

Extended spectrum AmpC and metallo-beta-lactamases in *Serratia* and *Citrobacter* spp. in a disc approximation assay

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Abstract

Objectives: This study aimed to develop a novel model for detection of extended spectrum beta-lactamase (ESBL), AmpC and metallo-beta-lactamase (MBL) producing *Serratia* and *Citrobacter* species using cefoperazone sulbactam as well as other inducer-substrate combinations in a disc approximation assay. In the absence of molecular tools in developing countries, we attempted to standardize simple phenotypic techniques for detection of beta-lactamases to allow effective patient care in our countries. These techniques have been scarcely used in *Serratia* and *Citrobacter* spp., which are emerging as significant pathogens in our region.

Methodology: Clinical isolates of *Serratia* and *Citrobacter* were tested for ESBL production. Cefoperazone (CP)/cefoperazone sulbactam (CPS), piperacillin (PIP)/piperacillin-tazobactam (TZP) and ceftazidime (CAZ)/ceftazidime-clavulanic acid (CAZ-CLAV) combinations were compared for their ability to detect ESBL producers phenotypically. Multi-drug resistant strains were further tested for detection of inducible/derepressed AmpC mutants by a disc approximation assay. Isolates were screened for MBL production by Imipenem (IMI). MBL production was confirmed using Ethylenediaminetetraacetic acid (EDTA) in a double disc synergy assay and Hodge test. Minimal inhibitory concentration (MIC) was performed for CP, CPS and IMI by agar dilution method for all isolates of *Serratia* and *Citrobacter* spp.

Results: Thirty-three percent of isolates of *Serratia* spp. and 35.4% of *Citrobacter* spp. were ESBL producers. CPS was a more sensitive inducer of ESBL than TZP and CAZ/CAZ-CLAV. AmpC producers were detected in 25.6% of the isolates of *Serratia* spp. (40% inducible and 60% derepressed mutants) and in 35.4% of the isolates of *Citrobacter* spp. (33% inducible and 66% derepressed mutants). Six isolates (four class B and two class A) of *Serratia* and eight isolates (seven class B and one class A) of *Citrobacter* spp. were MBL producers. Multiple mechanisms co-existed in eight isolates of *Serratia* and 15 isolates of *Citrobacter* spp. CPS was more effective in identifying ESBLs and inducible AmpC producers as well as type 1 carbapenemases than TZP and CAZ-CLAV were able to identify inducible AmpC producers.

Conclusions: The high prevalence of ESBL, AmpC, and MBL in *Serratia* and *Citrobacter* species in this study suggests that detection of these by phenotypic methods in the absence of more specific molecular tests should be actively considered in not only developing countries but also in the developed world as this approach can lead to timely and appropriate antibiotic treatment. CPS may be advised due to the triple advantage of detection of all three types of beta-lactamases.

Key Words: ESBL, AmpC, metallo-beta-lactamases, *Serratia*, *Citrobacter*

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Introduction

Resistance to broad spectrum beta-lactams, mediated by extended spectrum beta-lactamase (ESBL), AmpC beta-lactamases (AmpC), and metallo-beta-lactamase (MBL) enzymes are an increasing problem worldwide [1]. Presence of the latter two enzymes in clinical infections can result in treatment failure if one of the second- or third-generation cephalosporins is used. The scenario worsens in cases of metallo-beta-lactamase production where the drugs of last

resort—the carbapenems—are rendered inactive. While clear-cut Clinical Laboratory Standards Institute (CLSI) guidelines are available in cases of ESBL, AmpC and MBL producing *Escherichia coli* and *Klebsiella pneumoniae*, guidelines regarding their detection in other members of the Enterobacteriaceae family have not been clearly elucidated [2]. Antimicrobial therapy of serious infections by other members of *Enterobacteriaceae* particularly, *Serratia* spp.

and *Citrobacter* spp., can be hampered due to their ability to produce a wide variety of beta-lactamases. Detection of these constitutes a diagnostic challenge. The ability of various beta-lactam antibiotics to induce AmpC synthesis varies, with some agents, such as ampicillin, cefoxitin and carbapenems, acting as strong inducers while ceftazidime, cefotaxime and ureidopenicillins serve as substrates of AmpC [3]. Carbapenems, though stable even in response to extended spectrum and AmpC beta-lactamases, are ineffective against acquired MBL of which IMP and VIM are being increasingly reported worldwide [4,5]. Few studies are available regarding these resistance mechanisms in *Serratia* and *Citrobacter* species.

