

AmpC β -lactamase producing bacterial isolates from Kolkata hospital

Suranjana Arora & Manjusri Bal

Section of Microbiology, Department of Physiology, University of Calcutta, University College of Science & Technology, Kolkata, India

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Background & objectives: The widespread use of β -lactam antibiotics has led to the development of resistance to this group of antibiotics in bacterial pathogens due to β -lactamase production. Information on such pathogens is not available from eastern region of India. This study was undertaken to determine the AmpC β -lactamase production in pathogens isolated from hospitalized patients in Kolkata.

Methods: Non-repeat clinical isolates (284) from pus, urine, sputum and other clinical specimens of hospitalized patients were taken. Disk agar diffusion (DAD) and minimum inhibitory concentration (MIC) with different β -lactam antibiotics, and double disc synergy test (DDST) with clavulanic acid and sulbactam were done. Disk antagonism test (DAT) and three-dimensional extract test (TDET) were conducted for phenotypic confirmation of AmpC and inducible AmpC β -lactamase production. Nitrocefin spot test and microiodometric assay of β -lactamase were also performed.

Results: Twenty seven isolates were found to be resistant to cefoxitin, a α -methoxy- β -lactam. Of these, 19 were observed to be AmpC β -lactamase producers and 4 were inducible AmpC β -lactamase producers by DDST, DAT and TDET. Remaining 4 were non AmpC β -lactamase producers. Of the 23 AmpC β -lactamase producers, the distribution of different species was as follows: *Escherichia coli* 11 (47.8%), *Pseudomonas aeruginosa* 4 (17.3%) *Klebsiella pneumoniae* 3 (13%), and *Klebsiella aeruginosa* 1 (4.3%).

Interpretation & conclusion: Our finding showed 6.7 per cent AmpC β -lactamase and 1.4 per cent inducible AmpC β -lactamase producing clinical isolates from Kolkata. AmpC β -lactamase producing bacterial pathogens may cause a major therapeutic failure if not detected and reported in time.

Key words AmpC β -lactamase - cefoxitin - clavulanic acid - inducible AmpC β -lactamase - sulbactam - three-dimensional extract test (TDET)

A common mechanism of bacterial resistance to β -lactam antibiotics is the production of β -lactamase enzymes that cleave the structural β -lactam ring of

these drugs. This is the predominant mechanism of β -lactam resistance in Gram-negative bacteria^{1,2}. Over the last two decades many new β -lactams have been

developed that were specifically designed to be resistant to hydrolytic actions of β -lactamase². However, with this new class of drug that has been used to treat patients, new types of β -lactamases emerged. AmpC β -lactamase is one of these new types of β -lactamases.

AmpC β -lactamases are cephalosporinases, which belong to the molecular class C as classified by Ambler in 1980³ and Group I under a classification scheme of Bush *et al*⁴. AmpC β -lactamases are more sensitive to inhibition by sulbactam than by clavulanate or tazobactam⁵. These are clinically significant as they may confer resistance to a wide variety of β -lactam drugs, including α -methoxy- β -lactams, narrow, expanded and broad-spectrum cephalosporins, aztreonam, a monobactam⁴ and most significantly β -lactam plus β -lactamase inhibitor combinations (*viz.*, ampicillin-clavulanic acid, piperacillin-tazobactam, *etc.*).

In many species, β -lactamases are normally produced at very low levels but are induced to several hundred fold higher by the presence of β -lactams (*viz.*, cefoxitin, cefotaxime, *etc.*) and certain β -lactam inhibitors (*viz.*, clavulanic acid)⁶. Inducible AmpC β -lactamases are such examples. Amoxicillin-clavulanic acid combination is commonly used in controlling β -lactamase producing pathogens, as clavulanic acid acts as an inhibitor to many β -lactamases. But in case of inducible AmpC β -lactamases, this type of drug can cause more harm than help.

β -lactamase producing bacteria can cause serious therapeutic failure if not detected on time. Though the clinicians treat infections based on the results of antibiotic susceptibility tests available, the number of infections caused by AmpC β -lactamase producing organisms is on the rise and pose a threat to the patients due to treatment failure. A few groups have reported the occurrence of β -lactamases producing bacteria from northern and southern regions, but there are not much data available on these pathogens from Kolkata or anywhere in the eastern region. The present study was therefore undertaken to find out the presence of AmpC and inducible AmpC type of β -lactamases producing clinical isolates from hospitalized patients in Kolkata using standard methods presently available for their detection. It may be mentioned here that

currently there is no clear consensus regarding guidelines for performing tests for the phenotypic screening or confirmatory tests for the isolates that harbour AmpC β -lactamases^{7,8}.

