESBL-producing isolates was based on positive ESBL screening test results following the CLSI guidelines [11]. All patients who had infections due to ESBL producers were reviewed for their demographics, including age, sex, hospital unit where they received medical service, and the type of clinical specimen. This study was approved by the ethics committee of the institution.

Antimicrobial susceptibility testing and ESBL detection

In vitro susceptibility testing of all isolates to a wide range of antimicrobials, including both beta-lactams and non-beta-lactams, was performed using the automated VITEK Gram Negative Susceptibility System with cards GNS 206 and 121 (bioMérieux, Vitek Inc, Hazelwood, USA). Isolates reported as ESBL positive, using the automated system, were designated as ESBL screen-positive and were further subjected to a confirmatory test. Confirmation of the ESBL phenotype was performed using the combination disk method based on the inhibitory effect of clavulanic acid according to the CLSI criteria [11]. Antimicrobial disks used were obtained from BD BBL Sensi-Disc (Becton Dickinson, Sparks, MD, USA). *E. coli* ATCC 25922 (negative control) and *K.*

Table 1. List of primers and the detectable ESBL genes in each gene group

Gene	primer	Amplicon	detectable genes*
SHV	SHV-F: CGCCTGTGTATTATCTCCCT SHV-R: CGAGTAGTCCACCAGATCCT	294 bp	1- 2, 2A, 5,8-9,11-13, 18, 24-27, 29-31, 33-38, 41-42, 44-46, 48, 50-52, 55, 57, 59- 60, 62-67, 69-83, 85- 86, 89, 92- 93, 95-97, 101-105, 108, 110, 120-123, 128-129, 133-137, 140-142, 145, 147-163, 165, 167
TEM	TEM-F: TTTCGTGTCGCCCTTATTCC TEM-R: ATCGTTGTCAGAAGTAAGTTGG	404 bp	1, 10, 15, 28, 30, 34, 47, 68, 70, 76-77, 79, 88, 95, 102, 104-107, 109, 124, 126-130, 132, 140, 143-144, 148, 158, 162, 166, 176, 186, 198, 201
CTX-M	CTX-M-F: CGCTGTTGTTAGGAAGTGTG CTX-M-R: GGCTGGGTGAAGTAAGTGAC	754 bp	1, 3, 10-12, 15, 22-23, 28-30, 32, 34, 36, 42, 52, 54-55, 57-58, 60-62, 71-72, 79-80, 82, 88, 96, 101, 108, 114, 117, 123, 132-133

*Gene numbers are cited according to the Lahey Clinic website (www.lahey.org/studies).

pneumoniae ATCC 700603 (positive control) were used for quality control processes as recommended by the CLSI [11]. The antibiotic susceptibility profiles of the ESBL-screen positive isolates to a panel of antimicrobials including amoxicillin/clavulanate, piperacillin, piperacillin/tazobactam, imipenem, cefazolin, cefepime, tetracycline, tobramycin, amikacin, gentamicin, cotrimoxazole, ciprofloxacin, and tigecycline were reported. Isolates shown to be resistant to at least three different classes of antimicrobial agents were determined to be multidrug resistant (MDR).

Detection of ESBL genes by PCR

All isolates positive in ESBL screening test were subjected to testing to detect the possible presence of *SHV, TEM* and *CTX-M* genes by conventional PCR. The primers and a list of the detectable genes of each gene group are listed in Table 1. A single colony of the isolated bacteria was emulsified in the 50 µl reaction mix, which contained 10 pmol of each primer, 10mM dNTPs mix (Qiagen, Hilden, Germany) and 2.5 U of Taq polymerase (Qiagen, Hilden, Germany) in 1x Taq polymerase buffer. A negative control (*E. coli* ATCC 25922) and a positive control sample (kindly provided by Dr. Atef Shibl) were included in every PCR run.

Amplification reactions were performed under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds with an extension at 72°C for 50 seconds, and a final extension for one cycle at 72°C for 5 minutes. The PCR product was then run on a 1.5 % agarose gel for detection of the amplified fragment.

