Original Article

Genotypes and cephalosporin susceptibility in extended-spectrum beta-lactamase producing enterobacteriaceae in the community

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Abstract

Introduction: Infections from extended spectrum beta lactamases (ESBLs) producing enterobacteriaceae are increasingly being reported in the community setting. These infections are often multidrug resistant, with clinical and epidemiological implications, and necessitate surveillance measures based on local data. In the present study ESBLs genotypes were correlated with susceptibility to cephalosporins among ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates acquired in the community.

Methodology: We investigated 28 *E. coli* and 24 *K. pneumoniae* isolates by PCR for the presence of bla_{SHV}, bla_{CTX-M}, and bla_{TEM}. Minimum inhibitory concentration (MIC) for cephalosporins was determined by use of E-tests.

Results: bla_{CTX-M} was detected in 46 (88.5%), bla_{SHV} in 13 (25%) and bla_{TEM} in18 (34.6%) of the isolates. Nineteen (36.5%) isolates had more than one genotype detected. Urine specimens provided most of the ESBL-producing isolates (71%) followed by respiratory specimens (11%). MIC_{50} for cefotaxime, ceftazidime, and ceftriaxone were at 60µg/ml, 13µg/ml, and 139µg/ml, respectively. There was a statistically significant association (p-value = 0.017) between bla_{SHV} and resistance to ceftazidime. Though other associations could be seen among the genotypes and susceptibility profiles of the three drugs, they were not statistically significant. Twenty-four (52.2%) of the bla_{CTX-M} isolates were sensitive and nine (19.6%) resistant to ceftazidime. For cefotaxime, 29 (63%) of bla_{CTX-M} isolates were resistant and two (4.3%) were sensitive.

Conclusion: The predominant ESBL genotype in the local community-acquired infections is bla_{CTX-M} , most of which involved the urinary tract. ESBL genes elevated MICs for the cephalosporins, but only bla_{SHV} could predict resistance to ceftazidime.

Key words: ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*; genotype; MIC

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Introduction

Extended spectrum beta-lactamase (ESBL) producing strains of *Enterobacteriaceae* have emerged as a major problem in hospitalized as well as community-based patients [1].These organisms are responsible for a variety of infections such as urinary tract infection (UTI), septicemia, hospital-acquired pneumonia, intra-abdominal abscess, brain abscess and device-related infections. Failure to recognize ESBL producers can have consequences including treatment failure in patients who receive inappropriate antibiotics and outbreaks of multidrug-resistant Gram-negative pathogens which require expensive control efforts [2,3]. In Africa, there are few reports on ESBL producers in community settings where the spread of EBLs could have grave implications for already strained health-care systems. Beta-lactamases remain the most important contributing factor to beta-lactam resistance in Gram-negative pathogens, and their increasing prevalence, as well as their alarming rates of evolution (since the first ESBL was described in 1983, more than 200 ESBLs have been characterized) seems to be directly linked to the clinical use of novel sub-classes of beta-lactams [4]. Cephalosporins are beta-lactam antibiotics that cover a broad range of organisms. The third-generation cephalosporin class is marked by stability to the common beta-lactamases of Gram-negative bacilli, and these compounds are highly active against Enterobacteriaceae.

The Aga Khan University Hospital (AKUH) is a private tertiary institution located in Nairobi, Kenya, with twelve satellite clinics spread across the country which send specimens for culture to the main laboratory. It attends mainly to the middle class in the main hospital as well as to low-income earners in the satellites. Among the Enterobacteriacea, *Escherichia* *coli* and *Klebsiella pneumoniae* are the two most isolated organisms in the hospital's microbiology section and ESBL-producing isolates make up about 8% of the two bacteria species. Cephalosporins are among the most prescribed antibiotics in the hospital. In the year 2009 about 250,000 doses of ceftriaxone were consumed in the outpatient department at AKUH and this number is rising. Currently no information exists on ESBL genotypes present in the community served by AKUH. Previous studies done locally have looked at the CTX-M phenotype only, in a small number of hospitalized neonates in a different hospital, and patients with urinary tract infections [5,6]. Knowledge of the genotypes' prevalence in a certain locality is important in surveillance of the spread in antibiotic resistance since the genotypes of resistance among ESBL-producing Enterobacteriaceae differ from one geographic region to another. Locally, there is increasing and widespread use and misuse of broad spectrum antibiotics in medical facilities and for self-treatment [7] which could lead to a rise in ESBL isolates in the local community. The association of ESBL genotypes with cephalosporin resistance may be used to guide therapy where infections with ESBL Enterobacteriaceae are suspected and susceptibility testing results are not immediately available.

