ORIGINAL ARTICLE

Occurrence of extended-spectrum beta-lactamase, AmpC and MBLase producers among multidrug-resistant Enterobacteriaceae causing urinary tract infection in a tertiary health-care teaching hospital

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ABSTRACT

Introduction: Emerging multidrug resistance continues to be a major concern in healthcare settings. The aim of the study was to determine the resistance pattern of multidrug-resistant (MDR) Enterobacteriaceae causing urinary tract infections in our hospital and to report the occurrence of extended-spectrum beta-lactamase (ESBL), AmpC and metallo-beta-lactamase (MBL) production in them. **Materials and Methods:** Out of 280 MDR strains collected over a period of one year, 130 strains of *Escherichia coli* (96), Klebsiella spp. (31) and Enterobacter spp. (3) resistant to the second- and third-generation Cephalosporins were selected for further testing. Cefotaxime, Cefotaxime-Clavulanic acid, Ceftazidime, Ceftazidime-Clavulanic acid and Cefepime, Cefepime-Clavulanic acid Etest strips, Cefoxitin and Cefotetan with Boronic acid and Imipenem/Imipenem-EDTA Etest strips were used to detect ESBLs, AmpC and MBLs. Multiplex polymerase chain reaction (PCR) was done to detect plasmid-mediated AmpC genes. **Results:** Among 130 Cefoxitin-resistant strains, Cefoxitin-Boronic acid inhibitor method detected AmpC phenotype in 116 (89.2%) isolates. The overall occurrence of AmpC (*n* = 280) was 116 (41.42%). 92 (32.8%) isolates were found to be ESBL producers by the Clinical and Laboratory Standards Institute confirmatory method. ESBL production was detected in 107 (38.2%) more isolates by Cefepime/Cefepime-Clavulanic acid Etest. MBL producers were relatively low in our study 5 (1.8%). PCR detected CIT genotype (CMY-2) in 13 isolates (4.6%). **Conclusion:** This study reveals high prevalence of AmpC and ESBL co-carriage suggesting plasmid-mediated spread, indicates the need for surveillance of resistance mechanisms and takes necessary measures to control the emergence of MDR organisms.

Key words: AmpC genotype, extended-spectrum beta-lactamase, multidrug resistance, urinary tract infection

INTRODUCTION

Urinary tract infection (UTI) is one of the most common diseases present in both the community and hospital setting. It accounts for 35% of nosocomial infections[1] and approximately one million hospitalisations worldwide.[2] In community practice, UTI is the second most common infection. Approximately 40% of females and 12% of males experience at least one symptomatic infection during their lifetime.[3]

It is difficult to accurately assess the incidence of UTI because it is not a notifiable disease. The increasing prevalence of UTI by antibiotic-resistant Enterobacteriaceae makes empirical treatment of these infections difficult. The various mechanisms of drug resistance among pathogens include extended-spectrum beta-lactamase (ESBL) production, AmpC production, efflux mechanism and porin deficiency. Production of ESBL and AmpC beta-lactamases

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is the most common mechanisms of resistance to third-generation Cephalosporins. ESBLs are able to hydrolyse Oxymino-cephalosporins and Monobactams but not Cephamycins and are susceptible to Beta-lactam inhibitors. AmpC confers resistance to all third-generation Cephalosporins, Cephamycins, Monobactams, shows variable resistance to Beta-lactam inhibitors and is usually susceptible to the fourth-generation Cephalosporins.[4] Genes for ESBL are located on plasmids, while genes for AmpC are either plasmid or chromosomally encoded. Transmissible plasmids have acquired genes for AmpC enzymes which can appear in bacteria lacking or poorly expressing chromosomal AmpC gene such as *Escherichia coli*, Klebsiella spp. and *Proteus mirabilis*.[5]

Carbapenems are often considered as the last resort antibiotics in the treatment of infections by multidrug-resistant (MDR) Enterobacteriaceae. However, during the last few years, Carbapenem resistance has been increasingly reported among this group and is largely attributed to the production of metallo-beta-lactamases (MBLs).

