ORIGINAL ARTICLE

Concurrent occurrence of Amp C and Cefotaxime (CTX)-M inclinical isolates of enterobacteriaceae

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ABSTRACT

Enterobacteriaceae producing both Amp C beta lactamases and extended-spectrum beta lactamases (ESBLs) have been increasingly reported worldwide. While the phenotypic tests for ESBL is standardised and used widely, it is not so for Amp C. When they coexist they may mask each other's detection phenotypically. We undertook this study to detect the concurrent occurrence of Cefotaxime (CTX)-M and plasmid Amp C in clinical isolates of Enterobacteriaceae by phenotypic and genotypic methods. One hundred clinically significant isolates of *Escherichia coli* (*E. coli;* 43), *Klebsiella pneumoniae* (*K. pneumoniae;* 43) and *Proteus mirabilis* (*P. mirabilis;* 14) were included in the study. Antibiotic susceptibility testing to various classes of antimicrobials was performed by disc diffusion using Clinical Laboratory Standards Institute (CLSI) guidelines. Isolates were screened for production of ESBL by CLSI method and Amp C beta lactamase by inhibitor based method using boronic acid and cloxacillin. Polymerase chain reaction (PCR) was performed for the detection of plasmid Amp C genes and bla_{CTX-M}. Plasmid Amp C genes were detected in 27 isolates which included CIT (Origin Citrobacter freundii): 14; DHA (Dhahran Hospital in Saudi Arabia): 12; EBC (Origin Enterobacter cloacae): 1. Bla_{CTX-M} was detected in 51 isolates. Both coexisted in one *E. coli* and two *K. pneumoniae*. In one of the *K. pneumoniae* isolate, all phenotypic tests employed were negative. A high degree of cross resistance to other classes of antimicrobials was observed. Carbapenem resistance was noted in 21 isolates. The concurrent occurrence of Amp C and CTX-M is not common in clinical isolates of Enterobacteriaceae. Phenotypic tests perform poorly when these enzymes are coproduced.

Key words: CTX-M, enterobacteriaceae, plasmid Amp C

INTRODUCTION

Beta lactamases have become synonymous with antimicrobial resistance in gram-negative bacteria. Till date, more than 1,000 beta- β-lactamases have been characterised.[1] Extended-spectrum beta lactamase (ESBL) producing gram-negative bacteria are a serious clinical concern across the world since they cause a variety of infections with the risk of therapeutic failure. Much has been published on ESBLs and their impact. In Asia, among ESBLs, Cefotaxime (CTX)-M type beta lactamases play a major role. These are natural ESBLs that exhibit a striking substrate preference for CTX (and Ceftriaxone) over Ceftazidime. During the past decade, CTX-M-type ESBLs have undergone a rapid and global spread leading to the 'CTX-M pandemic', thus outnumbering the classic TEM (Temoneira) and SHV (Sulphydryl variable types).[2] Amp C beta lactamases belong to Ambler class C, hydrolyse cephamycins and are not inhibited by beta lactamase inhibitors.[3] Both Amp C and CTX-M producers also carry resistance genes for other antibiotics namely fluoroquinolones and aminoglycosides, thus further limiting the choice of therapy. Presence of Amp C and/or CTX-M when combined with a loss of porin results in resistance to carbapenems. So far, simultaneous occurrence of CTX-M type ESBLs and plasmid-mediated AmpC beta lactamases in gram-negative bacteria has not been widely investigated.[4-6] This study was carried out to determine the concurrent occurrence of CTX-M and plasmid Amp C in clinical isolates of *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Proteus mirabilis* (*P. mirabilis*).

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MATERIALS AND METHODS

The study protocol was approved by the Institutional Ethics Committee

Clinical isolates

The study was carried out in a 1,600 bedded university teaching hospital for a period of 6 months (August 2010

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to January 2011). It included a total of 100 non-duplicate, clinically significant third-generation cephalosporin-resistant isolates belonging to Enterobacteriaceae family, namely *E. coli*, *K. pneumoniae* and *P. mirabilis,* isolated from specimens obtained from hospitalised patients. The study isolates were identified up to species level by standard biochemical tests and/or Microscan Walk Away 96 and gram-negative panels (Siemens Healthcare Diagnostics Inc — Sacramento CA, USA). They were obtained from clinical specimens such as blood, exudative specimens and respiratory secretions. All urine isolates and those isolates which were deemed as colonizers were excluded from the study. Sample size was calculated for a prevalence of ESBL of 72% and Amp C of 27%, and a total of 100 isolates during the study period were selected for molecular characterisation of CTX-M and Amp C.