We sought to detect ESBL genotypes and correlate these with susceptibility to cephalosporins among ESBL-producing *E. coli* and *K. pneumoniae* isolates in the local community infections.

Methodology

This was a cross-sectional study using isolates processed in a consecutive manner during the study period. The setting of the study was at the Aga Khan University Hospital, Nairobi, which, as a referral facility has a wide catchment and is quite representative on the trends in the Kenyan urban population.

Patients' characteristics

There were 29 males and 23 females who provided the specimens from which the above isolates were obtained. The ages ranged from two to 90 years with a mean of 44 years.

Specimen source

Urine formed the majority of the specimens (37 of the 52 total specimens). Other specimens were respiratory (6), pus (4), skin (screening) swabs (4), and stool (1).

Isolates

During the period March 2009 to February 2010, 350 multidrug resistant *E. coli* and *K. pneumoniae* isolates cultured from clinical specimens submitted to the microbiology laboratory, AKUH, were screened for ESBL production by the double disk diffusion method on Mueller-Hinton Agar using the Clinical Laboratory Standards Institute (CLSI) guidelines [8]. Fifty-two isolates which met the criteria of being community acquired (infections that are acquired by persons who have not been hospitalized or had a medical procedure preceding 30 days at time of specimen collection) were confirmed to be ESBL producers by the combined disc method (Becton Dickinson, New Jersey, USA) containing ceftazidime (CAZ 30 µg) or cefotaxime (CTX 30 µg) in combination with clavulanate (CLA 10 µg). The presence of ESBL was determined by a $\geq$ 5 mm increase in zone diameters for CAZ/CLA and CTX/CLA compared with those for CAZ and CTX, respectively [8]. There were 28 *E. coli* and 24 *K. pneumoniae* identified by API 20E (bioMerieux, Marcy-l'Etoile, France). All 52 isolates were sensitive to cefoxitin (30 µg) by the disk diffusion method, since cefoxitin resistance along with oxyimino-beta-lactam resistance raises the suspicion of an AmpC-type enzyme [9]. Thirty-seven were from urine, six from sputum, four from pus, four from skin and one from stool.

Molecular characterization

DNA extraction from the isolates was performed using the QIAmp DNA extraction minikit (QIAGEN) per the manufacturer's instructions. A suspension of the isolate was enzymatically lyzed using proteinase K and purified by the use of ethanol-containing washes, followed by the elution of the DNA with distilled water. The quantity and quality of the DNA was confirmed with spectrophotometry (NanoDrop ND1000, Thermo Fisher Scientific Inc, Delaware, USA).

PCR methods

PCR for three beta-lactamases genes, bla_{SHV}, bla_{CTX-M}, and bla_{TEM}, was performed using generic primers sourced from Bioneer Corporation, South Korea (Table 1).

Amplification reactions were performed in a volume of 50 µl containing 1.25 units Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.2 µM of each forward and reverse primers, 200 µM of each dNTP, 12.5 µl of PCR water, and 2.5 µl of DNA template. The

Table 1. Primers used for detection of SHV, CTX-M & TEM genes

Primer		Nucleotide Sequence	Amplicon size	Thermocycling conditions [Reference]
bla_{SHV}	F	5'-CGC CGG GTT ATT CTT ATT TGT CGC-3'	1016 bp	12
bla_{SHV}	R	5'-TCT TTC CGA TGC CGC CGC CAG TCA-3'		
bla_{CTX-M}	F	5'-TTT GCG ATG TGC AGT ACC AGT AA-3'	544 bp	10
bla_{CTX-M}	R	5'-CGA TAT CGT TGG TGG TGC CAT A-3'		
bla_{TEM}	F	5'-CTT CCT GTT TTT GCT CAC CCA-3'	717 bp	11
bla_{TEM}	R	5'-TAC GAT ACG GGA GGG CTT AC-3'		