Material & Methods

Bacterial isolates: A total of 284 non-repeat clinical isolates collected from patients admitted to various wards during February 2002 to April 2003 [90 (31.7%) from pus, 132 (46.5%) from urine, 57 (20.1%) from sputum, 5 (1.8%) from others specimens such as burns, catheter, throat swab, ear discharge, gastric lavage fluid, and peritoneal fluid] from five hospitals in Kolkata, namely Calcutta Medical College and Hospital (CMC), Nil Ratan Sarkar Medical College (NRS), Seth Sukhlal Karnani Memorial Hospital (SSKM), R.G. Kar Medical College (RGK) and School of Tropical Medicine (STM) were included in this study.

Media and chemicals: Ampicillin (A, Lyka Labs, India), amoxicillin (Am, Wyeth Lederle Ltd, India), amoxicillin/clavulanic acid (Am/CA, Ranbaxy Laboratories, India) aztreonam (Ao, Hi-media, Mumbai), cefactam (Cfs, Aurobindo Pharma Ltd., India), cefotaxime (Ce, Alkem Laboratories Ltd, India), cefpodoxime (Cep, Universal Medicare Pvt, India), ceftriazone (Ci, Wockhardt, India), ceftazidime (Ca, Glaxo, India), cefoxitin (Cn, Hi-media, Mumbai), cefpirome (Cpm, Alkem, India), cephalixin (Cpl, Glaxo, India), ciprofloxacin (Cf, Ranbaxy, India), clavulanic acid (CA, Glaxo Smith Kline, UK), co-trimoxazole [Co, (sulphamethazole/trimethoprim), Welcome, India], gentamicin (G, Nicolas Piramal, India), imipenem (IPM, Ranbaxy Laboratories, India), piperacillin/tazobactam (Pt, Hi-media, Mumbai), tetracycline (T, Hoesct, India), were used in this study. Antibiotic solutions were prepared in sterile water. For disc agar diffusion (DAD), minimum inhibitory concentration (MIC) and double disk synergy test (DDST) Muller-Hinton broth (MHB, Hi-media, Mumbai) and agar agar (Qualigen Fine Chemicals, India) were used.

Disc agar diffusion method (DAD): The test bacterium, taken from an over-night culture (inoculated from a single colony) was freshly grown for 4 h and with this culture a bacterial lawn was prepared on MHA plate. Filter paper disks of 6 mm

size were used to find out antibiotic susceptibility pattern against 10 antibiotics (concentration in μg) [A (10), Am (20), Ao (30), Cf (5), Cn (30), Co (sulphamethazole/trimethoprim, 1.25/23.75), G (10), T (30), Cpl (30) and IPM (10)], four third generation cephalosporins [Ca (30), Ci (30), Ce (30), Cep (10)], one fourth generation cephalosporin [Cpm (30)], three β -lactam+ β -lactamase inhibitor combination, viz., amoxicillin-clavulanic acid combination [Am/CA, (20/10)], piperacillin-tazobactam combination [Pt, (100/10)] and cefaperazone-sulbactam [Cfs (75/30)] combination following Kirby-Bauer method⁹. The disks were prepared according to manufacturer's instruction. Strains resistant to cefoxitin (zone diameter less than 18 mm) were suspected to be AmpC β -lactamase producers.

Minimum inhibitory concentration (MIC): The MIC of the antibiotics was determined by two-fold serial broth dilution method¹⁰.

Double disk synergy test (DDST): In DDST, synergy was determined between a disk of cefotaxime (30 μg) and a disk of cefotaxime plus CA (30 μg + 10 μg) which were placed at a distance of 20 mm apart on a lawn of culture of the suspected β -lactamase producing clinical isolates on MHA. Disks containing clavulanic acid were prepared by applying 10 μl of a 1000 $\mu\text{g}/\text{ml}$ clavulanic acid stock solution to each disk. The test organism was considered to produce β -lactamase if the zone size around the cefotaxime plus clavulanic acid increased >5 mm in comparison to the third generation cephalosporin (Ce) disk alone. This increase occurred because the β -lactamases produced by the isolates were inactivated by clavulanic acid. The NCCLS guidelines do not have any standard tests for isolates, that produce AmpC β -lactamase⁸. If the increase in zone size was <5 mm or there was no increase in zone size after addition of clavulanic acid in DDST, then the DDST was done with cefotaxime and cefactam (cefperazone/sulbactam, Cfs, 75/30 μg). Here cefactam was used as a source of sulbactam. If the zone of inhibition increased on using sulbactam, the isolates were considered to be AmpC β -lactamase producers¹¹.