Many clinical laboratories have problems detecting ESBLs, plasmid, and chromosomal AmpC and MBL in *Serratia* and *Citrobacter* spp. [2]. Confusion exists about the importance of these resistance mechanisms, optimal test methods, and appropriate reporting conventions. In this study we studied drug resistance with reference to ESBL, AmpC, and MBL in *Serratia* spp. and *Citrobacter* spp., which are silently emerging as significant pathogens in this region. There is a paucity of data pertaining to these mechanisms of resistance in *Serratia* and *Citrobacter* species from India in particular and the world in general. Antimicrobial therapy of serious infections caused by *Serratia* and *Citrobacter* species can pose a problem as these organisms may possess not only a variety of ESBLs but also inducible and derepressed AmpC beta-lactamases as well as MBL producers. It is increasingly being noted that clavulanic acid, though recommended by the CLSI, is a suboptimal inhibitor of ESBLs for those isolates that produce inducible AmpC beta-lactamases [6]. We compared the three beta-lactamase inhibitors—clavulanic acid, sulbactam, and tazobactam—in combination with ceftazidime, cefoperazone, and piperacillin respectively for their potential to detect ESBL production. Many methods for the detection of ESBL, AmpC and MBLs have been proposed but they are usually technically demanding, time-consuming,

difficult to interpret, or may require specialized, hard-to-obtain reagents [7].

The lack of molecular diagnostic techniques for regular use in clinical microbiology laboratories of developing countries led us to develop a disk approximation assay for the detection of beta-lactamases. We devised a proactive approach to detect on a daily basis ESBL, AmpC, and MBL in *Serratia* and *Citrobacter* species in all clinical specimens. We attempted to make detection of these markers of drug resistance part of the daily activities of a diagnostic microbiology laboratory because doing so would go a long way in the timely management of seriously ill patients.

Materials and Methods

All consecutive isolates of *Serratia* spp. and *Citrobacter* spp. from various clinical specimens were studied for their drug resistance profiles for a period of 6 months (from September 2007 to February 2008) in the Department of Microbiology, Jawaharlal Nehru Medical College, Aligarh Muslim University, AMU, Uttar Pradesh, India. Thirty-nine consecutive isolates of *Serratia* species and 110 isolates of *Citrobacter* species were studied. A single isolate per patient was considered for further study. The strains were genetically unrelated as determined by their sensitivity profiles, which differed from each other. These isolates were obtained from surgical site infections (57), orthopaedic infections (36), urinary tract infections (28), cerebrospinal fluid (5), sputum (11), and ear and eye samples (12). Isolates were identified by standard biochemical techniques [8].

Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing was performed by disc diffusion method by the Kirby Bauer technique according to CLSI guidelines on Mueller Hinton agar [9]. The isolates were tested against amikacin (30 µg), gentamicin (10 µg), gatifloxacin (5 µg), ofloxacin (5 µg), ceftriaxone (30 µg), cefoperazone (75 µg), cefoperazone-sulbactam. (75 µg, 1:1) cefixime (5 µg) and

netilmicin (30 µg). All discs were obtained from HiMedia, India.

Detection of extended spectrum beta lactamases

Ceftriaxone and cefoperazone were used as screening agents while cefoperazone sulbactam was used for confirmation of ESBL. This combination is not routinely used to confirm the production of an ESBL but we standardized this combination against amoxicillin-clavulanic acid. The latter combination was more sensitive than the former in identifying ESBL producers (unpublished data). The cut-off zone of cefoperazone for screening of possible ESBL was kept at ≤ 22 mm and that of ceftriaxone was as recommended by CLSI (< 25 mm). Confirmation was done on the same day by noting the potentiation of the activity of cefoperazone (CP) in the presence of cefoperazone-sulbactam (CPS) [9]. An increase in diameter of ≥ 5 mm was considered positive for ESBL detection. All ESBLs thus identified were retested using piperacillin (PIP)/piperacillin-tazobactam (TZP) and Ceftazidime (CAZ)/ ceftazidime-clavulanic acid (CAZ-CLAV) combinations as well as by MIC reduction method using CP/CPS, ceftriaxone/ceftriaxone-sulbactam combinations.