Antibiotic resistance is directly proportional to the use and misuse of antibiotics. Therefore, isolating the causative organisms and determining their susceptibility pattern are mandatory for helping the selection of an antibiotic for effective treatment. Keeping this in view, we conducted this study to fulfil the following objectives:

- To study the occurrence of MDR Enterobacteriaceae in patients with community and hospital-acquired UTI in our hospital (noncritical areas)
- To determine antimicrobial susceptibility pattern and ESBL, AmpC and MBLase producers among these isolates.

MATERIALS AND METHODS

The study was conducted in the clinical microbiology laboratory of Government T. D. Medical College, Alappuzha, Kerala, for one year from July 2012 to June 2013 after approval by the Institutional Ethical Committee.

During the one-year study, urine samples of patients attending the Outpatient Department and admitted in the wards with UTI were screened for MDR Enterobacteriaceae. Two hundred and eighty non-duplicate isolates thus obtained were identified by standard microbiological procedures, and susceptibility testing was done and interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Among them, 130 isolates showing resistance to one or more extended-spectrum Cephalosporins and Cefoxitin by Kirby-Bauer disk diffusion method (CLSI 2009)[6] were selected and screened for ESBL, AmpC and MBL production as follows.

Extended-spectrum beta-lactamase testing

ESBLs were detected by CLSI method using Cefotaxime (30 μg) and Ceftazidime (30 μg), along with Cefotaxime-Clavulanic acid (30, 310 μg) and Ceftazidime-Clavulanic acid (30, 10 μg) combination discs[7] (HiMedia, India) respectively.

Klebsiella pneumoniae ATCC 700603 and *E. coli* ATCC 25922 were used as ESBL positive and negative controls, respectively. ESBL production was inferred if the inhibition zone increased by 5 mm towards the combination disc in comparison to third-generation Cephalosporin disc alone.

Inhibitor-based method for AmpC detection[8]

The difference in inhibition zone for Cefoxitin (30 μg) and Cefotetan (30 μg) discs (HiMedia, India) alone and in combination with Phenylboronic acid (400 μg) was determined. Boronic acid was prepared by dissolving 120 mg of Phenylboronic acid in 3 ml Dimethyl sulfoxide, to which 3 ml of sterile distilled water was added. Discs were prepared by dispensing 20 μl of stock solution on Cefoxitin and Cefotetan discs. An increase in zone diameter of >5 mm in the presence of Phenylboronic acid compared with Cefoxitin[9,10] and Cefotetan tested alone was considered to be positive for the presence of AmpC beta-lactamase.

Detection of extended-spectrum beta-lactamase production by Etest

The Etest strip Cefepime/Cefepime-Clavulanate (bioMerieux SA, France) containing Cefepime (minimum inhibitory concentration [MIC] 0.25–16 μg/ml) and Cefepime plus 4 mg/L Clavulanic acid (MIC 0.064–4 mg/L) was used to detect production of ESBL.[11]

Results were read either as MIC values, presence of phantom zones or deformation of inhibition ellipses.

The isolates were considered to be ESBL producers if:

- The MIC ratio for Cefepime/Cefepime-Clavulanate was ≥8
- Presence of phantom zone, deformation or ellipse even if MIC ratio is <8 or cannot be read.

Detection of metallo-beta-lactamase

The Etest MBL strip containing a double-sided seven-dilution range of Imipenem (IP) (4–256 μg/ml) and IP (1–64 μg/ml) in combination with a fixed concentration of EDTA (IPI) was used for MBL detection. The test was done according to the manufacturer's instructions

(Etest technical manual, bioMerieux SA, France). MIC ratio of ≥8 for the two reagent sides, a phantom zone between IP/IP inhibitor and deformation of either ellipse was indicative of MBL production.

Molecular detection and characterisation of AmpC resistance

Multiplex polymerase chain reaction (PCR) was done to detect the most common plasmid-mediated AmpC genes - ACC, FOC, MOX, DHA, CIT and EBC reported in previous literature.[12,13]

Preparation of template DNA

A single colony of each isolate from MacConkey agar plate was inoculated into 100 µl of 0.5M TrisEDTA (pH8). After mixing thoroughly by vortexing, the cells were incubated at 100°C for 10 min and cellular debris was removed by centrifugation at 17310 g for 5 min. Two microlitres of the supernatant was used as the DNA template for PCR amplification.

PCR was performed with a final volume of 50 µl in 0.5 ml thin-walled tubes.