Investigation of ESBL confirmatory negative (non-confirmed) isolates for AmpC production

The non-confirmed isolates were subjected to a phenylboronic acid (PBA) disk confirmation test. PBA disks were prepared as follows: 120 mg of PBA was dissolved in 3 ml dimethylsulfoxide (DMSO). Three milliliters of sterile distilled water were added to this solution to create a stock solution; 20 µl of the stock solution were dispensed onto each of 30 µg cefoxitin and blank disks (6 mm in diameter). These disks had been obtained from Oxoid Inc. (Nepean, ON, Canada). The disks were allowed to dry for 30 minutes and then used immediately. Isolates were tested for susceptibility to cefoxitin with and without PBA. Susceptibility test was performed on Mueller-Hinton agar according to the standard disk diffusion method [11]. An increase of ≥ 5 mm in zone diameter in the presence of PBA compared to cefoxitin tested alone was considered to represent a positive test for the presence of an AmpC beta-lactamase [22].

The non-confirmed isolates were also screened for reduced susceptibility for cefoxitin using a 30 µg disk on Mueller-Hinton agar according to the standard disk diffusion method [11]. Cefoxitin intermediate or resistant isolates (zone diameter ≤ 18mm) were re-evaluated using Mueller-Hinton agar supplemented with 200 µg/ml cloxacillin (an AmpC inhibitor) [23]. Isolates that converted to susceptible were considered positive for AmpC production. Reproducibility of the AmpC assays was determined by performing them in triplicate on consecutive days.

Transconjugation experiments

Transconjugation experiments were performed using *E. coli* strain K12 ER2738 (New England BioLabs, Essex, USA) as the recipient. This strain is

Table 2. Patients' demographic data and specimen types in relation to type of bacterial isolate

		E. coli **(n = 139)**		***K. pneumoniae*** **(n = 90)**		***K. oxytoca*** **(n = 2)**		***P. mirabilis*** **(n = 5)**		**Total (n = 236)**	
		No.	%	No.	%	No.	%	No.	%	No.	%
Sex	Male	59	42.4	66	73.3	0	0	4	80	129	54.7
	Female	80	57.6	24	26.7	2	100	1	20	107	45.3
Age	≤ 20	25	18.0	10	11.1	0	0	2	40	37	15.7
	21- 40	23	16.5	25	27.8	1	50	1	20	50	21.2
	41- 60	26	18.7	17	18.9	0	0	0	0	43	18.2
	> 60	65	46.8	38	42.2	1	50	2	40	106	44.9
Hospital unit[a]	Ward (inpatient)	89	64	49	54.4	0	0	5	100	143	60.5
	ICU (inpatient)	15	10.8	30	33.3	1	50	0	0	46	19.5
	OPD(outpatient)	20	14.4	8	8.9	0	0	0	0	28	11.9
	ER (outpatient)	15	10.8	3	3.3	1	50	0	0	19	8.1
Specimen type	WS	48	34.5	34	37.8	0	0	1	20	83	35.2
	Urine	54	38.8	16	17.8	1	50	1	20	72	30.5
	Blood	12	8.6	11	12.2	1	50	0	0	24	10.2
	Sputum/chest fluid	6	4.3	10	11.1	0	0	1	20	17	7.2
	CSF	0	0	3	3.3	0	0	1	20	4	1.7
	Others	19	13.7	16	17.8	0	0	1	20	36	15.2

ICU = intensive care unit, OPD = outpatient department, ER = emergency room. WS = wound swab, CSF = cerebrospinal fluid
The outpatient units include both OPD and ER
The inpatients units include both ward and ICU
[a] Significant association between outpatient units and frequency of isolation of ESBL-producing *E. coli* (p value= 0.01), similar association between inpatient units and frequency of isolation of ESBL-producing *K. pneumonia* (p value = 0.02)