Disk antagonism test (DAT): The disk antagonism test was used to detect the inducibility of β -lactamase. Disks of inducing agent cefoxitin (Cn) and cephalosporins (Cpm, Ca, Ci and Ce) were placed

on the surface of the test bacterial lawn on MHA plates on a lawn of bacterial culture of the suspected inducible AmpC β -lactamase producers separated by 15 mm. The plates were examined after overnight incubation at 37°C¹². If blunting of the cephalosporin disks adjacent to the cefoxitin disks occurred, the organisms were considered to produce inducible AmpC β -lactamase.

Three-dimensional extract test (TDET): 50 μl of a 0.5 McFarland bacterial suspension prepared from an overnight MHB was inoculated into 12 ml of MHB and the culture was grown for 4 h at 35°C. The cells were concentrated by centrifugation, and crude enzyme preparations were made by sonicating the pellets at 8 μm (in Soniprep, UK) for 15 sec (two cycles) with 10 sec cooling in between sonications; this was repeated four times. The surface of a MHA plate was inoculated with *Escherichia coli* strain ATCC 25922 (obtained from Central Drug Laboratory, Kolkata) as described in DAD. A 30 μg cefoxitin (Cn) disk was placed at the center of the inoculated agar. With a sterile scalpel blade, a slit beginning at 5 mm from the edge of the Cn disk was cut in the agar in an outward radial direction. By using a pipet, 25 to 30 μl of enzyme preparation was dispensed into the slits, beginning near the disk and moving outward. Slit overflow was avoided. The inoculated media were incubated overnight at 35°C. Enhanced growth of the surface organism at the point where the slit intersected the zone of inhibition due to Cn was considered a positive TDET result and was interpreted as evidence for the presence of AmpC β -lactamase⁷.

Nitrocefin spot test: The crude enzyme was prepared by centrifuging overnight culture of the organisms at 10,000 r.p.m at 4°C for 10 min. The pellets were then sonicated at 8 μm (in Soniprep, UK) for 15 sec (two cycles) with 10 sec cooling in between sonications. The sonicated material was centrifuged again to obtain the enzyme; 10 μl of enzyme was incubated with 50 μl of 1.5 mM nitrocefin (Calbiochem, San Diego, USA; working solution prepared according to the manufacturer's instruction) in the well of a microtitre plate for 30 min at room temperature. The presence of β -lactamase was detected if colour of nitrocefin changed from yellow to reddish-orange¹³.

Microiodometric determination of β -lactamase activity: Microiodometric determination was done

according to the method of Sykes and Nordstrom¹⁴ using benzylpenicillin and cefotaxime as substrates.

Induction of AmpC β -lactamase by clavulanic acid: For testing, induction of AmpC β -lactamase by clavulanic acid, 10 μ g/ml of clavulanic acid was added to MHB in which the test bacteria were grown overnight at 37°C in a gyratory shaker. Enzyme was prepared from this overnight growth as described previously. The enzymes thus obtained were tested by nitrocefin spot test and microiodometric determination of β -lactamase activity.

Isoelectric focusing: Analytical isoelectric focusing was performed with some of the β -lactamase extracts, by the method of Mathew *et al*¹³. The ampholine range used were pH 3.5-10. The samples were loaded on the gel and given a pre-run of 20 min at 15 mA at constant volts of 1 Watt and then run for 60 min at 50 mA on LKB Multiphor II isoelectric focusing apparatus (Pharmacia, Sweden). β -lactamases with known isoelectric point (pI) were focused as control: 15 μ l of TEM 1 (pI 5.4) obtained from *E. coli* J53 RI (Dr Reddy's Laboratory, Hyderabad) and 10 μ l of SHV-18 (pI 7.8) obtained from *Klebsiella pneumoniae* ATCC 700603 (Dr Reddy's Laboratory, Hyderabad) were used. Gels were stained with 10⁻⁴ M nitrocephin. The pI of the enzyme was indicated by a reddish-orange band on the gel bed at a specific position.

Results

Of the 284 non-repeat clinical isolates tested by DAD, 27 (9.5%) were resistant to ceftazidime which was used as a primary selection criterion of AmpC β -lactamases. MIC as well as DDST done on these 27 isolates with Ce, Ce/CA and Cfs showed that 19 (12 from urine, 3 from pus and 4 from sputum and throat swabs) were resistant to inhibition by clavulanic acid but were inhibited by sulbactam (Table I, Fig.1) and tazobactam. Four isolates showed decreased zone of inhibition with clavulanic acid suggesting production of β -lactamases induced by clavulanic acid (Table II). Of the 27 isolates, the remaining four were non-producers of AmpC β -lactamase. All the 23 isolates, which produced AmpC β -lactamase, exhibited high level of resistance to the antibiotics tested by DAD. Seventeen of the AmpC β -lactamase producers were resistant to ciprofloxacin. Ceftazidime showed

sensitivity in 9 (39%) of the 23 isolates tested positive for AmpC β -lactamase production. Cefpodoxime resistance was seen in all 23 isolates.

