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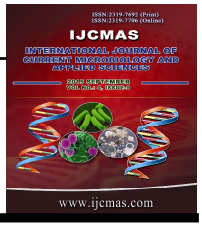

Original Research Article

Prevalence of AMPC Beta Lactamases among Gram Negative Bacterial Isolates in a Tertiary Care Hospital

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Keywords

AmpC β-lactamases, Phenotypically, Gram negative organisms, Cefoxitin resistance, Multidrug resistance

A B S T R A C T

Gram negative bacilli producing AmpC β lactamases are often responsible for multidrug resistance that is not always detected in routine antimicrobial susceptibility tests as there are no Clinical Laboratory Standards Institute (CLSI) or other approved criteria for AmpC detection, this remains a serious clinical concern. Hence it is necessary to know the prevalence of AmpC mediated resistance in a hospital setting so as to enable the clinician to select the appropriate antibiotic regimens. An attempt was made to detect the prevalence of AmpC β lactamases in gram-negative bacilli. This prospective study was conducted in the Department of Microbiology at VIMS & RC. A total of 256 Gram negative clinical isolates were obtained from various clinical samples from out patients and in patients admitted into various wards and intensive care units. All the isolates were identified based on the microscopy, the colony morphology and the biochemical reactions according to the standard guidelines. Antimicrobial Susceptibility Testing was performed by modified Kirby Bauer's disc diffusion method. AmpC screening was done using cefoxitin disk diffusion method, screen positives were phenotypically confirmed by disk approximation test, AmpC disk test and three-dimensional test (3D) 256 gram negative isolates were obtained from various clinical specimens. 84 were from the out patients and 172 from inpatients. *E. coli* was isolated more frequently (32%) followed by *Klebsiella pneumoniae* (27%), *Pseudomonas aeruginosa* (21%), Acinetobacter species (14%), Proteus mirabilis (3%) and *Citrobacter freundii* (3%). 68% (174/256) of the isolates were cefoxitin resistant by disc diffusion method and 32% (82/256) were sensitive. Among 174 screen positive isolates Amp C was confirmed in 59% (103/174) of the isolates, 84 (82%) were from inpatients and 19 (18%) from out patients. Amp C production was predominant in Acinetobacter baumannii (61%), followed by *Klebsiella pneumoniae* (42%), *Pseudomonas aeruginosa* (41%), *Citrobacter freundii* (38%), *E. coli* (30%) and Proteus mirabilis (25%). All Amp C producing isolates were sensitive to imipenem, however high level resistance was demonstrated to the other antibiotics used. Isolates producing AmpC β-lactamases raise special concerns as these isolates have been responsible for several nosocomial outbreaks and high rate of therapeutic failure, identification of these enzymes may aid in hospital infection control and help the physician to restrict the indiscriminate use of antibiotics thus decreasing the selective pressure, which generates antibiotic resistance. This study highly recommends performing screening and phenotypic tests for AmpC detection in a routine diagnostic laboratory work, however sequencing and typing the strains may be required to give a better insight into the genetic relatedness and the molecular epidemiology of this resistance mechanism.