resistant to tetracycline and was confirmed to be sensitive to ceftazidime by disc diffusion test. It was also found to be negative for the ESBL genes by PCR. Only *K. pneumonia* (donor) strains, which are sensitive to tetracycline, were used in this assay. An overnight culture of 1 ml was prepared from each bacterial isolate in LB broth (Difco, Detroit, USA). Equal volumes (500 µl) of the donor and the recipient strains were mixed together and incubated overnight at 37°C with shaking. After centrifugation for 5 minutes at 4000 rpm, the supernatant was removed and the precipitated bacterial pellet was reconstituted in 1 ml sterile distilled water and inoculated onto MacConkey agar plates (Difco, Detroit, USA) containing 100 µg tetracycline and 4 µg ceftazidime (Oxoid, Nepean, Canada). Colonies that showed resistance to ceftazidime were first confirmed as *E. coli* using the API system. The transfer of any of the ESBL genes to the *E. coli* strain K12 was further analyzed by PCR. Co-transfer of resistance to non-beta-lactam antibiotics, namely gentamicin, tobramycin and co-trimoxazole, from the donor isolates to the recipient strain were investigated by comparing the antibiotic susceptibility pattern of the recipient *E. coli* strain K12 after the experiment to the antibiotic susceptibility pattern of the donor isolates. Throughout the study, results were interpreted using CLSI criteria [11].

Statistical analysis

Data were recorded in computer using SPSS for Windows version 14.0 (SPSS Inc, Chicago, IL, USA). Results were cross-tabulated to examine the relationships between the variables. Statistical analysis was performed using the χ^2-square test of association and Fisher's exact test as appropriate. Odd ratios and a 95% confidence interval were performed as appropriate. Frequency tables were performed as descriptive statistics. P-value of less than 0.05 was considered significant in all statistical analysis.

Results

Prevalence of ESBL-producing isolates and patient demographics

During the study period, clinical specimens obtained from patients yielded 5,256 isolates [*E. coli* (n = 3308), *K. pneumoniae* (n = 1470), *K. oxytoca* (n =

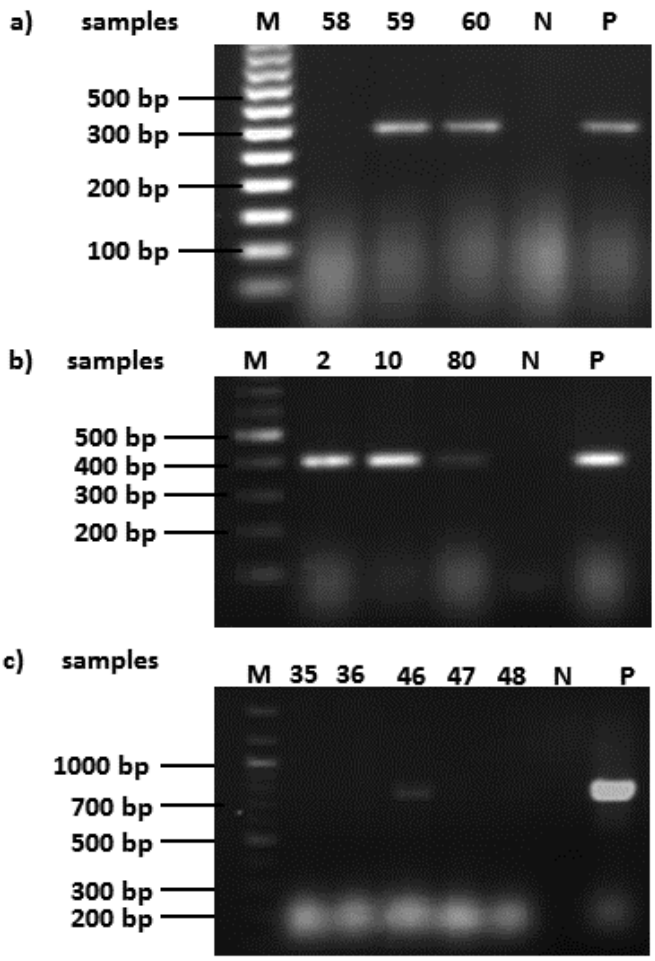
47), other *Klebsiella species* (n = 29), *P. mirabilis* (n = 359), *P. vulgaris* (n = 23) and other *Proteus species* (n = 20)] that were screened for ESBL phenotype. Out of 5,256 isolates, 253 (4.8%) were ESBL screen-positive with a detection rate of 2.7% (71/2584) in 2007-2008, which increased to 6.8% (182/2672) in 2008-2009. The ESBL phenotype was detected in 4.7% (154/3,308), 6.3% (92/1,470), 4.3% (2/47) and 1.4% (5/359) of *E. coli, K. pneumoniae, K. oxytoca*, and *P. mirabilis* respectively. *K. pneumoniae* was the most frequent ESBL-producing species.

