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Detection of Inducible and Non-inducible (constitutive) AmpC β-lactamase-producing Gram-Negative Bacteria among Family Enterobacteriaceae by Two Phenotypic Methods-Disk Antagonism Test (DAT) and AmpC disk Test at a Tertiary Care Hospital, Himachal Pradesh, India

A. K. Ashok*, S. C. Jaryal, K. Thakur, A. Sood, P. K. Gupta, and S. Thakur

Department of Microbiology, Dr. Rajendra Prasad Govt. Medical College Kangra at Tanda, Himachal Pradesh, India
**Corresponding author*

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A B S T R A C T

Gram-negative bacilli particularly among family Enterobacteriaceae produce AmpC β-lactamases which are often responsible for multidrug resistance. Their detection is problematic in routine as there are no Clinical Laboratory Standards Institute (CLSI) or other approved criteria for screening and detection of AmpC β-lactamases. So, it is a matter of great concern. Also only some studies have shown a few phenotypic methods which are comparatively easy and cheap. There has not been done such study in our state Himachal Pradesh. So it was decided to perform such types of studies at our institute. Aims & Objectives: To detect inducible and non-inducible (constitutive) AmpC β-lactamases by Disk Antagonism Test (DAT) and AmpC Disk methods. Material and Methods: Out of total 9105 clinical samples received in the Department of Microbiology for culture and sensitivity, 1200 samples were identified by colony morphology, Gram staining and Biochemical reactions as belonging to family Enterobacteriaceae. These samples were isolated from urine, pus, blood, and others. These isolates were screened by cefoxitin disk test. Those organisms sensitive to cefoxitin disc test were subjected to Disk antagonism test (DAT) & those resistant to it were subjected to AmpC disk test. Results: Of the 1200 isolates 575 were cefoxitin sensitive and 625 were cefoxitin resistant. Of 625 cefoxitin-resistant isolates, 48 (4.0%) isolates were found to be positive for AmpC β−lactamase production by the phenotypic method AmpC disc test. Out of 575 cefoxitin sensitive strains, 28(4.87%) revealed the presence of inducible AmpC β−lactamase by Disk antagonism test. Conclusions: The test DAT to know inducible AmpC β-lactamase and AmpC disk test to know plasmid-mediated AmpC β-lactamase is simple, less time consuming as well as cost effective and has a reliable approach.

Introduction

β-lactamases are heterogeneous bacterial enzymes that cleave the β-lactam ring of penicillins and cephalosporins to inactivate the antibiotic. (Allen *et al*., 2006) Extended spectrum β-lactamases (ESBLs) are enzymes that mediate resistance to third generation cephalosporins (3 GCs) and monobactams (aztreonam) but do not affect cefamycins (cefoxitin, cefotetan, etc) or carbapenems (imipenem, meropenem, ertapenem, doripenem etc.). They are inhibited by β-lactamase inhibitor

combinations (BLIs) such as clavulanic acid, sulbactam and tazobactam. Therefore, any strain resistant to 3GC but sensitive to β-lactam/β-lactam inhibitor combination (BL/BLI) is likely to contain ESBL. (Paterson and Bonomo, 2005) AmpC class β-lactamases (AmpC) are cephalosporinases which are not inhibited by clavulanic acid, tazobactam & sulbactam. They degrade penicillins, monobactams, and β-lactam inhibitors. (Jacoby, 2009) There are 2 types of classifications of β-lactamases. According to Bush-Jacoby-Medeiros functional classification, AmpCs belong to Group1 and according to Ambler Structural / Molecular classification they belong to Class C. (Bush *et al*., 1995)

Production of β-lactamase is the most common mechanism of antibiotic resistance among Gram-negative bacilli. Among the β-lactamases, the most common is production of ESBLs and AmpCs. (Coudron *et al*., 2000) AmpC β-lactamase production is frequently accompanied by multidrug resistance. Resistance caused by plasmid-mediated AmpC β-lactamase is less common than the production of ESBLs, but may be more difficult to detect. (Hsieh *et al*., 2015)

AmpC β-lactamases are of two types: Plasmid-mediated and Chromosomal-mediated. The chromosomally mediated β-lactamase production takes place mainly through the expression of the AmpC gene which is either constitutive or inducible. In most of the genera of the family *Enterobacteriaceae*, AmpC is inducible. The plasmid-mediated AmpC enzymes except for DHA enzymes are almost always expressed constitutively.[4] Majority of AmpC β-lactamases are chromosomally mediated (unlike ESBLs which are plasmid-mediated). In general, plasmid-encoded AmpC beta-lactamases are expressed constitutively. Plasmid-mediated AmpC β-lactamases have arisen through transfer of chromosomal gene for AmpC β-lactamases on to the plasmid. (Akhter, 2015) Chromosomal AmpC beta-lactamases are usually inducible, while, except for DHA (Docosahexanoic acid) enzymes, plasmid-mediated AmpC enzymes are uninducible.

The detection of AmpCs is important as they are concerned with treatment failure & their prevalence is increasing worldwide. The detection of AmpC production can be done by phenotypic and molecular methods. Different phenotypic AmpC detection tests have been described in the literature. Enzyme extraction methods have traditionally been cited as the optimum phenotypic detection method for AmpC activity. However, these are labour-intensive and not suitable for routine clinical use. Inhibitors of the AmpC enzyme are well described and include boronic acid compounds, cloxacillin, and novel inhibitors such as Syn2190. (Manual of workshop on detection of beta-lactamases, 2011) The use of disk approximation tests by Kirby-Bauer testing to detect inducible AmpC activity has also been described, using one antibiotic as an inducing substrate and a second antibiotic as a reporter substrate. A standard test for the detection of the plasmid-mediated AmpC enzyme is necessary. The phenotypic tests done namely Disk antagonism test (DAT) and AmpC Disk test were comparatively easier, cheaper and could readily be done in even small set up. Molecular techniques like Polymerase chain reaction (PCR) are costly and not available in every set up.