AmpC β -lactamase production was confirmed by performing TDET. All the suspected 19 isolates were found to be positive for TDET, *i.e.*, there was growth along the slit within the zone of inhibition of ceftazidime (Fig. 2). Inducibility of the β -lactamases was further recognized by DAT, which demonstrated blunting of specific cephalosporin (cefotaxime) disks adjacent to the ceftazidime disks (Fig. 3).

AmpC β -lactamase production (including inducible AmpC β -lactamases) was seen in 23 (8.1%) isolates. AmpC β -lactamases were produced by *E. coli* 11 (47.8%), *P. aeruginosa* 4 (17.3%), *K. pneumoniae* 3 (13%) and *K. aeruginosa* 1 (4.3%). Of the inducible AmpC β -lactamase producing isolates, 3 were isolated from urine samples (*K. pneumoniae* CMC-40, *Proteus vulgaris* CMC-90 and *P. aeruginosa* NRS-3) and 1 from burn patient (*P. aeruginosa* NRS-226) (Tables I, II).

All AmpC β -lactamase producers were found to be positive by nitrocefin spot test. β -lactamase activity from all the 23 isolates were also tested using benzylpenicillin and cefotaxime as substrates following the microiodometric assay. The specific activity of β -lactamase enzyme varied from one organism to another but all were positive for β -lactamase production. Four isolates were found to be producing inducible AmpC β -lactamase which was also confirmed enzymatically. Addition of clavulanic acid in the growth medium induced the production of inducible AmpC β -lactamase in case of both the substrates, benzylpenicillin and cefotaxime, from 1.1 fold to 16 fold, confirming that clavulanic acid is an inducer of this enzyme *in vivo* (Table III).

Isoelectric focusing showed that both TEM-1 (pI 5.4) and SHV-18 (pI 7.8) type of β -lactamase enzymes were the predominant types of enzyme present in the AmpC β -lactamases (Figs 4, 5). In case of some samples including the reference strain *E. coli* J53RI, a band is seen at the point of application (Figs 4, 5); this may be due to aggregation of proteins which did not migrate under the experimental conditions applied.

Table I. Clinical isolates showing AmpC β -lactamase production

Clinical isolate	Source of sample	DAD (resistant to)	MIC (μ g/ml)		DDST (mm)		
			Ce*	Cf/S	Ce*	Ce/CA**	Cf/S
<i>Escherichia coli</i> STM-70	Urine	A,Am,Cpl,Cf , Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA	128	16	10	10	20
<i>E. coli</i> RT-11	Catheter	A,Am,Cpl,Cf,Co,G,T,Cep,Ci, Ce, Ao, Am/CA	128	16	8	10	17
<i>E. coli</i> NRS-55	Urine	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA,	128	8	8	8	15
<i>E. coli</i> CMC-67	Pus	A,Am,Cpl,Cf,Co,G,T,Ce,Cep,Ci, Ao, Am/CA,	>256	16	6	6	22
<i>E. coli</i> CMC-68	Urine	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA	128	16	10	10	18
<i>E. coli</i> CMC-28	Urine	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA,	>256	16	6	6	19
<i>E. coli</i> NRS-29	Urine	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep, Ao, Am/CA,	>256	16	6	6	16
<i>E. coli</i> CMC-100	Urine	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA,	>256	16	6	6	15
<i>E. coli</i> RGK-2	Urine	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA,	>256	16	6	6	16
<i>E. coli</i> STM- 9	Urine	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA,	>256	16	6	6	20
<i>E. coli</i> STM-4	Urine	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA,	>256	8	6	6	18
<i>Klebsiella pneumoniae</i> STM-107	Throat swab	A,Am,Cpl,Cf,Co,G,T,Ce,Cep,Ci, Ao, Am/CA	>256	16	10	6	20
<i>K. pneumoniae</i> CMC-10	Sputum	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA	>256	16	6	6	21
<i>K. pneumoniae</i> CMC-242	Sputum	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA	>256	16	6	6	10
<i>K. pneumoniae</i> NRS -83	Throat swab	A,Am,Cpl, T,Cep, Ao, Am/CA	>256	16	6	6	20
<i>K. aerogenes</i> NRS-26	Urine	A,Am,Cpl,Cf,Co,G,T,Ce,Cep,Ci, Ao, Am/CA	128	8	9	10	17
<i>Pseudomonas aeruginosa</i> CMC-3	Burns	A,Am,Cpl, G,T,Ca,Ce,Cep, Ao, Am/CA	>256	16	6	6	16
<i>P. aeruginosa</i> SSKM_1	Burns	A,Am,Cpl,Cf,Co,G,T,Ce,Cep,Ci, Ao, Am/CA	>256	16	6	6	34
<i>P. aeruginosa</i> NRS-264	Urine	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci, Ao, Am/CA	>256	8	11	11	17