Of the 253 ESBL screen-positive isolates, 17 were excluded due to incomplete data. A total of 236 isolates were further investigated. The age of patients from which ESBL producers were isolated ranged from 1.8 months to 92 years old with an average of 48.89 years ± 26.20 STD. A large number of patients (44.9%) was over 60 years old (Table 2). Of the 236 ESBL-producing isolates, 189 (80.0%) were from the inpatient departments [143/236; 60.5% from the ward (both medical and surgical units) and 46/236; 19.5% from the ICU]. The isolation rate of *E. coli* was significantly higher among the outpatient (35/47; 74.4%) than the inpatient (104/189; 55.0%) population, p value = 0.01, whereas *K. pneumonia* was isolated at a significantly higher frequency among the inpatient than the outpatient population (79/189; 41.8% versus 11/47; 23.4%, p = 0.02). Moreover, the isolation rate of *K. pneumoniae* from the ICU (65% of ICU isolates) was significantly higher than that of *E. coli* (p < 0.0001). *E. coli* predominated among urinary (54/72; 75%) as well as among wound swab isolates (48/83; 57.8%), whereas *K. pneumoniae* was isolated from the majority of CSF as well as sputum/chest fluid specimens (3/4, 75% and 10/17, 58.8% respectively) (Table 2).

Susceptibility of ESBL-producing isolates to antimicrobial agents

The resistance rates to ceftazidime, cefotaxime, ceftriaxone and aztreonam among *E. coli* isolates were 97.8%, 100%, 98.6%, and 98.5% respectively, and among *K. pneumoniae* isolates were 96.6%, 97.7%, 95.3% and 97.7% respectively. The resistance rate to the fourth-generation cephalosporine, cefepime, was 95.7% among *E. coli* and 91.8% among *K. pneumoniae* isolates. All *E. coli* and *K. pneumoniae* isolates were resistant to both piperacillin and cefazolin. Regarding the beta-lactam/beta-lactamase inhibitor combinations, the proportion of isolates showing resistance to amoxicillin/clavulanate (68.5%) was significantly higher than that showing resistance

Figure. Examples of PCR results obtained for a) SHV gene group, b) TEM gene group and c) CTX-M gene group. M stands for marker, which is a 50bp marker in case of a) and a 100bp marker in case of b) and c). N stands for negative control and P for positive control.



to piperacillin/tazobactam (41.1%; p = 0.001). Resistance to a panel of non-beta-lactam agents was also demonstrated. The proportions of ESBL-producing *E. coli* and *K. pneumoniae* isolates showing resistance to non-beta-lactam agents, including ciprofloxacin, cotrimoxazole, tobramycin, tetracycline, gentamicin and amikacin, were comparable, with 82.7%, 72.5%, 74.8%, 77.7%, 56.8% and 17.9% for *E. coli* respectively and 80.2%, 86.4%, 75.9%, 69.8%, 60.2% and 28.4% for *K. pneumoniae,* respectively. Up to 8.0% of *E. coli* and 6.8% of *K. pneumoniae* isolates were resistant to imipenem. Tigecycline showed the highest activity against ESBL-producing isolates, where all *E. coli* isolates were susceptible and only 3.3% of *K. pneumoniae* isolates were resistant. Up to 96.6% of ESBL isolates expressed the MDR phenotype.