Introduction

The rapid emergence of antibiotic resistance among the hospital pathogens is a serious threat to the management of infectious diseases. Beta-lactam antibiotics are the most frequently used antimicrobials for the empirical therapy. Production of beta-lactamases is one of the strategies adopted by the bacteria to develop resistance to beta-lactam class of antibiotics (Col Naveen Grover *et al.*, 2013). AmpC β lactamases and/or extended spectrum β lactamases (ESBLs) are of particular concern. AmpC b-lactamases are clinically significant because they may confer resistance to penicillins, cephalosporins, oxyimino-cephalosporins (e.g., ceftriaxone, cefotaxime, and ceftazidime), cephamycins (e.g., cefoxitin and cefotetan) and monobactams. AmpC β lactamase activity is not affected by the ESBL inhibitor clavulanic acid. In the Ambler structural classification of β lactamases AmpC enzymes belong to class C, while in the functional classification scheme of Bush *et al.*, they were assigned to group 1. Genes for AmpC b-lactamases are commonly found on the chromosomes of several members of the family Enterobacteriaceae, including *Enterobacter*, *Shigella*, *Providencia*, *Citrobacter freundii*, *Morganella morganii*, *Serratia marcescens*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter* sp. AmpC β lactamase production is chromosome or plasmid mediated. Chromosomal *AmpC* genes are expressed constitutively at a low level. Some Enterobacteriaceae, such as *Enterobacter* species, *Citrobacter* spp., and *Serratia* spp., carry an inducible AmpC gene. In these cases, the gene is strongly induced by b-lactams, such as cefoxitin and imipenem, with expression mediated by the regulator *AmpR*. Mutations in the repressor gene *AmpD* may lead to overproduction of AmpC β lactamases. Plasmid-based *AmpC* genes are expressed constitutively in most cases. However, some plasmid-carried *AmpC* genes, such as the *DHA-1* gene, are inducible by b-lactams, with expression regulated similar to that of inducible chromosomal *AmpC* genes. All plasmid-carried *AmpC* genes are considered to be of significant clinical relevance. AmpC overproduction in addition to porin mutations of the outer membrane can reduce susceptibility to carbapenems, in particular in plasmid mediated AmpC producers. Porins are chemically selective and transport only one group of molecules, or may be specific for one molecule. B-lactam and fluoroquinolone antibiotics must pass through porins to reach their targets in gram negative bacteria. Bacteria can develop resistance to these antibiotics by mutating the gene that encodes the porin – the antibiotics are then excluded from passing through the outer membrane. Although reported with increasing frequency in case isolates, the true rate of occurrence of plasmid mediated AmpC β lactamases in Klebsiella pneumoniae, *E. coli* and *Proteus mirabilis* remains unknown. These organisms usually exhibit multidrug resistance that is not always detected in routine antimicrobial susceptibility tests as there are no Clinical Laboratory Standards Institute (CLSI) or other approved criteria for AmpC detection (El-Hady and Adel, 2015; Laghawe Avinash *et al.*, 2012). It is necessary to know the prevalence of AmpC mediated resistance in a hospital setting so as to enable the clinician to select the appropriate antibiotic regimens. With this background this study was undertaken to detect the prevalence of AmpC β lactamases in gram-negative bacilli at our institute.

Materials and Methods

This prospective study was conducted in the Department of Microbiology at VIMS & RC. A total of 256 clinical isolates (*E. coli* (n=82), *Klebsiella pneumoniae* (n=69),

Pseudomonas aeruginosa (n=54), *Acinetobacter baumannii* (n=36), *Proteus mirabilis* (n=8), *Citrobacter freundii* (n=8)) were obtained from various clinical samples such as urine, pus, sputum, tracheal aspirate, catheter tip, aspirated fluids and blood from out patients and in patients admitted into various wards and intensive care units.

Processing of samples

All the samples were aseptically inoculated on to Blood and Mac Conkey agar plates and incubated overnight at 37°C. Urine was inoculated on hichrome (urochrome media). All the isolates were identified based on the colony morphology and biochemical reactions according to the standard guidelines (Koneman *et al.,* 2006).

Antimicrobial susceptibility testing

Routine disc diffusion susceptibility testing was performed by modified Kirby Bauer's disc diffusion method using discs of standard potency.

Cefotaxime-30 μg; ceftazidime-30 μg; ceftriaxone-30 μg; cefoxitin (30), Amikacin-10 μg; amoxycillin-20 μg; gentamycin-10 μg; Tetracycline-30 μg; imipenem-30 μg; ciprofloxacin-5 μg; Aztreonam-30 μg; cotrimoxazole-1.25/23.75 μg. The results were interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI).

AmpC screening using the disk diffusion method (Mohamudha Parveen *et al.*, 2010; Supriya Upadhyay *et al.*, 2010)

Cefoxitin resistant strains were detected using cefoxitin disk 30 μg. Isolates with zone diameters less than 18 mm were considered AmpC screening positive and were selected for phenotypic AmpC confirmatory tests

Phenotypic AmpC confirmation methods

Disk approximation test (El-Hady and Adel, 2015; Supriya Upadhyay *et al.*, 2010; Michael Dunne and Hardin, 2005)

Used for the detection of inducible AmpC production. A test isolate (with a turbidity equivalent to that of 0.5 McFarland standards) was spread over a Mueller Hinton agar plate. Cefotaxime (30μg) and cefoxitin (30μg) disks were placed 20 mm apart from centre to centre. Isolates showing blunting of the cefotaxime zone of inhibition adjacent to the cefoxitin disk were screened as positive for AmpC β-lactamase (Figure 1).