Each reaction contained 25 µl of Emerald AMP GT PCR Master Mix (TakaRa, Bio USA); 0.6 µM primers MOXMF, MOXMR, CITMF, CITMR, DHAMF and DHAMR; 0.5 µM primers ACCMF, ACCMR, EBCMF and EBCMR; and 0.4 µM primers FOXMF and FOXMR [Table 1]; 2 µl of template DNA was added to 48 µl of the master mix. Amplification reaction was carried out as follows: Initial denaturation at 94°C for 1 min followed by thirty cycles of denaturation at 94°C for 1 min, primer annealing at 64°C for 30 s, primer extension at 72°C for 1 min and final extension step of 7 min at 72°C. Fifteen microlitres of PCR product was loaded on to 2% agar gel, electrophoresed and stained with Ethidium bromide (5 µg/ml). The bands were visualised by GELDOC (Syngene). Negative controls were PCR mix with water.

AmpC genotype standard strains A7 (ACC), A9 (CMY-2) and PMG252 (FOX-5) (provided by Anand Manoharan, Department of Infectious Diseases, CMC, Vellore) were used as PCR positive controls.

RESULTS

During the one-year study period, 280 consecutive, non-repetitive, MDR Enterobacteriaceae were isolated from urine samples of patients with both community- and hospital-acquired UTI. The antibiotic resistance pattern of these isolates is shown in Figure 1. There were 130 isolates showing resistance to one or more extended-spectrum Cephalosporins and Cefoxitin by Kirby-Bauer disk diffusion method (CLSI 2009).[6] These were selected and screened for ESBL, AmpC and MBL production [Table 2].

Among 130 Cefoxitin-resistant isolates, AmpC phenotype was detected in 116 isolates (89.2%) by inhibitor-based

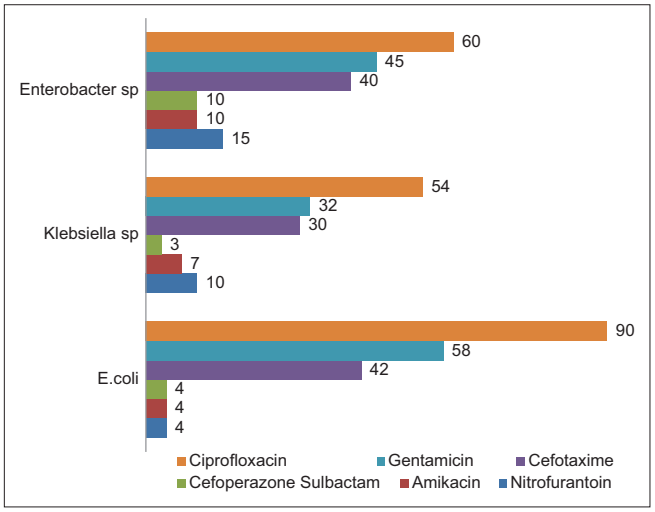

Figure 1: Antibiotic resistance pattern of multidrug-resistant isolates in the study obtained by disk diffusion method

Table 1: Primers used in multiplex polymerase chain reaction

Target gene	Primers	Sequences (5′-3′)	Amplicon size (bp)
MOX-1, MOX-2, CMY-1	MOXMF	GCT GCT CAA GGA GCA CAG GAT	520
CMY-8 to CMY-11	MOXMR	CAC ATT GAC ATA GGT GTG G TG C	
LAT-1 to LAT-4, CMY-2	CITMF	TGG CCA GAA CTG ACA GGC AAA	462
To CMY-7, BIL-1	CITMR	TTT CTC CTG AAC GTG GCT GGC	
DHA-1, DHA-2	DHAMF	AAC TTT CAC AGG TGTGCT GGG T	405
	DHAMR	CCG TAC GCATAC TGG CTT TGC	
ACC	ACCMF	AAC AGC CTC AGC AGC CGG TTA	346
	ACCMR	TTC GCC GCA ATC ATC CCT AGC	
MIR-IT ACT-1	EBCMF	TCG GTA AAG CCG ATG TTG CGG	302
	EBCMR	CTT CCA CTG CGG CTG CCA GTT	
FOX-1 to FOX-5b	FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190
	FOXMR	CAA AGC GCG TAA CCG GAT TGG	

Cephamycins (CMY), Cefoxitin (FOX) and Moxalactam (MOX) or Latamoxef (LAT), AmpC type (ACT) or Ambler class C (ACC), Miriam Hospital Providence RI (MIR-1) or Dharan Hospital in Saudi Arabia (DHA), BIL-1 named after the patient (Bilal)

method using Boronic acid. The overall occurrence of AmpC in the study was found to be 41.42% (116/280).