Table 3. Distribution of the three studied ESBL genes among bacterial isolates

	E. coli (n = 139)		*K. pneumonia* (n = 90)		*K. oxytoca* (n = 2)		*P. mirabilis* (n = 5)		Total (n = 236)			
	Pos	%	Pos	%	Pos	%	Pos	%	Pos	%	Neg	%
Any CTX-M[a]	106	76	82	91	2	100	3	60	193	82	43	18
Any SHV	30	22	77	86	2	100	0	0	109	46	127	54
Any TEM	70	50	43	48	1	50	1	20	115	49	121	51
Only CTX-M[b]	38	27	3	3	0	0	2	40	43	18	193	82
Only SHV	3	2	4	4	0	0	0	0	7	3	229	97
Only TEM	13	9	3	3	0	0	0	0	16	7	220	93
Any CTX-M+ SHV	26	19	72	80	2	100	0	0	100	42	136	58
Any CTX-M+ TEM	56	40	39	43	1	50	1	20	97	41	139	59
Any SHV+TEM	15	11	33	37	1	50	0	0	49	21	187	79
Only CTX-M+ SHV	12	9	40	44	1	50	0	0	53	22	183	78
Only CTX-M+ TEM	42	30	7	8	0	0	1	20	50	21	186	79
Only SHV+TEM	1	1	1	1	0	0	0	0	2	1	234	99
CTX-M+ SHV+TEM	14	10	32	36	1	50	0	0	47	20	189	80
At least one ESBL gene	123	88	90	100	2	100	3	60	218	92	18	8

[a] The word "any" means the presence of the indicated gene regardless of the presence or absence of the other genes
[b] The word "only" means the presence of the indicated gene merely

ESBL confirmatory test

Out of 236 screen-positive isolates, 21 (8.9%) gave negative confirmatory test results as evident by the lack of clavulanate enhancement with any of the tested substrates (non-confirmed isolates). The screen-positive, non-confirmed profile was observed in 9.4% (13/139) of *E. coli,* 6.7% (6/90) of *K. pneumoniae*, and 40% (2/5) *P. mirabilis* isolates.

Molecular detection of ESBL genes

An example of the PCR results obtained during the study is shown in the Figure. Out of 236 isolates, 218 (92%) were positive for at least one of the studied genes (Table 3). CTX-M were the most prevalent ESBL genes among the study isolates (193/236, 82%), as they were the most frequently detected genes within both *E. coli* and *K. pneumoniae* (76% and 91% respectively) (Table 3). The least frequently detected genes were SHV in *E. coli* (22%) and TEM in *K. pneumoniae* (48%). SHV genes were preferentially detected in *Klebsiella species*: 86% of *K. pneumoniae* and 100% of *K. oxytoca* compared to the 22% detection rate in *E. coli* and 0% in *P. mirabilis*. Out of 218 PCR-positive isolates, 47 (22%) harbored the three gene groups simultaneously. Co-presence of all three gene groups was significantly more encountered among *K. pneumoniae* (32/47, 68%) than *E. coli* isolates ($p < 0.0001$) (Table 3). There were 18 (8%) PCR-negative isolates (16 *E. coli* and 2 *P. mirabilis*). No significant difference was detected between inpatient and outpatient isolates regarding the distribution of the studied genes.

Comparing the genetic detection profile between the confirmed and the non-confirmed isolates, it was found that the presence of TEM genes alone was significantly associated with non-confirmed isolates ($p = 0.001$) (Table 4). In contrast, the presence of any CTX- M ($p = 0.00002$), any SHV ($p = 0.03$), or any combination of both enzymes ($p = 0.023$), resulted in a positive ESBL confirmatory test in the majority of cases (Table 4). The combination of the three-gene group was encountered much more frequently among confirmed than non-confirmed isolates (21.4% versus 4.8%); however, the difference was statistically not significant. PCR-negative results were significantly more encountered among the non-confirmed compared to the confirmed isolates ($p = 0.003$) (Table 4).

The non-confirmed isolates are most likely harboring AmpC genes

In total, 21 isolates gave positive ESBL screening test results and negative ESBL confirmatory test results. Out of these 21 isolates, 16 showed the presence of at least one of the ESBL genes by PCR (Table 5). As the co-presence of AmpC genes with ESBL genes might obscure the results of the ESBL confirmatory test, we attempted to phenotypically identify the presence of AmpC genes in the non-

Table 4. Comparison between ESBL- confirmed and non-confirmed isolates regarding the distribution of ESBL genes