thermocycling conditions were as described previously [10-12]: bla_{SHV} at 94°C for 5 minutes followed by 30 cycles at 94°C for 30 seconds, 68°C for 60 seconds, and 72°C for 60 seconds, with a final extension of 72°C for 10 minutes; bla_{TEM} at 94°C for 2 minutes followed by 30 cycles of 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute with a final extension at 72°C to 7 minutes; bla_{CTX-M} at 94°C for 2 minutes followed by 30 cycles of 95°C for 20 seconds, 51°C for 30 seconds, 72°C for 30 seconds with a final extension at 72°C to 3 minutes .

PCR products were resolved by electrophoresis on 1% agarose gels at 100 V run for 1 hour and visualized using an UV transilluminator (BioDoc-It Imaging System, UVP-Upland, USA).

Antimicrobial testing

Minimum inhibitory concentration (MIC) was determined by E-tests (AB Biodisk, Solna, Sweden) for the three following cephalosporins chosen on the basis of their being commonly used: ceftriaxone, ceftazidime and cefotaxime. A suspension with a turbidity matching 0.5 MacFarland standard was made by suspending pure bacteria colonies grown overnight for 12 hours in peptone water. This was plated on a Petri dish containing Mueller-Hinton Agar and the E-test strips placed per the manufacturer's instructions. The negative control was *E. coli* American Type Culture Collection (ATCC) 25922 (non-ESBL producer) and the positive control *K. pneumoniae* K6 ATCC 700603. The inoculum was incubated for 16 hours and the MIC read. Categorical assignment into sensitive, intermediate resistance, and resistant was conducted using the CLSI breakpoints [8].

Statistical analysis

Data were collected and stored in an Excel database (Microsoft, Washington, USA) and analyzed using the Statistical Package for Social Sciences (SPSS® 17.0, IBM SPSS, New York, USA). The associations among the bacteria isolates, ESBL genotype and the MICs for the three drugs were determined using the Fisher's Exact Test. A p value of < 0.05 (2 sided) was taken as statistically significant. The study was approved by the Aga Khan University Hospital Ethics and Research Committee.

Results

The isolates' characteristics with the genotypes detected and MICs for the three Cephalosporins are shown in Table 2.

Genotypes

The distribution of the three genotypes detected among the 52 isolates was as follows: bla_{SHV} 13 (25%), blaCTX$_{-M}$ 46 (88.5%), and bla_{TEM} 18 (34.6%). A gel electrophoresis image of the predominant genotype is shown in Figure 1.

The genotypes occurred singularly in 33 (63.5%) of the isolates and in several gene combinations among the remaining 19 (36.5%). bla_{CTX-M} + bla_{TEM} was the most frequent gene combination found in nine (17.3%) of the isolates followed by a combination of the three genes in six (11.5%).

When the genotypes were analyzed by the bacteria species, bla_{CTX-M} was predominant in the two species; bla_{SHV} was almost exclusive to *K. pneumoniae* and was detected in 12 (50%) of the isolates as opposed to only one (4%) *E. coli* isolate. Figure 2 shows that bla_{TEM} was also detected in more *K. pneumoniae* isolates (11; 46%) than in *E. col,* (7; 25%) .

Antibiotic sensitivity

The MIC_{50} for the three cephalosporins were as follows: cefotaxime 60 µg/ml, ceftazidime 13 µg/ml, and ceftriaxone, 139 µg/ml. When the antibiotic