In inhibitor-based method, we also compared the sensitivity of Cefoxitin (30 μg) and Cefotetan (30 μg) as screening drug for AmpC detection. Cefoxitin was found to be more sensitive than Cefotetan in detecting AmpC phenotype [Table 3].

ESBL production in the study group by CLSI phenotypic confirmatory method was found to be 32.8% (n = 92). We also tested the performance of Cefepime-Clavulanate ESBL Etest strip for detection of ESBLs in the study group producing AmpC enzymes. This method detected more number of ESBL positive isolates, i.e., 107 (38.2%) as compared to CLSI confirmatory method [Table 4].

In the study, five Carbapenem-resistant strains 1.8% (5/280) were found to be MBL producers by IP/IPI Etest.

Multiplex PCR done on 130 Cefoxitin-resistant isolates revealed CIT (CMY-2) genotype in eight *E. coli* and five Klebsiella spp. [Figure 2].

Table 2: Bacterial isolates screened (n=130)

Bacterial isolates	Number
Escherichia coli	96
Klebsiella spp.	31
Enterobacter spp.	3

Table 3: Number of isolates positive by inhibitor based method to Cefoxitin and Cefotetan (n=130)

Inhibitor based method	Number (%)
Inhibitor-based method positive by Cefoxitin	116 (89.2)
Inhibitor-based method positive by Cefotetan	102 (78.4)

Table 4: AmpC and extended-spectrum beta-lactamase test results for Enterobacteriaceae isolates studied

Organism	Inhibitor based test for AmpC	ESBL combined disc test	ESBL Etest
Escherichia coli (96)			
Positive	88	70	80
Negative	8	2	3
Klebsiella spp. (31)			
Positive	27	18	22
Negative	4	1	1
Enterobacter spp. (3)			
Positive	1	1	1
Negative	2	0	0
Total	130	92	107

ESBL: Extended-spectrum beta-lactamase

DISCUSSION

In India, the prevalence of ESBL and AmpC beta-lactamases producers vary among various hospitals and even between different sites of infection such as UTI, wound infection and septicaemia, of which UTI is the most common infection. However, most of the hospitals are ignorant to the prevailing antibiotic susceptibility patterns. This may result in inappropriate prescription of antibiotics. In view of these issues, our study was designed to assess the antimicrobial susceptibility pattern of MDR Enterobacteriaceae causing UTI and also to study the occurrence of ESBL, AmpC and MBL production among them.

The susceptibility pattern of uropathogens in the study shows a higher rate of susceptibility to Cefoperazone-Sulbactam (90%–97%), Amikacin (90%–95%) and Nitrofurantoin (90%–95%) compared to other antibiotics.

The reduced susceptibility to Quinolones and third-generation Cephalosporins is probably due to their empirical use in our region. Nitrofurantoin seems to be the only promising oral antibiotic effective in this scenario.

The exact prevalence of AmpC production is unknown due to the lack of simple and reliable methods for detection in a routine laboratory. Although there are no CLSI guidelines for phenotypic methods to screen and detect AmpC activity in Enterobacteriaceae, several methods have been developed for the detection of plasmid-mediated AmpC, namely, the three-dimensional test,[14] modified double-disk test,[15] AmpC disk test,[16] IBM employing Boronic acid,[8] and modified three-dimensional.[17] Reduced susceptibility to Cefoxitin is one of the screening methods for putative AmpC enzyme detection.