Since cefoperazone-sulbactam is used extensively in our facility, we used it along with cefoperazone in our first-line drugs to initially establish ESBL status. CPS not only has been found to have a better efficacy than TZP in our patients, but it is also cheaper than TZP. As there is a mounting problem of AmpC beta-lactamases in our region, clavulanic acid is being reported as a suboptimal inhibitor of ESBLs. Sulbactam is unlikely to cause this problem and could be a better alternative to clavulanic acid for detection of ESBLs [6]. This was our rationale for using cefoperazone-sulbactam.

Detection of inducible and derepressed AmpC beta lactamase

Isolates resistant to ceftriaxone, cefixime, cefoperazone and cefoperazone sulbactam, and cefoxitin were tested for AmpC production.

Induction of C synthesis was Amp based on the disc approximation assay using several inducer substrate combinations. The assay was performed according to the CLSI guidelines [9]. Inducer/substrate discs were placed on the surface of Mueller Hinton agar plates (HiMedia) at a distance of 25 mm from center to center using the template. A distance of 27 mm and 28 mm from center to center was also evaluated. Strains were considered stably derepressed if resistance was observed to all substrate drugs.

Quality control of the performance of the assay was accomplished with positive control (*Pseudomonas aeruginosa* ATCC 27853) and negative control (*Escherichia coli* ATCC 25922). Strains were considered inducible if a positive test was obtained with any of the inducer/substrate combinations. A test was considered positive if the zone of inhibition was reduced by ≥ 2 mm on the induced side of the substrate disc. Strains were considered stably derepressed if resistance was observed to all substrate drugs.

Imipenem (10 µg) and cefoxitin (30 µg) were used as inducers of AmpC. Discs of 100/10 µg piperacillin-tazobactam (PIP-TZP), 30 µg cefotaxime (CTX), 100 µg piperacillin, (PIP) 30 µg ceftazidime (CAZ), 30/10 µg ceftazidime-clavulanic acid (CAZ-CLAV), 75 µg (1:1) cefoperazone-sulbactam (CPS), (30 µg) cefepime, and 30 µg cefoxitin (FOX) were substrates to Imipenem (IMI). TZP and CTX were used as substrates to FOX. On the same template, CTX, CAZ, and PIP were used as substrates for the detection of ESBL using CPS, CAZ-CLAV, and PIP-TZP by double-disc diffusion test. The discs were placed at a distance of 20 mm.

Detection of Metallo-beta-lactamases

In the same template, sensitivity of the isolate to Imipenem was tested. Type A MBL was detected if a potentiating zone of Imipenem was observed towards a beta lactam/beta-lactamase inhibitor. If the zone of Imipenem was reduced to ≤ 16 mm or heaping occurred, or if the zone was > 16 but ≤ 20 , we tested the isolate

for MBL production. Hodge test and Double Disc synergy test using EDTA were used for detection of MBL. The method was as described by Lee *et al.* [10].

Hodge test

A modification of the Hodge test was used [10]. The indicator organism, *Escherichia Coli* ATCC 25922 at a turbidity of 0.5 McFarland standards, was used to swab inoculate the surface of Mueller Hinton agar plates and the test strain was heavily streaked from the centre to the plate periphery. After the plate was allowed to stand for 15 minutes at room temperature, a 10 µg Imipenem disc (HiMedia) was placed at the centre and the plate was incubated overnight. The presence of a distorted inhibition zone was interpreted as a positive result for carbapenem hydrolysis screening. Alternatively, 20 µl of 50m M zinc sulfate solution was added to an Imipenem disk, to see if better results are obtained.

Double Disc Synergy Test (DDST) for MBL detection

Test strains were adjusted to McFarland 0.5 standard and used to inoculate Mueller Hinton agar plates. A 10 µg Imipenem disk was placed on the plate and a blank filter paper disk was placed at a distance of 10 mm (edge to edge). To the blank disc, 10 µl of a 0.5 M EDTA solution (ca 1,900 µg of disodium salt, dehydrate) was added. After overnight incubation, the presence of even a small synergistic inhibition zone was interpreted as positive.