Table 2. Bacteria isolates' characteristics, genotypes and MICs

						MIC (µg/ml)		
Isolate no	**Species**	**Specimen source**	**Genotype(s)**			**cefotaxime**	**ceftazidime**	**ceftriaxone**
1	*k. pneumoniae*	urine	$bla_{\text{CTX-M}}$			24	8	64
2	*k. pneumoniae*	urine	bla_{SHV}			1.5	1	6
3	*E. coli*	urine	$bla_{\text{CTX-M}}$			256	128	256
4	*k. pneumoniae*	urine	$bla_{\text{CTX-M}}$			32	16	128
5	*E. coli*	urine	$bla_{\text{CTX-M}}$			16	4	48
6	*E. coli*	pus	$bla_{\text{CTX-M}}$			256	8	256
7	*E. coli*	urine	$bla_{\text{CTX-M}}$			128	12	256
8	*k. pneumoniae*	skin	$bla_{\text{CTX-M}}$			256	256	256
9	*k. pneumoniae*	urine	$bla_{\text{CTX-M}}$	bla_{SHV}		96	12	256
10	*E. coli*	urine	$bla_{\text{CTX-M}}$			16	3	24
11	*k. pneumoniae*	respiratory	$bla_{\text{CTX-M,}}$	bla_{TEM}	bla_{SHV}	256	64	256
12	*E. coli*	urine	$bla_{\text{CTX-M}}$	bla_{TEM}		64	16	128
13	*k. pneumoniae*	respiratory	$bla_{\text{CTX-M}}$	bla_{SHV}		96	16	256
14	*E. coli*	urine	$bla_{\text{CTX-M}}$			48	4	128
15	*E. coli*	urine	$bla_{\text{CTX-M}}$			3	0.75	32
16	*E. coli*	urine	$bla_{\text{CTX-M}}$			4	1.5	6
17	*E. coli*	urine	$bla_{\text{CTX-M}}$			64	8	128
18	*E. coli*	urine	$bla_{\text{CTX-M}}$			96	8	256
19	*k. pneumoniae*	urine	$bla_{\text{CTX-M}}$	bla_{SHV}	bla_{TEM}	128	32	256
20	*E. coli*	urine	$bla_{\text{CTX-M}}$			256	16	256
21	*E. coli*	urine	$bla_{\text{CTX-M}}$	bla_{TEM}		24	4	64
22	*k. pneumoniae*	respiratory	$bla_{\text{CTX-M}}$			24	8	128
23	*E. coli*	urine	$bla_{\text{CTX-M}}$	bla_{SHV}		64	12	128
24	*k. pneumoniae*	respiratory	$bla_{\text{CTX-M}}$			64	256	256
25	*k. pneumoniae*	respiratory	$bla_{\text{CTX-M}}$			256	64	256
26	*E. coli*	urine	$bla_{\text{CTX-M}}$			128	12	256
27	*E. coli*	urine	$bla_{\text{CTX-M}}$			32	6	64
28	*E. coli*	urine	$bla_{\text{CTX-M}}$			32	8	192
29	*k. pneumoniae*	respiratory	$bla_{\text{CTX-M}}$			64	8	128
30	*E. coli*	urine	$bla_{\text{CTX-M}}$			12	3	32
31	*k. pneumoniae*	pus	$bla_{\text{CTX-M}}$			48	4	256
32	*E. coli*	urine	$bla_{\text{CTX-M}}$			48	8	96
33	*E. coli*	urine	$bla_{\text{CTX-M}}$			32	8	256
34	*E. coli*	urine	$bla_{\text{CTX-M}}$	bla_{TEM}		64	16	96
35	*k. pneumoniae*	urine	bla_{SHV}			48	12	256
36	*k. pneumoniae*	urine	$bla_{\text{CTX-M}}$	bla_{TEM}		64	64	256
37	*E. coli*	stool	$bla_{\text{CTX-M}}$	bla_{TEM}		128	128	256
38	*E. coli*	urine	bla_{TEM}			192	16	256
39	*k. pneumoniae*	urine	bla_{SHV}			192	24	256
40	*k. pneumoniae*	urine	$bla_{\text{CTX-M}}$	bla_{TEM}		32	8	64
41	*k. pneumoniae*	urine	bla_{SHV}	bla_{TEM}		256	256	256
42	*k. pneumoniae*	skin	$bla_{\text{CTX-M}}$	bla_{SHV}	bla_{TEM}	32	6	48
43	*E. coli*	urine	$bla_{\text{CTX-M}}$			64	8	96
44	*k. pneumoniae*	skin	bla_{TEM}			128	16	192
45	*E. coli*	urine	$bla_{\text{CTX-M}}$			48	8	96
46	*k. pneumoniae*	urine	$bla_{\text{CTX-M}}$	bla_{SHV}	bla_{TEM}	32	16	128
47	*k. pneumoniae*	pus	$bla_{\text{CTX-M}}$	bla_{SHV}	bla_{TEM}	48	12	128
48	*E. coli*	urine	$bla_{\text{CTX-M}}$			48	16	96
49	*E. coli*	urine	$bla_{\text{CTX-M}}$	bla_{TEM}		48	8	96
50	*E. coli*	pus	$bla_{\text{CTX-M}}$	bla_{TEM}		32	8	96
51	*k. pneumoniae*	urine	$bla_{\text{CTX-M}}$	bla_{SHV}	bla_{TEM}	32	12	128
52	*k .pneumoniae*	skin	$bla_{\text{CTX-M}}$	bla_{TEM}		96	32	256