Antimicrobial susceptibility testing

Antibiotic susceptibility test was done by disc diffusion method as per the CLSI guidelines.[7] The drugs tested were Ceftazidime (30 μg), CTX (30 μg), Cefepime (30 μg), Ciprofloxacin (5 μg), Amikacin (30 μg), Piperacillin — Tazobactam (100/10 μg), Cefoxitin (30 μg), Imipenem (10 μg) and Meropenem (10 μg) [Himedia laboratories, Mumbai, India].

Phenotypic tests

ESBL screening

Disc diffusion

Isolates were tested for ESBL production by the CLSI method using Ceftazidime and CTX (30 μg) disks, with and without Clavulanic Acid (10 μg) (Himedia Laboratories, Mumbai, India). A difference in inhibition zones of ≥5 mm for at least one extended-spectrum Cephalosporin-Clavulanic Acid combination versus the corresponding extended-spectrum Cephalosporin alone was considered indicative of ESBL production.[7]

Minimum inhibitory concentration

Minimum Inhibitory Concentration (MIC) to CTX, with and without Clavulanic acid, was determined by agar Dilution method in accordance with CLSI guidelines incorporating suitable controls *E. coli* ATCC 25922 and *K. pneumonia* ATCC 700603.[7]

Screening tests for Amp C production

Amp C production was tested by inhibitor-based disc diffusion method using Cloxacillin and boronic acid as inhibitors. Disks containing 30 μg of Cefoxitin alone and two Cefoxitin disks, one impregnated with Cloxacillin (200 μg) and the other with boronic acid (400 μg) were used. The strains were inoculated on Mueller-Hinton agar using McFarland 0.5 inocula and incubated at 35°C for 16-18 h. A difference in the Cefoxitin-Cloxacillin inhibition zones vs. the Cefoxitin alone zones of ≥4 mm and a difference of ≥5 mm for Cefoxitin boronic acid vs. Cefoxitin alone was considered indicative for plasmid Amp C production.[8]

Genotypic tests

Multiplex PCR for Amp C genes

Amp C genes were detected by multiplex PCR using the method described by Pérez-Pérez and Hanson with some modifications.[9] Deoxyribonucleic acid (DNA) template was prepared by boiling the bacterial cell suspension for 10 min. The primers used are listed in Table 1.

The concentration of the primers in the PCR mixture was 0.6 μM primers MOXMF, MOXMR, CITMF, CITMR, DHAMF, DHAMR; 0.5 μM primers ACCMF, ACCMR, EBCMF, EBCMR and 0.4 μM primers FOXMF and FOXMR.

Table 1: Primers used for amplification

Targets	Primer	Sequences (5′ to 3′)	Amplicon size (bp)
CTX-M	CTX-M F	TTTGCGATGTGCAGTACCAGTAA	544
	CTX-M R	CGATATCGTTGGTGGTGCCATA	
MOX-1,MOX-2, CMY-1, CMY-8 to CMY-11	MOX MF	GCT GCT CAA GGA GCA CAG GAT	520
	MOXMR	CAC ATT GAC ATA GGT GTG GTG C	
LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGG CCA GAA CTG ACA GGC AAA	462
	CITMR	TTT CTC CTG AAC GTG GCT GGC	
DHA-1, DHA-2	DHAMF	AAC TTT CAC AGG TGT GCT GGG T	405
	DHAMR	CCG TAC GCA TAC TGG CTT TGC	
ACC	ACCMF	AAC AGC CTC AGC AGC CGG TTA	346
	ACCMR	TTC GCC GCA ATC ATC CCT AGC	
MIR-1T, 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	

Cefotaximase-M: CTX-M; moxalactam: MOX; cephamycin: CMY; latamoxef: LAT; Bilal: BIL; Dhahran Hospital in Saudi Arabia: DHA; ACC: Ambler class C; Miriam Hospital in Providence: MIR; Amp C type: ACT; cefoxitin: FOX; Citrobacter freundii (origin): CIT; Enterobacter cloacae (origin): EBC

The PCR conditions consisted of an initial denaturation step at 94°C for 3 min followed by 25 cycles of DNA denaturation at 94°C for 30 s, primer annealing at 64°C for 30 s and primer extension at 72°C for 1 min and a final extension step at 72°C for 7 min. Around 5 µl aliquots of PCR product were analysed by gel electrophoresis with 2% agarose. Gels were stained with ethidium bromide and visualised by ultraviolet (UV) transillumination.