	Confirmed isolates (n = 215)		Non-confirmed isolates (n = 21)		Total	P value	O.R.
	No.	%	No.	%			
Any CTX-M	183	85.1	10	47.6	193	0.00002	6.3 (2.5-17.7)
Any SHV	104	48.4	5	23.8	109	0.03	3.0 (0.98-9.7)
Any TEM	105	48.8	10	47.6	115	NS	
Only CTX-M	43	20.0	2	9.5	43	NS	
Only SHV	6	2.8	1	4.8	7	NS	
Only TEM	11	5.1	5	23.8	16	0.001	0.17 (0.5-0.65)
Any CTX-M+ SHV	96	44.7	4	19.0	100	0.023	3.4 (1.0-12.5)
Any CTX-M+ TEM	92	42.8	5	23.8	97	NS	
Any SHV+TEM	48	22.3	1	4.8	49	NS	
Only CTX-M+ SHV	50	23.3	3	14.3	53	NS	
Only CTX-M+ TEM	46	21.4	4	19.0	50	NS	
Only SHV+TEM	2	0.9	0	0.0	2	NS	
CTX-M+SHV+TEM	46	21.4	1	4.8	47	NS	
PCR negative	13	6.0	5	23.8	18	0.003	4.86 (1.3-17.3)

NS. Not significant

Table 5. Phenotypic detection of AmpC β-lactamase in ESBL non-confirmed isolates

Test	Positive		Negative	
	No.	%	No.	%
PBA disk confirmation test	8	38	13	62
Cefoxitin + cloxacillin in Mueller-Hinton agar	10	48	11	52
PBA test & Cefoxitin + cloxacillin in Mueller-Hinton agar	7	33	14	67
At least one ESBL gene present	16	76	5	24

Table 6. Co-transfer of resistance to non β-lactams from donors to the recipient strain

Antimicrobial	Sensitive	Resistant	Transferred resistance	
			Nr.	%
Amikacin	11	0	0	0
Gentamicin	9	2	1	50
Tobramycin	2	9	9	100
Co-trimoxazole	0	11	10	91

confirmed isolates. We used the phenyl boronic acid (PBA) test and cefoxitin/±cloxacillin test, which employ the AmpC inhibitors PBA and cloxacillin, for detection of AmpC production. Out of 21 screen-positive, non-confirmed isolates, 8 (38%) were positive for PBA by the disk confirmation test. Ten of the non-confirmed cefoxitin intermediate/resistant isolates converted to susceptible to cefoxitin in the presence of cloxacillin in Mueller-Hinton agar (Table 5), whereas 7 isolates demonstrated positive results with both tests. The latter were comprised of five *E. coli*, one *K. pneumoniae*, and one *P. mirabilis*. Two of the five *E. coli* isolates were negative for all the studied ESBL genes.

Transconjugation assay

Only ESBL-producing *K. pneumonia* strains that are resistant to ceftazidime and sensitive to tetracycline were used to test the ability of the ESBL genes to be transferred to the tetracycline resistant and ceftazidime sensitive recipient strain, *E. coli* K12 ER2738. Out of 37 *K. pneumonia* strains, 11 (29.7%) could transfer their ceftazidime resistance to the recipient strain. The presence of the ESLB genes in the recipient strain was confirmed by PCR. All ESBL genes present in the donor strains were faithfully transferred to the recipient strain. Furthermore, resistance to tobramycin, co-trimoxazole, and gentamicin were co-transferred to the transconjugant *E. coli* K12 strain at a rate of 100%, 91%, and 50% respectively (Table 6).

Discussion

In the current era of increasing use of broad-spectrum antimicrobial agents, the incidence of ESBL-producing Enterobacteriaceae has increased worldwide at an alarming rate [1]. At present, the major challenge to infection control teams is the prevention of the emergence and spread of ESBL-producing Enterobacteriaceae.

Besides being associated with high morbidity and mortality, therapeutic options for treatment of infections involving ESBLs have also become increasingly limited. The efficacy of extended spectrum cephalosporines is compromised while co-resistance to co-trimethoxazole, aminoglycosides and fluoroquinolones has been reported [9].

Epidemiologic and descriptive data on ESBL-producing isolates in hospitals from Saudi Arabia are limited. The overall prevalence of ESBL producers was found to vary greatly in different geographical areas and in different institutes within the kingdom. The current study is the first report focusing on both the distribution of ESBL-producing Enterobacteriaceae and detection of ESBL genes in the Eastern Province in Saudi Arabia.