A, Ampicillin; Am, amoxicillin; Cpl, cephalixin; Cf, ciprofloxacin; Co, cotrimoxazole; G, gentamycin; T, tetracycline; Ca, ceftazidime; Ce, cefotaxime; Cep, cefpodoxime; Ci, ceftazidime; Cpm, ceftazidime; IPM, imipenem; Am/CA, amoxicillin/clavulanic acid; Cf/S, cefperazone/sulbactam; Pt, piperacillin/tazobactam (all the above isolates were sensitive to Pt, piperacillin/tazobactam; Cpm, ceftazidime and IPM, imipenem)

*Ce, Cefotaxime added 30 μ g/disc; **CA, clavulanic acid added in constant amount of 10 μ g/disc for DAD and DDST, 4 μ g/ml for MIC

DAD, Disc agar diffusion; DDST, double disc synergy test; MIC, minimum inhibitory concentration

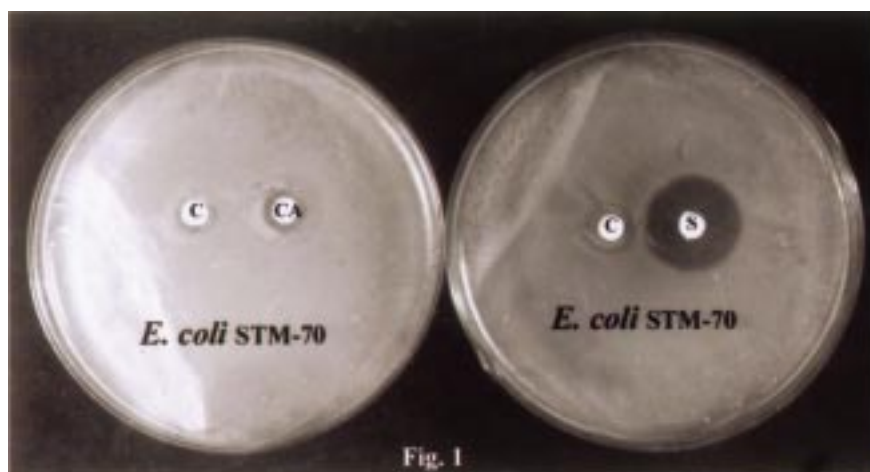


Fig. 1. Double disk synergy test done on *E. coli* STM 70; C, cefotaxime disk; CA, cefotaxime + clavulanic acid disk; S, ceftaxum (cefperazone + sulbactam) disk; CA with C shows no synergy, whereas C with S shows enhanced zone of inhibition indicating production of AmpC β -lactamase.

Table II. Clinical isolates showing inducible AmpC betalactamases production

Clinical isolates	DAD (Resistant to)	Source of sample	MIC ($\mu\text{g/ml}$)			DDST (mm)	
			Ce*	Cfs	Ce*	Ce/CA**	Cfs
<i>Klebsiella pneumoniae</i> CMC-40	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci,Cn,Ao,Am/CA	Urine	32	128	12	06	16
<i>Pseudomonas aeruginosa</i> NRS-3	A,Am,Cpl,Cf,Co,G,T,Ce,Cep,Ci,Cn,Ao,Am/CA	Urine	64	>256	16	06	21
<i>P. aeruginosa</i> NRS-226	A,Am,Cpl,Cf,Co,G,T,Ca,Ce,Cep,Ci,Cn,Ao,Am/CA	Burns	08	>256	14	10	21
<i>Proteus vulgaris</i> CMC-90	A,Am,Cpl,Cf,Co,G,T,Ce,Cep,Ci,Cn,Ao,Am/CA	Urine	128	>256	22	18	24

*Ce, Cefotaxime added 30 μg /disc; **CA, clavulanic acid added in constant amount of 10 μg /disc for DAD and DDST and 4 μg /ml for MIC