AmpC disk test (Avinash *et al.*, 2012; Coudron *et al.*, 2003)

Used for the detection of plasmid mediated AmpC production. 0.5 McFarland suspension of ATCC *E. coli* 25922 was inoculated on the surface of Mueller-Hinton agar plate. A 30 μg cefoxitin disc was placed on the inoculated surface of the agar. A sterile plain disc inoculated with several colonies of the test organism was placed beside the cefoxitin disc almost touching it, with the inoculated disk face in contact with the agar surface. After overnight incubation at 37°C, the plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (positive result) or the absence of a distortion, indicating no significant inactivation of cefoxitin (negative result) (Figure 2).

Three-dimensional test (3D) (Gude *et al.*, 2012; Coudron *et al.*, 2000)

Plasmid mediated AmpC production was further confirmed by this method. This method is a modified Hodge test described by Coudron *et al.* (2000). A lawn of *E. coli* ATCC 25922 was inoculated on a Mueller-

Hinton agar plate. After the agar surface dried, a 30-µg cefoxitin disk was placed at the center and 20 µl of a 0.5 McFarland suspension of the test isolate was dispensed into a radial slit performed in the plate. After overnight incubation at 37°C, enhanced growth of the surface organism in the inhibition zone along with the test strain was interpreted as evidence for the presence of AmpC β-lactamase (Figure 3).

Results and Discussion

In this study 256 gram negative isolates were obtained from various clinical specimens such as urine (n=97), pus (n=56), sputum (n=38), tracheal aspirate (n=29), aspirated fluids (n=18), catheter tip (n=13) and blood (n=5).

Among the 256 gram negative isolates 84 were from the out patients and 172 from inpatients.

E. coli was isolated more frequently (32%) followed by *Klebsiella pneumoniae* (27%), *Pseudomonas aeruginosa* (21%), *Acinetobacter baumannii* (14%), *Proteus mirabilis* (3%) and *Citrobacter freundii* (3%).

Of the 256 gram negative isolates 68% (174/256) were deemed cefoxitin resistant by disc diffusion method and 32% (82/256) were sensitive.

Among 174 screen positive isolates Amp C was confirmed by disc antagonism test, AmpC disk test and Three-dimensional test in 59% (103/174) of the isolates (Table 1).

Of the 103 Amp C positive isolates 84 (82%) were from inpatients and 19 (18%) from out patients.

Highest percentage of Amp C was reported from pus (61%), followed by tracheal aspirate isolates (48%), aspirated fluids (44%), catheter tip (38%), urine (36%), blood (20%) and sputum (16%) (Table 2).

Amp C production was predominant in *Acinetobacter baumannii* 22/36 (61%), followed by *Klebsiella pneumoniae* 29/69 (42%), *Pseudomonas aeruginosa* 22/54 (41%), *Citrobacter freundii* 3/8 (38%), *E. coli* 25/82 (30%) and *Proteus mirabilis* 2/8 (25%) (Table 3).

All Amp C producing isolates were sensitive to imipenem, however high level resistance was demonstrated to the other antibiotics used.

Drug resistance poses a therapeutic problem not only in the hospital settings, but also in the community as most of the bacteria have acquired resistance to multiple antibiotics. Antimicrobial drug resistance is emerging world wide as a major public health problem. Selective pressure of misuse and overuse of antibiotics in the hospitals has resulted in the emergence and dissemination resistant bacteria in many areas of hospitals. The various mechanisms of drug resistance in Gram negative bacteria include extended spectrum b-lactamases (ESBL) production, AmpC β lactamase production, efflux mechanism and porin deficiency. In the clinical laboratory settings, the commonly detected enzymes causing resistance are AmpC β lactamases and ESBLs. Clinical relevance of AmpC β lactamases lies in the fact that they confer resistance to both narrow and broad spectrum cephalosporins, β lactam/ β lactamase inhibitor combinations and aztreonam. Detection of AmpC is important to improve the clinical management of patients suffering from infections and would also provide us with sound epidemiological data. However, there are no clinical and laboratory standard institute guidelines for detection of AmpC mediated resistance in gram negative

clinical isolates and hence, it usually poses a problem due to misleading results, especially so in phenotypic tests (Col Naveen Grover *et al.,* 2013; El-Hady and Adel, 2015).