PCR for CTX-M

The primers used were CTX-M F-5´-TTTGCGATGTGCAGTACCAGTAA-3´ and CTX-M R-5´ CGATATCGTTGGTGGTGCCATA-3´ [Table 1]. Plasmid DNA isolation was done using 1.5 ml of overnight grown culture by alkali lysis method. The PCR conditions used were initial denaturation of 94°C for 2 min followed by 35 cycles of 95°C for 20s, 510°C for 30s, 72°C for 30s of annealing and a final extension of 72°C for 3 min. PCR product of 544 bp was visualised by agarose gel electrophoresis.[10]

RESULTS

Among the 100 study isolates, there was a high degree of cross resistance to the other antimicrobials tested namely amino glycosides, fluoroquinolones, 4th generation cephalosporins and beta lactam beta lactamase inhibitor combinations. Notably 21 isolates exhibited resistance to carbapenems. The antimicrobial susceptibility pattern of the study isolates is shown in Table 2.

Phenotypic tests

ESBL screening test by disc diffusion was positive in 70 isolates

All isolates exhibited a high degree of resistance to CTX as determined by the MIC. However, a fourfold decrease in MIC on addition of clavulanic acid was observed in 72 isolates.

AmpC screening test

Resistance to Cefoxitin was exhibited by 58 isolates. Using Cloxacillin and boronic acid, AmpC screening was positive in 49 and 22 isolates, respectively.

Genotypic tests

PCR for Amp C beta lactamases

Out of the 100 study isolates, plasmid Amp C genes were detected in 27 isolates which included *E. coli* (13), *K. pneumoniae* (10) and *P. mirabilis* (4). They were obtained from blood (8), exudative specimens (17) and respiratory secretions (2). The Amp C families detected were CIT (14), DHA (12) and EBC (1). Of the 27 Amp C producers, three isolates harboured bla_{CTX-M}. The distribution of plasmid Amp C genes in the study isolates is shown in Table 3. In a proportion of isolates (31) which were Cefoxitin resistant, none of the Amp C genes looked for were detected.

Out of the 27 PCR-positive isolates, 18 were cefoxitin resistant and 9 were cefoxitin susceptible. Comparative results of the phenotypic tests and PCR is shown in Table 4. The specificity, sensitivity, positive and negative predictive values of the screening and phenotypic tests considering PCR as the 'gold standard' is depicted in Table 5.

PCR for bla_{CTX-M}

Bla_{CTX-M} alone was detected in 51 isolates; *E. coli* (25), *K. pneumoniae* (20) and *P. mirabilis* (6). All the CTX-M producers exhibited positive results in the screening test (Disc diffusion and MIC determination). Of these, 34 were cefoxitin resistant, 12 were inhibited by boronic acid and 22 were inhibited by Cloxacillin Sequencing of representative CTX-M positives was done and they were found to be CTX-M 15 type.

Table 2: Antimicrobial susceptibility pattern

Antimicrobial agent (All 100 were Cefotaxime resistant)	Number of study isolates (*n* = 100)		
	Susceptible	Intermediate	Resistant
Cefoxitin (30 µg)	42	—	58
Amikacin (30 µg)	59	16	25
Ciprofloxacin (5 µg)	20	13	77
Cefepime (30 µg)	12	2	86
Piperacillin-tazobactam (100/10 µg)	26	26	48
Meropenem (10 µg)	77	2	21
Imipenem (10 µg)	78	1	21

Table 3: Distribution of plasmid Amp C genes in the study isolates

Organism	Plasmid Amp C (27)		
	CIT (14)	DHA (12)	EBC (1)
Escherichia coli	9	4	—
Klebsiella pneumoniae	4	5	1
Proteus mirabilis	1	3	—

DHA: Docosahexaenoic acid

Table 4: Comparative results of the phenotypic tests and PCR

Amp C Family	Phenotypic test in Cefoxitin resistant PCR positive (18)			Cefoxitin susceptible PCR positive (9)
	Inhibition by BA & Cloxacillin	Not inhibited by BA & Cloxacillin	Inhibited by Cloxacillin alone	
CIT (14)	4	1	5	4
DHA (12)	4	1	2	5
EBC (1)	0	0	1	0

DHA: Docosahexaenoic acid; PCR: Polymerase chain reaction; BA: Boronic acid

The overall distribution of plasmid Amp C and CTX-M is given in Table 6. *Bla* $_{CTX-M}$ alone was detected in 51 and plasmid Amp C alone in 24.