Minimum Inhibitory Concentrations

Minimum Inhibitory Concentrations (MICs) were tested for cefoperazone, cefoperazone-sulbactam, ceftriaxone, ceftriaxone-clavulanic acid and Imipenem by agar dilution technique according CLSI guidelines for all isolates [11].

Statistical analysis

Sensitivity and specificity were calculated.

Results

Out of a total of 1,923 single patient isolates of the family *Enterobacteriaceae*, 39 *Serratia* spp (13 (33.3%) *Serratia marcescens* and (66.6%) other species) and 110 *Citrobacter* spp. (12 (10.9%) *Citrobacter freundii*, 16 (14.5%) *Citrobacter koseri* and 81 (74%) *Citrobacter amalonaticus*) were isolated.

Serratia species

ESBL detection: Among the 39 isolates of *Serratia* spp., 13 (33.3%) were ESBL producers. Twelve (92%) were identified as ESBL producers using CPS as an inducer, and one additional isolate was identified as an ESBL producer by TZP. TZP, however, missed two. CAZ-CLAV identified five ESBL producers only (Table 1). CP/CPS sensitivity in detection of ESB was 92.31%, specificity was 94.87%, PIP/TZP sensitivity was 84.62%, specificity was 89.74%, sensitivity of CAZ/CAZ-CLAV alone was 45.45%, and specificity was 76.92%. Combined CP/CPS+PIP/TZP sensitivity was 100%, specificity 100% CP/CPS+CAZ/CAZ-CLAV sensitivity was 92.31%, specificity 94.87% and PIP/TZP+CAZ/CAZ-CLAV sensitivity was 84.62% and specificity 89.74%.

ESBL production was confirmed by comparing the difference in MIC of CPS and CP. MIC₅₀ of CPS was 512 and the MIC range was 2,048 –(4 mg/L) while the MIC₅₀ of CP was 2,048 and the MIC range was > 4096-32mg/L respectively. MIC₅₀ of ceftriaxone-clavulanic acid was 512mg/L and the range was 4,096-16 while MIC of ceftriaxone was 4,096-64 mg/L. The difference in the MIC ratio of CPS and CP, as well as ceftriaxone and ceftriaxone clavulanic acid, was eightfold. A threefold difference was considered positive for ESBL production. There was 100% correlation of ESBL detection by CPS and TZP induction and by MIC.

AmpC detection: Fourteen isolates of *Serratia* spp. (35.8 %) were resistant to all the first-line drugs. Ten (25.6 %) of these were AmpC producers, four (40 %) being inducible

Table 1. Comparison of ESBL detection by cefoperazone /cefoperazone-sulbactam, piperacillin/piperacillin-tazobactam and ceftazidime/ ceftazidime-clavulanic acid

Combination	No. of ESBL producers detected		No. of ESBL producers missed	
	<i>Serratia</i>	<i>Citrobacter</i>	<i>Serratia</i>	<i>Citrobacter</i>
CP/CPS alone	12	37	1	2
PIP /TZP alone	11	35	2	4
CAZ /CAZ-CLAV alone	5	30	8	9
CP/ CPS + PIP/ TZP	13	39	0	0
CP/CPS+CAZ/ CAZ-CLAV	12	37	1	2
PIP/TZP +CAZ/CAZ-CLAV	11	35	2	4

Table 2. Comparison of ESBL detection by cefoperazone /cefoperazone sulbactam, piperacillin/piperacillin-tazobactam and ceftazidime/ ceftazidime-clavulanic acid

Combination	No. of ESBL producers detected		No. of ESBL producers missed	
	<i>Serratia</i>	<i>Citrobacter</i>	<i>Serratia</i>	<i>Citrobacter</i>
Cf/Cfs alone	12	37	1	2
PIP /TZP alone	11	35	2	4
CAZ /CAZ-CLAV alone	5	30	8	9
Cf/ Cfs + PIP/ TZP	13	39	0	0
Cf/Cfs+CAZ/ CAZ-CLAV	12	37	1	2
PIP/TZP +CAZ/CAZ-CLAV	11	35	2	4

and six (60%) being derepressed mutants. Four ESBL producers were also positive for AmpC. The majority of inducible AmpC (50%) were detected by flattening of the zone of Cfs towards Imipenem. All were resistant to cefoxitin and sensitive to cefepime (Table 2). No appreciable difference in results were noticed when distance between the inducer and substrate was increased to 27 and 28 mm.