Table 3. Association between ESBL genotype and antibiotic sensitivity profile

Genotype		Cefotaxime			Fisher's Exact Test	Ceftazidime			Fisher's Exact Test	Ceftriaxone			Fisher's Exact Test
		sensitive	intermediate	resistant	P value	sensitive	intermediate	resistant	P value	sensitive	intermediate	resistant	P value
SHV	Y	1 (7.7%)	3 (23.1%)	9 (69.2%)	0.88	2 (15.4%)	7 (53.8%)	4 (30.8%)	0.017	1 (7.7%)	0 (0%)	12 (92.3%)	0.532
	N	2 (5.1%)	12 (30.8%)	25 (64.1%)		23(59.0%)	9(23.1%)	7(17.9%)		1 (2.6%)	3 (7.7%)	35 (89.7%)	
CTX-M	Y	2 (4.3%)	15 (32.6%)	29 (63.0%)	0.16	24 (52.2%)	13(28.3%)	9 (19.6%)	0.236	1 (2.2%)	3 (6.5%)	42 (91.3%)	0.247
	N	1 (16.7%)	0 (0%)	5 (83.3%)		1 (16.7%)	3 (50.0%)	2 (33.3%)		1 (16.7%)	0 (0%)	5 (83.3%)	
TEM	Y	0 (0%)	6 (33.3%)	12 (66.7%)	0.63	5 (27.8%)	7 (38.9%)	6 (33.3%)	0.085	0 (0%)	0 (0%)	18 (100%)	0.467
	N	3 (8.8%)	9(26.5%)	22 (64.7%)		20 (58.8%)	9(26.5%)	5 (14.7%)		2 (5.9%)	3 (8.8%)	29 (85.3%)	

Y = gene present
N = gene absent

sensitivity profiles of the three drugs were compared among the two bacteria species, there were no differences among the two with regard to cefotaxime and ceftriaxone with p values of 1.00 and 0.309 respectively. The ceftazidime sensitivity profile, however, was different in the two bacteria types with *K. pneumoniae* showing more resistance than *E. coli* ($p = 0.011$). As shown in Table 3, in the comparison of the genotype and antibiotic sensitivity, only the presence of bla_{SHV} had a significant association with respect to the ceftazidime sensitivity profile (p value= 0.017).

Other trends, however, could be seen. Twenty-four (52.2%) of the isolates with bla_{CTX-M} were sensitive to ceftazidime compared to only one (16.7%) in those without, and 44 (96%) of bla_{CTX-M} containing isolates were resistant to cefotaxime while five (83.3%) without the gene were also resistant. The 18 (100%) isolates that had bla_{TEM} were resistant to ceftriaxone.