Compared to regional data, the prevalence of 4.8% ESBL producers reported in this study is on the lower edge of the spectrum. Within the Arabian Gulf region, reported ESBL detection rates range from 4.8% to 55% [19,20,24-34]. This variation could be attributed to differences in time of collection of isolates and differences in study populations and designs. However, and remarkably, we observed a double increase in ESBL detection rate during the second year of the study compared to the first year. This finding should emphasize the need for accurate *in vitro* detection of ESBLs to guide therapy selection and to allow efficient infection control interventions.

High prevalence of ESBL among *K. pneumoniae* isolates has been detected in numerous studies, as illustrated in a review by Paterson and Bonomo [1]. Similarly in this study, *K. pneumoniae* was the most frequently encountered ESBL-positive species and was particularly detected in inpatients. Our results showed that the majority of ESBL isolates (189; 80%) were obtained from inpatients, indicating that ESBL-associated infections in our population remain essentially nosocomial in nature and their dissemination to the community is not very significant. This finding is consistent with other regional and international data [32,35,5]. However, unless strict infection control measures are enforced, there is a high likelihood that these ESBL producers can be trafficked from the hospital into the community. On the contrary, *E. coli* was isolated at a significantly higher rate among the outpatient than the inpatient population. This finding is in line with the results of another study from the same area [25] and also in keeping with the global recognition of *E. coli*, particularly those harboring the CTX-M genes [8,36], as the major source of ESBL in the community. The 17.7% fecal carriage of ESBL producing *E. coli* reported recently from Saudi Arabia [37] indicates that ESBL-producing *E. coli* are circulating within the community and are associated with infections emerging from these settings.

Up to 19.5% of ESBL isolates were from the ICU, which can be explained by the higher use of invasive devices as well as the selective pressure imposed by inappropriate use of newer beta-lactams being routinely prescribed for ICU patients. Similar results were reported by other investigators [5]. Additionally, urine samples were the second major source for

ESBL-producing isolates. This observation has significant implications for empirical management of patients with urinary tract infection using third-generation cephalosporins or fluoroquinolones, especially as we also found high levels of ciprofloxacin resistance in the ESBL isolates identified.

The overall resistance rate of the studied isolates was alarmingly high to most antibiotics tested. Moreover, 96.6% of isolates expressed the MDR phenotype. The high level of fluoroquinolone resistance observed in this study has been previously documented [32,35]. However, reports from two other areas in Saudi Arabia showed a lower resistance rate (11%; 9%) to ciprofloxacin among ESBL-producing isolates [19,20]. Even carbapenems, which are considered the drugs of choice for therapy of serious ESBL-associated infections [9,35,38], showed lack of activity against 8.0% of *K. pneumoniae* and 6.8% of *E. coli* isolates. This finding is in agreement with data reported from other regional as well as global studies [24,34,38], which may imply that increased use of carbapenems could potentially further select resistant strains. New findings indicate that the spread of CTX-M type ESBLs, especially in *E. coli*, may provide a favorable background for selection of carbapenem resistance [39]. Nonetheless, some antibiotics still show hope in treating ESBL producing bacteria. Tigecycline showed the highest activity against ESBL-producing isolates. Amikacin remained active against 82% and 72% of *E. coli* and *K. pneumoniae* isolates in this study respectively. Clinically, this data is of concern as it indicates a limitation in the antibiotic choices available for treatment of such infections and emphasizes the judicious use of antimicrobials.