A, Ampicillin; Am, amoxicillin; Cpl, cephalixin; Cf, ciprofloxacin; Co, cotrimaxazole; G, gentamycin; T, tetracycline; Ca, ceftazidime; Ce, cefotaxime; Cep, cefpodoxime; Ci, ceftriazone; Am/CA, amoxicillin/clavulanic acid; Cfs, ceferperazone/sulbactam (all the above strains very sensitive to Pt, piperacillin/tazobactam; Cpm, ceftiprome and IPM, imipenem)

DAD, Disc agar diffusion; MIC, minimum inhibitory concentration; DDST, double disc synergy test

Table III. Specific activity of the clinical isolates producing inducible AmpC β -lactamase by microiodometric methods in the presence and absence of clavulanic acid

Clinical isolate	Specific activity					
	Benzylpenicillin as substrate			Cefotaxime as substrate		
	Without CA*	With CA*	Fold of increase in activity due to CA addition	Without CA*	With CA*	Fold of increase in activity due to CA addition
<i>Klebsiella pneumoniae</i> CMC-40	0.014	0.0479	3.4	0.0879	0.0995	1.13
<i>Pseudomonas aeruginosa</i> NRS-3	0.0094	0.1509	16.05	0.0623	0.3745	5.8
<i>P. aeruginosa</i> NRS-226	0.0471	.0735	1.5	0.0623	0.2811	4.5
<i>Proteus vulgaris</i> CMC-90	0.0868	0.0981	1.13	0.0698	0.0792	1.13

*CA, Clavulnic acid added 10 μg /ml *in vivo*

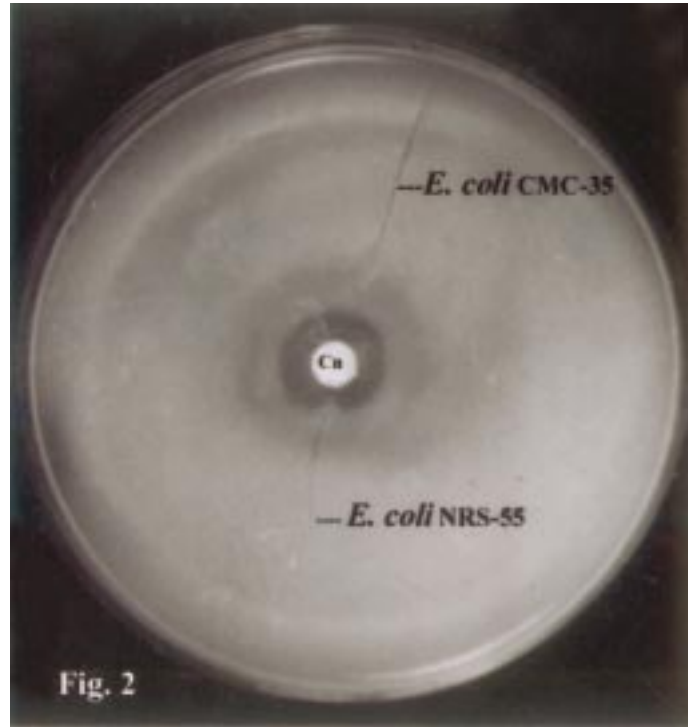


Fig. 2. Three dimensional extraction test. The disk placed in the middle is cefoxitin (Cn). One slit was filled with β -lactamase prepared from *E. coli* NRS 55. Regrowth along the slit in the zone of inhibition indicates production of AmpC β -lactamase by *E. coli* NRS 55. The other slit was filled with β -lactamase prepared from *E. coli* CMC 35. No growth was found in the case of *E. coli* CMC 35, a non-AmpC β -lactamase producer, which was used as negative control.