In this study, sizeable number of cefoxitin resistant isolates was not positive for AmpC production. Among 174 screen positive isolates Amp C was confirmed phenotypically in 59% (103/174) of the isolates, such a finding was also seen by Anand Manoharan *et al.* (2012) wherein only 36.5% cefoxitin resistant isolates were confirmed Amp C producers phenotypically, this warrants further investigation into the other mechanisms of resistance, this could be due to the inability of current phenotypic tests to accurately detect the type of transferable AmpC β lactamase which does not allow for the differentiation of multiple AmpC enzymes and it is possible that there is a limit to the amount of AmpC β lactamase that a bacterial cell can accommodate and still be a viable pathogen. The available tests are inadequate for organisms expressing plasmid-mediated AmpC β lactamases alone or in combinations with ESBLs. However the use of cefoxitin resistance as a screening agent/marker for AmpC production is quite reliable. The use of phenotypic methods in combination with cefoxitin as screening method may be a better tool for laboratory diagnosis and confirmation of AmpC producing Gram-negative bacteria (Anand Manoharan *et al.,* 2012).

In India the incidence of Amp C production among clinical isolates has been on steady increase over the past few years and ranges from 3.3–47% as seen by various authors, in the present study 40% were Amp C producers which is in accordance with the Indian scenario. Despite the phenotypic methods used, we were not able to distinguish between the chromosomal derepressed and plasmid mediated enzymes as this requires isoelectric focusing and genotypic methods (Col Naveen Grover *et al.,* 2013).

The high percentage of AmpC harbouring isolates was encountered in isolates from inpatients, showing its nosocomial importance. 61% isolates of pus, 48% of tracheal aspirate, 44% of aspirated fluids, and 38% of catheter tip isolates harboured AmpC enzymes reinforcing the fact that there is rampant spread of antibiotic resistance among the hospital strains (Upadhyay *et al.,* 2013; Varsha Gupta *et al.,* 2012).

Emerging evidence suggests that probably all *Acinetobacter baumannii* isolates produce a chromosomal Amp C enzyme. In the present study 61% of *Acinetobacter baumannii* isolates were found to harbour an Amp C enzyme. The reason for low occurrence could be that all the isolates may have *AmpC* genes, but these might not be expressed in all the isolates of *Acinetobacter baumannii*, they might have 'silent genes' or there might be low level expression of *AmpC* genes that was not detected by the present method (Vikas Manchanda and Singh, 2003). 41% of *Pseudomonas aeruginosa* isolates had Amp C enzymes as against high prevalence reported by Supriya Upadhyay *et al.* (2010) and Şerife Altun *et al.* (2013).

Pseudomonas aeruginosa may be intrinsically resistant or has acquired resistance to antibiotics due to permeability barrier of the cell surface, multidrug efflux pumps and production of β lactamases (AmpC β lactamase, extended spectrum β lactamases and metallo β lactamases). Michael Dunne and Hardin (2005) detected AmpC production in 77% isolates in *Citrobacter* species, *Citrobacter freundii*

having the highest percentage, *Citrobacter freundii* possess inducible AmpC β lactamases (AmpC) that can be upregulated by subinhibitory concentrations of certain β lactam antibiotics.

Table.1 Prevalence of *Amp C* in cefoxitin resistant cases

Total no of isolates	Cefoxitin		*Amp C* %
	Sensitive	Resistance	
256	82	174	103/174 (59%)

Of the 256 gram negative isolates 82 were cefoxitin sensitive and 174 were resistant. Of the 174 isolates 103 (59%) were AmpC producers

Table.2 Prevalence of Amp C beta lactamase in various clinical samples

S.l no	Sample	n	Amp C %
1	Urine	97	35 (36%)
2	Pus	56	34 (61%)
3	Sputum	38	6 (16%)
4	Tracheal aspirate	29	14 (48%)
5	Aspirated fluids	18	8 (44%)
6	Catheter tip	13	5 (38%)
7	Blood	5	1 (20%)
	Total	**256**	**103 (40%)**

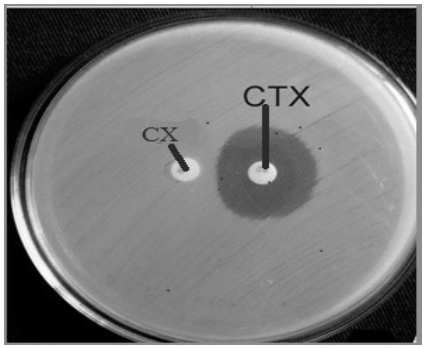
Highest percentage of Amp C was reported from pus (61%), followed by tracheal aspirate isolates (48%), aspirated fluids (44%), catheter tip (38%), urine (36%), blood (20%) and sputum (16%)