MBL detection: Five isolates were suspected to be MBL producers, of which four (10.2%) were confirmed to be class B metallo-beta-lactamase producers and the fifth was categorized as a derepressed mutant. However, it could also have had a porin deficiency. DDST using EDTA was far more sensitive 4/4 (100%) compared to the Hodge test 2/4 (50%). Addition of zinc sulphate did not significantly improve the result of the

Hodge test. Two isolates of *Serratia* displayed phenotypic expression of plasmid mediated KPC enzyme of class A carbapenemases, with CPS being the inducer in both cases.

The breakdown of different mechanisms of resistance is given in Table 3. MIC of Imipenem in these isolates was alarmingly high (40 – 20mg/L).

In eight isolates, multiple mechanisms of resistance were noticed. In four isolates, ESBL and AmpC co-existed; in two isolates, AmpC together with MBL were observed, while in the remaining two ESBL, AmpC and MBL occurred concurrently (Table 4).

Citrobacter species

ESBL detection: Thirty-nine (35.4%) of the *Citrobacter* spp. were identified as ESBL producers, of which CPS identified 37 (94.8%) while TZP identified 35 (89.7%) in all, two of which were not detected by Cfs. CAZ-CLAV identified 30 ESBL (76.9%) producers (Table 1). Sensitivity and specificity of CPS, TZP, and CAZ-CLAV was the same as that for *Serratia* spp. The MIC₅₀ of CPS was 512 mg/L and the MIC range was 4096 - 8 mg/L while the MIC₅₀ of CP was 4096 and the MIC range was > 4096-64mg/L respectively. The MIC₅₀ of ceftriaxone-clavulanic acid was 512mg/L and the range was 4,096-16, while MIC of ceftriaxone was 4,096-64 mg/L. The difference in MIC ratio of CPS and CP as well as of ceftriaxone-clavulanic acid and ceftriaxone was eightfold. A threefold difference was considered positive for ESBL production. There was 100% correlation of ESBL detection by CPS and TZP induction and by MIC.

AmpC Detection: Twenty-nine (26.3%) isolates were resistant to all first-line drugs. Eighteen (62%) of these were identified as AmpC producers, six (33.3%) being inducible AmpC producers (Table 2) while 12 (66.6 %) were derepressed AmpC mutants.

MBL Detection: Of 11 suspected MBL producers, seven (26.9%) were confirmed to be Class B MBL (DDST 100%: Hodge test 57%).

MIC of IMI ranged from 160 to 20 mg/L for these isolates. One isolate of Class A MBL was identified. Four additional DDST negative isolates were classified as derepressed AmpC producers and/or had porin loss. The breakdown of different mechanisms of resistance is given in Table 3.

Fifteen isolates exhibited multiple mechanisms of resistance. Three isolates were ESBL as well as inducible AmpC producers, and one was an ESBL as well as a derepressed AmpC producer. Inducible AmpC with MBL was seen in one isolate while derepressed AmpC and were found in seven isolates. All these mechanisms co-existed in two isolates, one of which had inducible AmpC resistance and the other had derepressed AmpC mutant. One ESBL producer was also an MBL producer (Table 4).

Discussion

The purpose of this investigation was to examine the status of ESBL, AmpC and MBL production among *Serratia* and *Citrobacter* spp. An in-depth study on these genera has not yet been conducted in northern India. This information can prove invaluable in exploring the treatment options of serious systemic infections by these pathogens. Furthermore, there is a need to develop a simple sensitive screening and confirmatory assay for ESBL, AmpC, and MBL detection which could be incorporated into the routine antimicrobial drug sensitivity testing in developing countries.

We performed initial screening of ESBL with ceftriaxone and cefoperazone and confirmation with CP/CPS on the same day. This combination was preferred as CPS had far superior therapeutic results than TZP in our hospital. We also compared the potential of CP/CPS for detection of ESBL with CAZ/CLAV and PIP/TZP. In this study, CP/CPS proved more sensitive and specific than the PIP/TZP combination, and far more superior to the CAZ/CAZ-CLAV combination. The advantage we had was that by administering the drugs currently recommended for use in our hospital, we could accurately arrive at the status of ESBL

Table 3. Comparison of detection of inducible or derepressed Amp C by various substrates in *Serratia* spp. and *Citrobacter* spp.