Three isolates carried both Amp C and *bla*$_{CTX-M}$ which included *E. coli* (1) and *K. pneumoniae* (2). The phenotypic tests for ESBL and Amp C were positive in two of them, but both phenotypic tests were negative in one PCR positive *K. pneumoniae* isolate.

In 22 study isolates, neither *bla*$_{CTX-M}$ nor plasmid AmpC was detected. All these isolates exhibited a positive ESBL screen test, 16 were resistant to cefoxitin and inhibition with boronic acid and Cloxacillin were positive in 2 and 11 isolates, respectively.

DISCUSSION

Enterobacteriaceae producing both AmpC beta lactamases and ESBLs have been increasingly reported worldwide. Since AmpC-producing organisms can act as hidden reservoirs for ESBLs, it is important for clinical microbiology laboratories to be able to detect ESBL production in these organisms on a routine basis. Co-existence of ESBL and Amp C may mask detection of each other, phenotypically. Though ESBL phenotypic testing is standardised and practiced in many laboratories, lack of a reliable phenotypic method for screening and detection of Amp C beta lactamases, makes the surveillance and characterisation of such strains problematic.[3,11] There are only few reports from India of the concurrent occurrence of CTX-M and Amp C beta lactamases in Enterobacteriaceae.[4-6] We undertook this study to detect the concurrent occurrence of CTX-M and plasmid Amp C in clinical isolates of Enterobacteriaceae.

Table 5: Sensitivity and specificity of the phenotypic detection methods for plasmid-mediated Amp C beta lactamase when compared with PCR

Test	Sensitivity %	Specificity %	Positive predictive value %	Negative predictive value %
Cefoxitin screening test	66.67	67.12	42.86	84.48
Boronic acid inhibition test	29.6	80.82	36.36	75.64
Cloxacillin inhibition test	59.25	54.79	32.65	78.43

PCR: Polymerase chain reaction

Table 6: Distribution of Amp C and CTX-M

Organism (*n*)	Amp C alone (24)	CTX-M alone (51)	Amp C plus CTX-M (3)	Neither Amp C nor CTX-M (22)
Klebsiella pneumoniae (43)	8	20	2	13
Escherichia coli (43)	12	25	1	5
Proteus mirabilis (14)	4	6	—	4

CTX: Cefotaxime

Plasmid Amp C genes were detected in 27 isolates of which three coproduced CTX-M. Of the 27, 9 were susceptible to cefoxitin. The inhibition test with boronic acid was positive in eight and with Cloxacillin in 16. Considering PCR as gold standard, we compared the sensitivity and specificity of the phenotypic tests to evaluate their utility in the clinical laboratory. The screening test and the phenotypic tests had low specificity and sensitivity but had better negative predictive value.

Of the 27 plasmid mediated Amp C producers (27%), 13 were *E. coli*, 10 were *K. pneumonia* and four were *P. mirabilis*. The prevalence of plasmid mediated Amp C varies widely in different parts of the world from 2% to 46%.[3,8] In Indian studies, the prevalence has ranged from 8% to 47%.[4,6,12-14]

We looked for the following plasmid Amp C types- Citrobacter freundii (origin): CIT; Dhahran Hospital in Saudi Arabia: DHA; ACC: Ambler class C; Enterobacter cloacae (origin): EBC; moxalactam: MOX; cefoxitin: FOX. The most common were the CIT family (14) followed by the DHA (12) and EBC (1). Notably FOX, MOX and ACC were not detected in any of the study isolates. *Bla*$_{CMY-2}$ is the most common plasmid mediated Amp C reported from most parts of the world. The other commonly reported Amp C is the DHA.[3,12] There are very few reports on the prevalence of different plasmid Amp C types in Indian isolates. In one study, out of 455 *E. coli* isolates, multiplex PCR detected 103 (22.6%) harbouring different families of Amp C gene with *bla* $_{CIT}$ being predominant.[14] Another study done on isolates from six different hospitals across India, reported plasmid Amp C genes in 92/241 (38.1%) isolates and the molecular types detected predominantly were DHA, CIT followed by MOX and ACC types.[12] Presence of multiple Amp C genes was not encountered in any of the study isolates. But, a similar study done in this centre in 2010, revealed multiple Amp C genes in single strains of *E. coli* and *K. pneumoniae*.[13] Since *K. pneumonia* and *P. mirabilis* do not possess chromosomal Amp C, their detection is presumed as plasmid borne. But in *E. coli* that are known to possess chromosomal Amp C, the distinction between plasmid and chromosomal Amp C is possible only by performing isoelectric focusing or by transfer experiments.[9] In a proportion of isolates (31), which were Cefoxitin resistant none of the Amp C genes looked for were detected. In *E. coli*, Cefoxitin resistance can be attributed to porin defects and /or chromosomal Amp C. In *K. pneumonaie a*nd *P. mirabilis* which are known to harbor only plasmid Amp C, Cefoxitin resistance is probably due to porin defects and/or novel Amp C.