Discussion

Our study used 52 isolates from community-acquired *E. coli* and *K. pneumoniae* infections for the detection of the common ESBL genotypes and to ascertain their association with the sensitivity profiles of three cephalosporin antibiotics. These isolates were recovered from patients in all the age groups from infants to the elderly. Males and females were represented in almost equal proportions and therefore could be said to be fairly representative of the community scenario. bla_{CTX-M} had the largest presence at 88.5% of all the isolates. This was also the case among the two bacteria species though the occurrence was higher in *E. coli* than in *K. pneumoniae* at 96% and 79%, respectively.

bla_{CTX-M} has been reported in several parts of the world as the predominant ESBL genotype, especially in *E. coli* among community-acquired infections and in nosocomial infections as well [13]. The regions include most countries in Europe and South America but other regions are also reporting it. Guo-Bao Tian showed that 35 (95%) out of 39 ESBL isolates he investigated in Phillipines General Hospital had bla_{CTX-M} [14]. Dearbhaile and Workeum demonstrated the same in Ireland and Korea, respectively [15, 16]. Pitout *et al*. found that most ESBL- producing isolates that cause community-acquired infections in Europe have been *E. coli* with bla_{CTX-M} [17] and Rodriguez-Bano found this to be so for lower urinary tract infections [18].

Our results showed similar findings to the above studies with bla_{CTX-M} as the predominant genotype found in 46 (88.5%) of the 52 isolates analyzed.

During the 1990s, TEM-ESBLs and SHV-ESBLs were dominant among ESBLs worldwide and CTX-M producing organisms were rarely recognized [13]. Their presence was inferred in surveillance studies because of higher levels of resistance to cefotaxime than to ceftazidime, a characteristic that is usually present in all CTX-M-producing isolates [19]. At that time, TEM-ESBLs and SHV-ESBLs were mainly associated with epidemic clones, and *K. pneumoniae* was the main carrier of the ESBL genes [20]. This is reflected to some extent by the above results in which bla_{SHV} and bla_{TEM} are still more dominant in *K. pneumoniae* (50% and 46%) as compared to *E. coli* (4% and 25% respecively).

While the presence of the ESBL genes generally was associated with varying degrees of resistance to the three cephalosporins, the presence of a particular genotype could not predict the susceptibility pattern to a particular drug with the exception of bla_{SHV}, which was associated with resistance to ceftazidime. Similarly, isolates that had bla_{CTX-M} were more sensitive to ceftazidime than those without (52.2% vs. 16.7%), though when isolates with intermediate resistance and those resistant to ceftazidime were included there was no statistical significance (Table 3). The susceptibility of bla_{CTX-M} containing isolates to ceftazidime has been documented by other authors who suggest that ceftazidime can be used in the treatment of community-acquired UTIs due to CTX-M ESBLs [21]. This presents a possible clinical application of genotyping ESBLs and for empiric therapy in UTIs suspected to be due to ESBL-producing *E. coli.* Half of the *K. pneumoniae* isolates carried bla_{SHV} which predicted resistance to ceftazidime, making it unsuitable for use as treatment in this species.

In a study conducted by Tribuddharat *et al*. on *K. pneumoniae* isolated from patients at a university hospital in Thailand, bla_{CTX-M} was associated with resistance to cefotaxime and ceftriaxone but not ceftazidime [22]. bla_{TEM} did not have any statistically significant association with resistance to any drug. Only two isolates had bla_{SHV} and seemed to correlate with the resistance to ceftazidime better than to cefotaxime and ceftriaxone [22]. The differences in the outcomes with regard to bla_{CTX-M} and association with resistance to cefotaxime and ceftriaxone could be explained by the latter study only using *K. pneumoniae*. The patient population was also hospital based and not community based as in the current study.

Limitations of the study

This study did have limitations because of the small numbers studied. The isolates processed only represented those patients who were referred to the laboratory for investigations. Those treated empirically without culture reports were omitted though it is difficult to tell what effect if any this would have had on the overall results.

These results are the findings from a single microbiology laboratory and need corroboration. A multicenter study might give a better picture of ESBL genotypes in the local community setting.

Conclusion

The CTX-M genotype dominates in the local community-acquired infections caused by ESBL-producing *E. coli* and *K. pneumoniae*. These infections tend to be mostly lower urinary tract infections, which is in agreement with previous studies [18, 23]. In our study only bla_{SHV} predicts resistance to ceftazidime. Isolates containing $bla_{CTX\text{-}M}$ were mostly susceptible to ceftazidime which could be used empirically in complicated UTIs suspected to be due to CTX-M producing *E. coli* as they rarely harbored bla_{SHV} in our study.

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