Criteria	Inducible AmpC by using impenem as inducer				Derepressed mutants of AmpC		
	Serratia n=4		Citrobacter n=5			Serratia n=6	Citrobacter n=12
	Detected	Missed	Detected	Missed			
Flattening of Cfs	2	0	3	1	Resistance to Ci, CTX CAZ	6	7
Flattening of TZP	1	0	1	2	Resistance to FOX	6	12
Flattening of CAZ-CLAV	0	0	1	1	Resistance to Cfs	4	10
Flattening of CAZ	1	0	0	0	Resistance to TZP	4	9
Flattening of CTX	0	0	0	0	Resistance to CAZ-CLAV	5	12
Flattening of PIP	0	0	1	0	Resistance to IMI	0	4
Flattening of FOX	0	0	0	0			
Resistance to FOX	4	0	0	0			
Sensitivity to Cpm	4	0	5	0			
Resistance to CPS	2	0	2	0			

Table 4 . Breakdown of the different resistance markers present in *Serratia* spp. and *Citrobacter* spp.

ESBL	Total n = 42	Serratia n = 16	Citrobacter n = 26
Inducible AmpC	11 (26.1)	4 (25%)	6 (19.2%)
Derepressed AmpC	18 (21.4%)	6 (37.5%)	12 (11.5%)
Class A carbapenemase	3 (7.1)	2 (12.5)	1 (3.8%)
Class B carbapenemase	11 (26.1)	4 (25%)	7 (26.9%)
Derepressed AmpC or Porin loss	5 (11.9)	1 (6.25%)	4 (15.3%)

Figures in parenthesis represent percentages.

production in these strains on day one of sensitivity testing. CPS is the preferred drug due to its superior cure rates and lower cost compared to TZP in our facility. The better efficacy of CPS could be because its activity remains unaffected in the presence of inducible AmpC. Clavulanic acid, though recommended by the CLSI, is a suboptimal inhibitor of ESBLs in strains producing inducible AmpC beta-lactamases. In organisms producing both ESBLs

and AmpC, clavulanate may induce hyperproduction of AmpC beta-lactamase, leading to hydrolysis of third-generation cephalosporins, thus masking any synergy arising from inhibition of ESBL [6]. Sulbactam is unlikely to cause this problem and appears to be a better alternative to clavulanic acid, not only for detection, but also for treatment of ESBLs. We used three beta-lactamase inhibitors to assess their potential to detect ESBL and AmpC. CPS was better at detecting both of these.

Up to 33.3% and 35.4% of single-patient *Serratia* and *Citrobacter* isolates respectively were ESBL producers. This high prevalence indicates that ESBL detection should be done on not only *E. coli* and *Klebsiella* but also on other species of *Enterobacteriaceae* routinely. Similar results have been reported from other studies in relation to *Citrobacter* [12,13]. Reports on drug resistance in *Serratia* spp., however, are scanty. ESBL isolates from India are completely dominated by the presence of *bla* CTX-M-15 genes [14]. This is manifested by the large-scale resistance to cefotaxime seen in our facility and we no longer use it as a first-line therapy (unpublished data).

Imipenem, which is a strong inducer of AmpC, was used for a dual purpose: detection of inducible AmpC as well as detection of non susceptible or resistant isolates to Imipenem. Cefoxitin was a poor AmpC inducer in this study. This observation was corroborated by a study conducted by Dunne Jr. *et al.* [15]. However, cefoxitin resistance helped indirectly to identify derepressed AmpC mutants. In this study, CPS was a more sensitive substrate for detection of inducible AmpC than TZP and CAZ-CLAV, and Imipenem was a better inducer than cefoxitin. Cefepime sensitivity in conjunction with cefoxitin resistance helped in identification of AmpC producers. Cefepime is more resistant to hydrolysis by AmpC than third-generation cephalosporin and can also be used in ESBL detection. In this study, cefepime was placed 20 mm away from CPS; however, it was not useful in detecting ESBLs, perhaps due to hyperproduction of AmpC or the presence of derepressed mutants. The exceptionally high MICs of the cephalosporins could be explained by the co-existence of both ESBL and AmpC [16]. Cefoxitin resistant isolates may produce AmpC type enzymes or possess porin changes; however, it must be recognized that these can co-exist with ESBL production [17].