Table.3 Production of Amp C beta lactamase in various organisms

Sl. no	Organism	No of isolates	Amp C %
1	*E. coli*	82	25 (30%)
2	*Klebsiella pneumoniae*	69	29 (42%)
3	*Pseudomonas aeruginosa*	54	22 (41%)
4	*Acinetobacter baumannii*	36	22 (61%)
5	*Proteus mirabilis*	8	2 (25%)
6	*Citrobacter freundii*	8	3 (38%)
	Total	**256**	**103 (40%)**

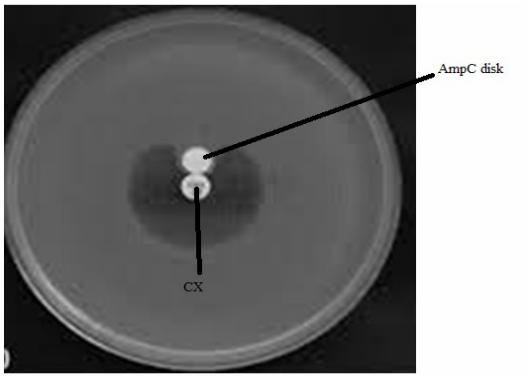
Among the gram negative isolates Amp C production was predominant in *Acinetobacter baumannii* (61%), followed by *Klebsiella pneumoniae* (42%), *Pseudomonas aeruginosa* (41%), *Citrobacter freundii* (38%), *E. coli* (30%) and *Proteus mirabilis* (25%).

Figure.1 Disk approximation test



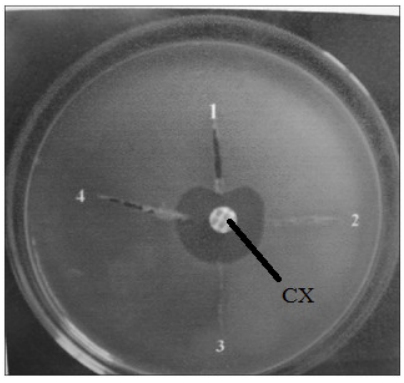
Isolates showing blunting of the cefotaxime zone of inhibition adjacent to cefoxitin disk are AmpC β lactamase producers.

Figure.2 AmpC disk test



AmpC disk test showing distortion of the inhibition zone around cefoxitin disk (CX) in the vicinity of the disk containing the test organism, indicating a positive test

Figure.3 Three-dimensional test (3D)



Organism showing clear distortion in the zone of inhibition strain 1 (positive control), 2 & 3 (test strains) and showing minimal distortion are AmpC producers and no distortion strain 4 (negative control) indicates non-AmpC producers.

Further, mutations can occur in the regulatory components of AmpC leading to a stable, hyperproduction of AmpC with concomitant high-level resistance to many classes of β lactam antibiotics, however in the present study 3/8 (38%) of *Citrobacter*

freundii had Amp C production, low prevalence could be due the low sample size, genetic level study could have possibly explained this ambiguity.

AmpC β lactamase producing *E. coli* and *Klebsiella pneumoniae* are being increasingly reported from many parts of the world incidence ranging from 14-49% for *E. coli* and17–58% for Klebsiella spp. In the present study the percentage of Amp C production in *E. coli* and *Klebsiella pneumoniae* was 30% and 42% respectively which is in accordance to the previous studies (Tanushree Barua *et al.,* 2013). The drug of choice remains carbapenems in Amp C producers as the β lactam / β lactamases inhibitor combinations fail even if these show sensitivity in vitro, the present study showed 100% sensitivity to imipenem

In conclusion, AmpC β lactamase producing bacterial pathogens may cause a major therapeutic failure if not detected and reported in time AmpC β lactamase. Identification of *AmpC* may aid in hospital infection control and help the physician to prescribe the most appropriate antibiotic, thus decreasing the selective pressure, which generates antibiotic resistance. Sequencing and typing the strains may be required to better understand the genetic relatedness and the molecular epidemiology of this resistance mechanism.

Conflict of interest: The authors declare that there are no conflicts of interest

References

Anand Manoharan, Madhan Sugumar, Anil Kumar, Hepzibah Jose, Dilip Mathai, 2012. ICMR-ESBL study group, Phenotypic & molecular characterization of AmpC β-lactamases among *Escherichia coli*, *Klebsiella* spp. & *Enterobacter* spp. from five Indian Medical Centers. *Indian J. Med. Res.*, March 2012, Pp. 359–364.

Clinical and Laboratory Standards Institute (CLSI), Performance Standards for Antimicrobial Susceptibility Testing. Approved Standards M100-S22.