PCR screening for TEM, SHV, and CTX-M showed that 92% of the isolates carried at least one of the beta-lactamase genes. It was notable also that 82% of ESBL-producing isolates carried CTX-M genes, which may justify the significantly lower proportion of isolates showing resistance to piperacillin/tazobactam (42%) compared to the amoxicillin/clavulanate resistance rate (68.5%), since CTX-M is better inhibited by tazobactam than by clavulanate [32]. The high prevalence of CTX-M is comparable to that reported from both regional and international studies [40,41]. SHV genes were detected in 86% of *K. pneumoniae* isolates compared to the 22% detection rate within *E. coli* isolates. Similar results were documented by other investigators [9]. TEM genes were detected in 49% of all ESBL producing isolates; however, not all TEM enzymes can be considered ESBL, and sequencing is therefore necessary for allele identification. Co-presence of CTX-M, SHV and TEM was encountered at a significantly higher rate among *K. pneumoniae* compared to *E. coli* isolates in this study ($p < 0.0001$). Coexisting ESBL genes are more likely to be located on one plasmid, as in our transconjugation assay the same ESBL gene pattern was transmitted faithfully from the donors to the recipient strain. Furthermore, co-transfer of resistance to other antibiotics also occurred. Similarly, in a study conducted in Brazil, 74.3% of ESBL-producing *K. pneumonia* isolates selected for conjugation experiments transferred ESBL genes by conjugation. The obtained transconjugants exhibited concomitant decreased aminoglycoside susceptibility [5]. This observation highlights the necessity of rapid and efficient infection control procedures. The presence of PCR-negative ESBL-producing isolates might be attributed to technical limitations with the present assays or to the existence of other beta-lactamases, given the recent rapid expansion of beta-lactamase types worldwide.

Out of the 236 isolates, 21 (8.9%) demonstrated an ESBL screen-positive non-confirmed profile using the conventional CLSI clavulanate confirmation test. This could be partly attributed to the presence of plasmid-borne AmpC beta-lactamases, which are not inhibited by clavulanic acid [17,42]. Isolates that coproduce both an ESBL and a high level of AmpC are becoming more common [15]. Currently, no standardized diagnostic test is recognized for the reliable screening and confirmation of the presence of AmpC [18]. However, the best approach to date is to screen for resistance to a cephamycin (*e.g.*, cefoxitin) along with the use of an AmpC beta-lactamase inhibitor [23]. We used PBA and cloxacillin as AmpC inhibitors. Out of the 21 isolates, eight tested positive for AmpC beta-lactamases using PBA with cefoxitin, while ten gave positive results using cloxacilin with cefoxitin. On comparing the results of both tests, seven isolates demonstrated consistent results. Pitout *et al*. documented 81% sensitivity and 98% specificity when PBA was used with cefoxitin [18]. Our results confirm Pitout's finding of reduced sensitivity of PBA with cefoxitin, as it failed to detect two isolates that have been detected on using cloxacillin with cefoxitin. On the other hand, the high specificity of that test (98%) may indicate that all isolates that tested positive are AmpC producers. Detection of AmpC genes using PCR is mandatory to confirm the production of AmpC beta-lactamase by these isolates.

In this study, the presence of TEM genes alone was significantly associated with non-confirmed isolates. A similar finding has been reported previously [42]. These results could be explained by either the presence of inhibitor-resistant TEM enzymes or the presence of non-ESBL TEM enzymes, as the molecular detection test captures many TEM genes, including the non-ESBL TEM-1. However, in the absence of sequence data, it is not possible to confirm the presence of such a non-ESBL TEM gene among our isolates. The CTX-M and SHV genes were detected in 47.6% and 23.8% of the non- confirmed isolates respectively, which should present a clear indication that the screening test itself is more meaningful than the confirmation test [42].

Conclusion

The data obtained from this study document the emerging threat of ESBL pathogens in our setting as aetiological agents of infection in both the hospital and the community. A high degree of antibiotic co-resistance among ESBL producers was observed, leaving few therapeutic options. Therefore, continued surveillance and judicious antibiotic usage together with the implementation of efficient infection control measures are absolutely required. To our knowledge, this is the first study regarding molecular characterization of ESBL genes in eastern Saudi Arabia. It clearly indicates that CTX-M genes are highly endemic in this region.

Genetic markers conferring resistance to aminoglycosides and cotrimoxazole were often transferred to the recipient strain along with ESBL genes in the conjugation process.

False negative ESBL confirmatory test results occurred at a considerable frequency. Further studies are needed for appropriate detection of concomitant ESBL and AmpC enzyme production among such isolates.

DNA sequencing of ESBL genes is recommended for a reliable epidemiological investigation of antimicrobial resistance. Additionally, further work is required to detect the prevalence of other ESBL-producing Gram-negative bacteria which are emerging as pathogens of concern in the clinical setting.

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