In this study, both Amp C and CTX-M were detected in 3 isolates only. In one *K. pneumoniae,* both ESBL and Amp C phenotypic tests were negative indicating that ESBL and Amp C were masking each other. The concurrent occurrence of *bla* Amp C families and bla_{CTX-M} geno groups has been observed and reported in literature. A study from Kolkata found co-existence of ESBL and Amp C in 7.2% of *E. coli* and 6.1% of *K. pneumoniae.* A few other studies observed this phenomenon in 1% of Klebsiella species and 8% of *E. coli*.[5,6] Apart from India, Korea and Singapore have also reported the occurrence of CTX-M and plasmid Amp C together in Enterobacteriaceae.[15,16]

Bla_{CTX-M} alone was detected in 51 isolates which included *E coli* (25), *K. pneumoniae* (20) and *P. mirabilis* (6). The ESBL screen test correlated well with the PCR results. Of these, 34 were cefoxitin resistant, 12 were inhibited by boronic acid and 22 were inhibited by Cloxacillin. In these isolates resistance to cefoxitin is possibly due to alteration in permeability of the cell, loss of porin or presence of other beta-lactamases.[8] CTX- M type ESBL has been widely reported in Enterobacteriaceae in South East Asia since its first finding in *Shigella sonnei, E. coli* and *K. pneumonaie* in 2001.[17] Dissemination of Enterobacteriaceae harbouring members of the CTX-M family has since been published from Asian countries.[2] The most prevalent of the CTX-M is the CTX-M 15 followed by CTX-M 3 and CTX-M14. Sequencing of the PCR products of representative bla_{CTX-M} positive isolates in this study revealed all of them to be CTX-M 15 type. In India, this type was first described in a small number of isolates from Delhi in 2000. One Indian study has reported that 44.4% of *E. coli* and 35.29% of *K. pneumoniae* carry $bla_{CTX-M15}$. Elsewhere, the prevalence of CTX-M 15 is between 45and 77.4%.[18-20]

In a proportion of the study isolates, neither plasmid Amp C nor bla_{CTX-M} were detectable by PCR (*K. pneumoniae* 13, *E. coli* 5 and *P. mirabilis* 4). All of them were ESBL screen test positive indicating the presence of other ESBLs like TEM and SHV. Sixteen were Cefoxitin resistant which included *E. coli* (5), *K. pneumoniae* (9) and *P. mirabilis* (1). The boronic acid inhibition test was positive in two *K. pneumoniae* and Cloxacillin inhibition test was positive in 11 (*E. coli* 5, *K. pneumoniae* 5 and *P. mirabilis* 1). All the *E. coli* (5) which did not harbor plasmid Amp C were resistant to Cefoxitin and were inhibited by Cloxacillin. This implies the presence of chromosomal Amp C in them. A positive phenotypic test in the absence of detectable plasmid Amp C is indicative of the presence of other Amp C types, or reflective of the poor performance of the phenotypic tests employed.[3,11]

The study isolates exhibited high cross resistance to Amikacin, Ciprofloxacin, Pipercillin Tazobactam and Cefepime. Amp C and ESBL phenotypes are known to carry multiple genes that confer resistance to other classes of antimicrobial agents. Carbapenem resistance was exhibited by 21 isolates. This could be attributed to carbapenemase production or Amp C /ESBL combined with porin loss.[11]

To conclude, this study found that co-existence of ESBL and plasmid Amp C occurs in 4.6% of *K. pneumoniae* (2/43) and 2.3% of *E. coli* (1/43). It was not encountered in *P. mirabilis* though a large number of isolates have to be tested to substantiate this finding. Hence, concurrent occurrence of ESBL and Amp C is not a common phenomenon in the Indian context. Identifying these enzymes has an impact on patient management because the two mechanisms may mask each other in phenotypic tests. Hence, PCR remains the mainstay for detection of these mechanisms. Production of either of these enzymes with porin defects leads to carbapenem resistance. Therefore, their identification is important for optimisation of treatment and containment measures.

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