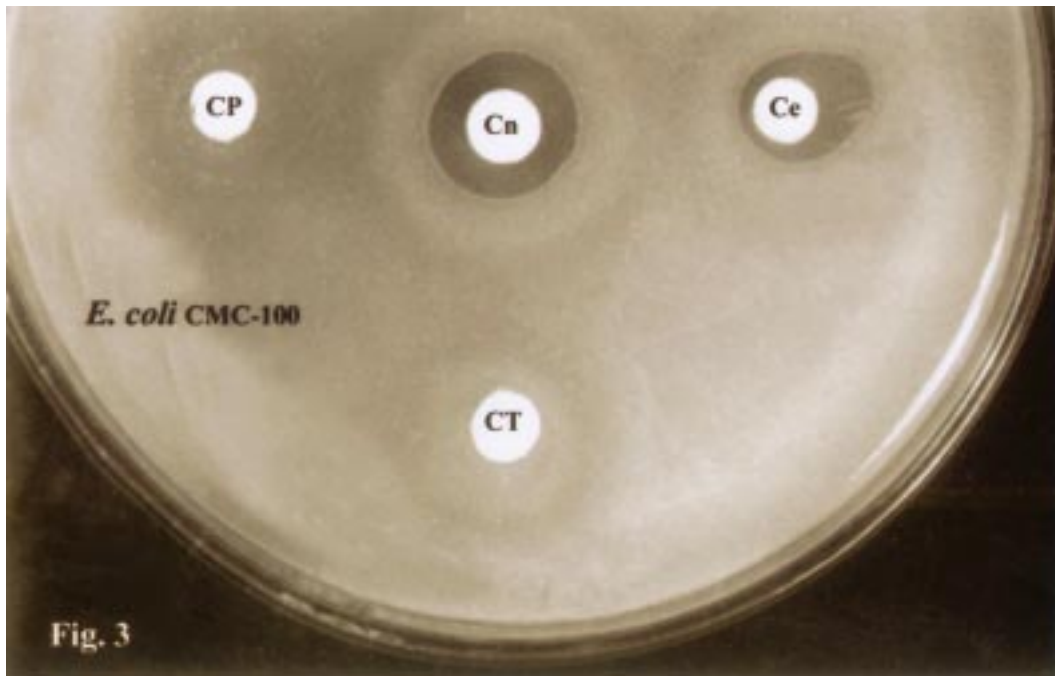


Fig. 3. Disk antagonism test done on *E. coli* CMC 100. The disk placed in the middle is cefoxitin (Cn); cefpodoxime (Cp) and ceftriazone (CT) showed no zone of inhibition on the side of cefoxitin (Cn) disk. But cefotaxime (Ce) disk on the right showed blunting of the zone of inhibition on the side of cefoxitin (Cn) disk. This confirms the presence of AmpC β -lactamase in *E. coli* CMC 100.

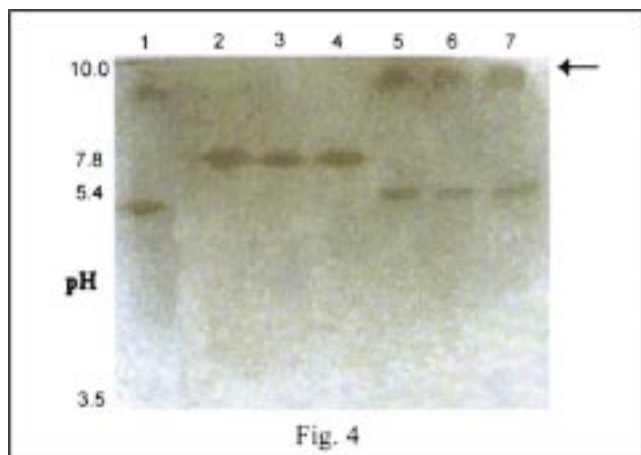


Fig. 4. Isoelectric focusing of β -lactamases using nitrocefin for detection. The enzyme in each lane was as follows: lane 1, *E. coli* NRS 29, pI <5; lane 2, *K. pneumoniae* CMC 40, pI 7.8; lane 3, *K. aeruginosa* NRS 26, pI 7.8; lane 4, *K. pneumoniae* ATCC 700603, pI 7.8, reference enzyme; lane 5, *E. coli* J53RI, pI 5.4, reference enzyme; lane 6, *E. coli* STM 70, pI 5.4; lane 7, *E. coli* CMC 67, pI 5.4. The arrow indicates the point of application of the samples.

Discussion

The efficacy of β -lactam group of antibiotics was reduced due to the production of β -lactamases by the resistant bacterial strains. Therefore, search for their inhibitors was initiated to protect the antibiotic activity *in vivo* against β -lactam resistant pathogens. Clavulanic acid, a naturally occurring β -lactam, had been the first such inhibitor, which is produced by *Streptomyces clavuligerus*¹⁵. Subsequently, a few more *viz.*, sulbactam, a penicillanic acid sulphone¹⁶, tazobactam, *etc.*, were found.

Plasmid mediated AmpC β -lactamase from *K. pneumoniae* isolates was first reported in 1989 from Seoul, South Korea¹⁷. Within 1998, nineteen types of plasmid mediated AmpC β -lactamases were reported from Algeria, France, Germany, Greece, India, Pakistan, Taiwan, Turkey, United Kingdom and United States¹⁸. The prevalence of AmpC β -lactamase enzyme was 2 per cent in *E. coli* and 17.1 per cent in *K. pneumoniae* in China¹⁹. Recently, AmpC-type β -lactamase producing *K. pneumoniae* were also reported from the Republic of Korea²⁰. Plasmid mediated inducible AmpC β -lactamases are still rare.