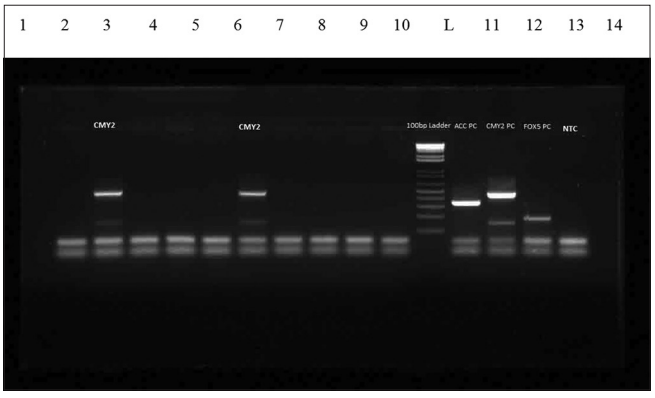

Figure 2: Characterisation of AmpC genes. A 100 bp DNA ladder (l). The amplified products for *Escherichia* coli (2) and *Klebsiella* (6) for CMY-2 are shown to the left of the ladder. Positive controls for ACC (11), CMY-2 (12) and FOX (13) with NTC are indicated on the right

In the present study, not all 130 Cefoxitin-resistant isolates were found to be positive for AmpC production by Boronic acid inhibitor methods. This can be explained due to the following reasons. First, Cefoxitin resistance is not only due to AmpC beta-lactamases production but also may be due to ESBLs and MBL production or non-enzymatic mechanism such as porin channel mutation. Second, Cefoxitin resistance can result from overexpression of chromosomal AmpC gene due to mutation in the promoter or attenuator regions. The other reason is that Cefoxitin has been demonstrated as a substrate to active efflux pump in clinical isolates.

The current study showed plasmid-mediated AmpC beta-lactamases in 116 (41.42%) of isolates. This is comparatively high as against Ratna *et al.*[18] and Manoharan *et al.* This higher prevalence may be due to exposure to previous empirical cephalosporin therapy which is a known selective pressure for increasing AmpC production among them.

The prevalence of plasmid-mediated AmpC resistance is unknown at the national level in many countries due to lack of adequate molecular studies. In spite of many phenotypic tests, PCR is considered the gold standard which is not available for routine diagnostic laboratories. In the study, only 13 (4.6%) isolates were found to be positive by multiplex PCR. This is in accordance with Manoharan *et al.* (5.2%). The 13 isolates showed positivity for CIT genotype (CMY-2). This may be related to geographical and epidemiological distribution of AmpC in our area. A Canadian study 2005 also reports CMY-2 gene in 13.5% of isolates.[19]

Discrepancy between genotypic and phenotypic tests may be due to the following reasons:

- The presence of more AmpC beta-lactamases genes that continue to expand beyond those contained in the six families of genes covered by PCR
- The other explanation is that the isolates were mostly likely to be hyper-producers due to over-expression of chromosomal AmpC gene
- False negative results may be explained by the fact that the genes are detected by PCR but not effectively expressed phenotypically.

Enterobacteriaceae-producing both AmpC and ESBL have been increasing reported worldwide. In this study, co-production was observed in 38.2% of isolates. This is comparatively higher than that reported by Devaraju[20] (24%) and Nasir *et al.*[21] (11.5%). Although the current CLSI guidelines recommend ESBL confirmation using synergy tests, this may be insufficient in situations where high-level expression of AmpC may mask recognition of ESBL. Cefepime is a more reliable detector in the presence of AmpC as it is stable to the enzyme and will thus demonstrate synergy arising from inhibition of ESBL by Clavulanate in the presence of AmpC. In our opinion, Cefepime-Clavulanate Etest is a suitable substitute test in such cases.

CONCLUSION

To conclude, 107 (38.2%), 116 (41.42%) and 5 (1.8%) of ESBL, AmpC and MBL producers were detected, respectively, among Enterobacteriaceae-causing UTI in our hospital. The predominant isolates obtained were *E. coli* and Klebsiella spp. Boronic acid inhibitor method using Cefoxitin reliably detected AmpC when compared to PCR in the present study. The most prevalent AmpC gene belongs to CMY-2. Cefepime-Clavulanate Etest strip is a valuable supplement to the current methods of ESBL detection among AmpC-producing isolates. The high co-carriage of AmpC and ESBL in this study is a cause of major concern. The dissemination of these plasmid-mediated resistance genes within the hospital is an important public health issue. Thus, the study advocates the necessity for continual surveillance of resistance mechanisms for better patient outcomes and thus avoids inappropriate therapy and reduces antibiotic resistance through better infection control practices.

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Conflicts of interest

There are no conflicts of interest

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