Among the AmpC producers it was noticed that the derepressed mutants far exceeded the inducible AmpC producers in this study. A total of 18 isolates were stably derepressed for AmpC

production, 6 (60 %) isolates of *Serratia* spp. and 12 (66.6 %) isolates of *Citrobacter* spp. Five others were suspected to be either derepressed AmpC mutants or may have had porin loss. This observation contradicts the study in Korea where the inducible AmpC producers were far higher (44%) compared to derepressed AmpC (29.6%) [18]. With respect to *Serratia* spp., no derepressed AmpC have been found in prior studies while a significantly lower prevalence was observed in *Citrobacter* spp. [15]. An outbreak of a single PFGE clone of *Serratia marcescens* in Napoli was found to be resistant to multiple antibiotics as well as produce inducible AmpC type of beta-lactamase enzyme [19]. Pfaller *et al.* have reported 38% in blood-stream isolates for the SCOPE surveillance programme [20]. Our numbers reflect organisms recovered from sources other than blood, which could account for the higher numbers. Inducible AmpC were significantly fewer in number than derepressed AmpC mutants in both genera (40 % in *Serratia* spp. and 33.3% in *Citrobacter* spp.). This is a first report on the presence of inducible and derepressed mutants of AmpC in *Serratia* and *Citrobacter* species from India. Although it is known that these genera harbor inducible AmpC enzymes [3,21], the unexpectedly higher prevalence of derepressed AmpC mutants in this study is alarming as it severely limits treatment options.

CAZ-CLAV, CPS and TZP were used to induce the phenotypic expression of the plasmid mediated KPC enzyme of class A carbapenemases. Two isolates of *Serratia* and one isolate of *Citrobacter* were identified as class A carbapenemases with KPC phenotype. A potentiation of the zone of Imipenem towards CPS was observed in all three, while no potentiation was noticed against TZP or CAZ-CLAV. To our knowledge, this is the first report of KPC phenotype of class A carbapenemase in *Serratia* and *Citrobacter* spp.

Four isolates of *Serratia* and seven isolates of *Citrobacter* were identified as class B carbapenemases using DDST, with EDTA as the chelating agent. EDTA enhancement was far

more sensitive (100%) than the Hodge test (54.5%). MIC of Imipenem was also found to be higher in these isolates ranging from 20 to 5mg/L. Simple and accurate tests are needed to detect MBL producing isolates, given the increasing prevalence of MBL producing gram negative bacilli in many countries [4,5].

Detection of MBL producing gram negative bacilli is crucial for optimal treatment of patients and to control the spread of resistance. Thus we believe that detection of MBL should become part of mainstream laboratory testing. In our study, a significant number of *Serratia* and *Citrobacter* isolates were class B MBL producers. This is the first report about prevalence of Class A and Class B carbapenemases in these genera from India.

Phenotypic detection of these resistance mechanisms, though not confirmatory, is faster, far more cost effective, less labour intensive, and does not require a high level of technical expertise. It is, therefore, easier to perform on a daily basis, not only in resource-poor countries but also in developed countries. The outcome of phenotypic detection of resistance mechanisms undoubtedly will be better patient care.

The algorithm we have developed lays stress on detection of ESBL on day one. Isolates resistant to beta lactam/beta lactam inhibitor should be selected for detection of AmpC beta-lactamases. Subsequently, MBLs should be looked for in Imipenem non-susceptible isolates. The template described can be used for detection of inducible and derepressed mutant of AmpC, class I MBL and non susceptible resistant isolates to Imipenem. If the isolates appear to be MBL producers, then DDST for confirmation of MBL should be performed. CPS was more sensitive and specific than TZP and CAZ-CLAV in detecting ESBLs, while the combinations of CPS/CP and TZP/PIP were 100% sensitive and specific. CPS proved useful in not only ESBL confirmation but also in detection of inducible AmpC producers, and in detection of KPC enzyme phenotype of class I MBL.

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