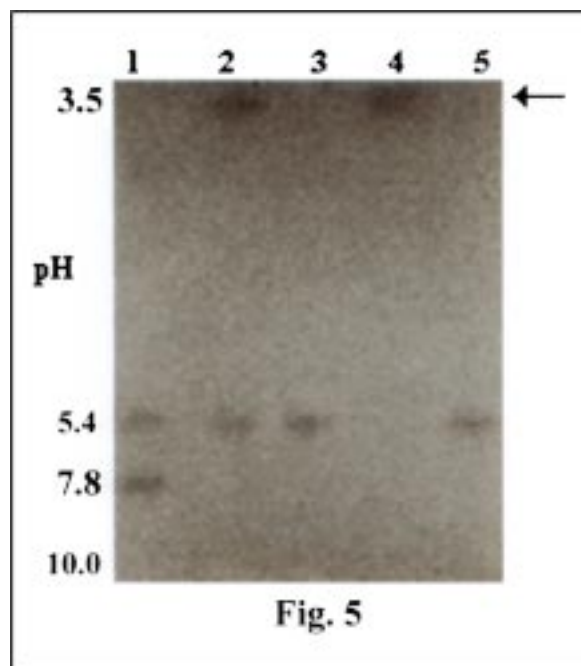


Fig. 5. Isoelectric focusing of β -lactamases using nitrocefin for detection. The enzyme in each lane were as follows: lane 1, *E. coli* J53RI, pI 5.4, and *K. pneumoniae* ATCC 700603, pI 7.8, both applied together in the same lane as reference enzymes; lane 2, *E. coli* CMC 55, pI 5.4; lane 3, *E. coli* STM 9, pI 5.4; lane 4, *E. coli* RGK 2, did not migrate; lane 5, *E. coli* STM 4, pI 5.4. The arrow indicates the point of application of the samples.

DHA-1 type of inducible AmpC β -lactamases were first reported from Saudi Arabia in 1998²¹ and later on from Taiwan in 2002¹². Six of the 51 isolates of Enterobacteriaceae were found to be inducible AmpC β -lactamase producers from Korea²². In Richmond, Virginia, USA, 2.6 per cent of *K. pneumoniae* were found to be AmpC β -lactamase producers²³. Plasmid encoded AmpC type β -lactamases were found in 8.5 per cent of the *K. pneumoniae*, 6.9 per cent of the *K. oxytoca* and 4.0 per cent of the *E. coli* collected from 25 US capital states and the district of Columbia²⁴.

In 2003, 20.7 per cent AmpC enzyme producers were found among Gram-negative bacteria in Guru Tegh Bahadur Hospital, Delhi²⁵. In the same year Subha *et al* found AmpC β -lactamase production in 24.1 per cent of *Klebsiella* spp. and 37.5 per cent of *E. coli* in Chennai²⁶. Shahid *et al* found 20 per cent of *P. aeruginosa* producing AmpC β -lactamase in Aligarh²⁷, and in Karnataka, 3.3 per cent of *E. coli*,

2.2 per cent of *K. pneumoniae*, 5 per cent of *C. freundii*, and 5.5 per cent of *E. aerogenes* (all urinary isolates) were found to harbour AmpC enzymes²⁸.

The phenotypic data generated in this study indicated that in Kolkata hospitals (6.7%) of the isolates were phenotypically confirmed to be AmpC β -lactamase producers and 1.4 per cent to be inducible AmpC β -lactamase producers which were less than that reported from Delhi²⁵, Chennai²⁶ and Aligarh²⁷ but more than that was found in Karnataka²⁸.

It has been reported that Amp C β -lactamases had pI values like that of TEM-1, SHV-11 and DHA-1 type of β -lactamases, which are pH 5.4, 7.6, and 7.8, respectively¹². In our study, we also found that many of the AmpC β -lactamases are having pI values in the range of pH 5.4 and pH 7.8.

This is perhaps the first report of AmpC β -lactamase and inducible AmpC β -lactamase producing bacteria from Kolkata, India. Clavulanic acid if used as an inhibitor of β -lactamase in the AmpC β -lactamase producing bacteria, can cause therapeutic failure. If the type of β -lactamase produced by the pathogen could be detected along with the antibiogram before administering the β -lactam drug to the patient, therapeutic failure might be avoided. These changes in the bacterial population represent evolutionary upgrades, which provide them a greater potential to resist β -lactam antibiotics and cause formidable therapeutic and diagnostic challenges.

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Reprint requests: Dr Manjusri Bal, Section of Microbiology, Department of Physiology, University of Calcutta University College of Science & Technology, 92, A.P.C. Road, Kolkata 700009, India
e-mail: manjusrb@vsnl.net