Col Naveen Grover, Brig A.K. Sahni, Col S. Bhattacharya (Retd.). 2013. Therapeutic challenges of ESBLS and AmpC beta-lactamase producers in a tertiary care center. *Med. J. Armed Forces India*, 6(9): 4–10.

Coudron, P.E., Hanson, N.D., Climo, M.W. 2003. Occurrence of extended-spectrum and AmpC β-Lactamases in bloodstream isolates of Klebsiella pneumoniae: Isolates harbor plasmid mediated Fox – 5 & ACT – 1 AmpC β Lactamases. *J. Clin. Microbial.*, 41: 772 –77.

Coudron, P.E., Moland, E.S., Thomson, K.S. 2000. Occurrence and detection of AmpC beta-lactamases among *Escherichia coli*, Klebsiella pneumoniae, and *Proteus mirabilis* isolates at a veterans medical center. *J. Clin. Microbiol.*, 38(5): 1791–1796.

El-Hady, S.A., Adel, L.A. 2015. Occurrence and detection of AmpC b-lactamases among Enterobacteriaceae isolates from patients at Ain Shams University Hospital. *Egypt. J. Med. Hum. Genetics*, 16: 239–244.

Gude, M.J., Seral, C., Sáenz, Y., González-Domínguez, M., Torres, C., Castillo, F.J. 2012. Evaluation of four phenotypic methods to detect plasmid-mediated AmpC β-lactamases in clinical isolates. *Eur. J. Clin. Microbiol. Infect. Dis.*, 31: 2037–2043.

Koneman, E.W., Allen, S.D., Janda, W.M., Schreckenberger, P.C., Winn, W.C.

2006. Color atlas and textbook of diagnostic microbiology,6th edn. Lippincott Williams Wilkins, Baltimore.

Laghawe Avinash R., Jaitly Neelam, K., Thombare Vilas, R. 2012. Prevalence of AMPC Beta-lactamase in Gram- negative bacilli. *JPBMS*, 20 (07).

Michael Dunne, W., Hardin, D.J. 2005. Use of several inducer and substrate antibiotic combinations in a disk approximation assay format to screen for AmpC Induction in patient isolates of *Pseudomonas aeruginosa*, Enterobacter spp., *Citrobacter* spp., and *Serratia* spp. *J. Clin. Microbiol.*, 43(12): 5945–5949.

Mohamudha Parveen, R., Harish, B.N., Parija, S.C. 2010. AMPC Beta lactamases among Gram negative clinical isolates from a tertiary hospital, South India. *Braz. J. Microbiol.*, 41: 596–602.

Şerife Altun, Zeliha Kocak Tufan, Server Yağcı, Ufuk Önde, Cemal Bulut, Sami Kınıkl and Ali Pekcan Demiroz, 2013. Extended spectrum beta-lactamases, AmpC and metallo beta-lactamases in emerging multi-drug resistant gram-negative bacteria in intensive care units. *Open Access Sci. Reports*, 2(4).

Supriya Upadhyay, Malay Ranjan Sen, Amitabha Bhattacharjee, 2010. Presence of different beta-lactamase classes among clinical isolates of *Pseudomonas aeruginosa* expressing AmpC beta-lactamase enzyme. *J. Infect. Dev. Ctries.*, 4(4): 239–242.

Tanushree Barua, Malini Shariff, S.S. Thukral, 2013. Detection and characterization of AmpC B-lactamases in Indian clinical isolates of *Escherichia coli, Klebsiella pneumoniae* and *Klebsiella oxytoca. Universal J. Microbiol. Res.*, 1(2): 15–21.

Upadhyay, S., Mishra, S., Sen, M.R. 2013. Co-existence of *Pseudomonas*-derived cephalosporinase among plasmid encoded CMY-2 harbouring isolates of *Pseudomonas aeruginosa* in north India. *Indian J. Med. Microbiol.*, 31(3): 257–260.

Varsha Gupta, Karthikeyan Kumarasamy, Neelam Gulati, Ritu Garg, Padma Krishnan, Jagdish Chander, 2012. AmpC β-lactamases in nosocomial isolates of *Klebsiella pneumoniae* from India. *Indian J. Med. Res.*, 136: 237–241.

Vikas Manchanda, Singh, N.P. 2003. Occurrence and detection of AmpC β-lactamases among Gram-negative clinical isolates using a modified three-dimensional test at Guru Tegh Bahadur Hospital, Delhi, India. *J. Antimicrobial Chemother.*, 51: 415–418.