Materials and Methods

The present cross sectional study was conducted in the Microbiology department of tertiary care centre Dr. Rajendra Prasad

Govt. Medical College Kangra at Tanda located in foothills of Northern Himalayan region of India. The study was conducted over a period of one year from March 2014 to April 2015.All the clinical samples processed during this period and isolates identified as a member of family *Enterobacteriaceae* were included in the study. All the clinical isolates were identified using standard microbiological procedures. (Allen *et al*., 2006)

The isolates were subjected to cefoxitin disk test as a screening test. (Chaudhary *et al*., 2008) Briefly, the isolates with zone diameter $\geq$ 18mm were considered sensitive and those less than 18mm as resistant. DAT test was performed on cefoxitin isolates while AmpC disc test was performed on cefoxitin resistant isolates. DAT test was done by the methods of Sanders *et al*. (Manual of workshop on detection of beta-lactamases, 2011) Briefly , the test isolate was exposed to disks of ceftazidime (30µg) and imipenem (10µg) placed 20mm apart (edge to edge) as shown in figure-1. It was incubated at 37° C for 18-24 hours. After overnight incubation flattening of the radius of the zone of inhibition around ceftazidime disk when produced indicated inducible AmpC production by the isolate.

For AmpC disk test a lawn culture of *Escherichia coli* ATCC 25922 was prepared on Mueller-Hinton agar plate as shown in figure-2. Sterile disk (6mm) moistened with 20µl Normal Saline was inoculated with several colonies of test organism. Inoculated disk was placed almost touching the cefoxitin disk (30µg) on the plate. It was incubated at 37° C for 18-24 hours. (Coudron, 2005) Afterovernight inoculation at 37°C, a positive test appeared as flattening or indentation of the zone of inhibition around the cefoxitin disk.

The study was approved by institutional ethical committee, Dr Rajendra Prasad Government Medical College & Hospital (DRPGMCH), Kangra at Tanda (Himachal Pradesh).

Results and Discussion

During one year period 9105 clinical samples were processed and 1200 bacterial isolates were identified as a member of family *Enterobacteriaceae* in 1094 samples (there were more than one bacterial isolates in some sample). The maximum number of samples were from urine 548(50.09%) followed by pus 382(34.92%) and blood 74(6.76%). Other samples included were sputum, endocervical swab, endotracheal tube, aural swab, anal swab, CVP line, tissue, stool, throat swab, cerebrospinal fluid (CSF), catheter tip, BM aspirate, ascitic fluid, pleural fluid, bronchoalveolar lavage, prostatic secretion. Different isolates were *E.coli* (66.08%) maximally followed by *Klebsiella species* (11.33%), *Citrobacter species* (10.17%), *Enterobacter species* (5.34%), *Proteus species* (3.50%), *Salmonella typhi* (1.08%), *Pantoea agglomerans* (1.00%), *Morganella morganii* (0.75%) and *Providencia species* (0.58%).Other species include *Serratia marcescens* (1) and *Salmonella paratyphi A*(1).*E. coli* followed by *Klebsiella* species were maximally isolated from urine. The maximum number of organisms isolated from pus and blood were *E.coli* followed by *Citrobacter spp.*

The screening test using cefoxitin disc (30µg) was sensitive in 47.92% (575/1200) of isolates and resistant in 52.08% (625/1200) of the isolates. Out of 1200 isolates, 48 (4%) were non–inducible AmpC producers shown by AmpC disk test. Similarly, out of 1200 bacterial isolates, 28 (2.33%) were inducible as shown by DAT.

Antimicrobial drug resistance is emerging

worldwide as a major public health problem.Misuse and overuse of antibiotics has resulted in the emergence and dissemination of resistant bacteria.There are many mechanisms of antibiotic resistance. AmpC β-lactamase production is one of the mechanisms of antibiotic resistance. The production of AmpC β-lactamase is mainly chromosomally mediated and also plasmid-mediated (Carmeli *et al*., 1999; Cavallo *et al*., 2002; Livermore, 2002).

AmpC detection is not routinely carried out in many microbiology laboratories. This could be due to lack of awareness, resources or facilities. This study was planned with a view to find the proportion of AmpC β-lactamases in various clinical isolates belonging to family En*terobacteriaceae* and also to detect inducible (chromosomal) and Plasmid mediated AmpC β-lactamases by two phenotypic methods. In all these AmpC producers**,** we were not able to distinguish between the chromosomal and plasmid mediated enzymes, as this requires genotypic confirmatory tests. (Yilmaz *et al*., 2007; Doi and Paterson, 2007)

Figure.1. Disk Antagonism Test (DAT)

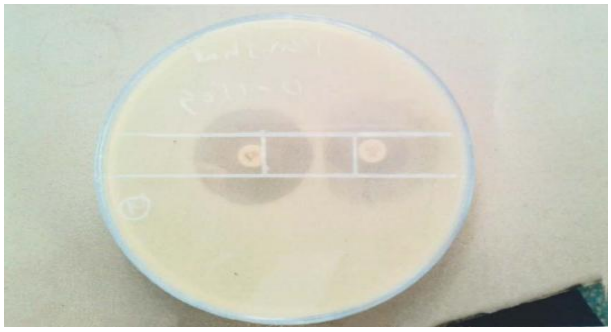
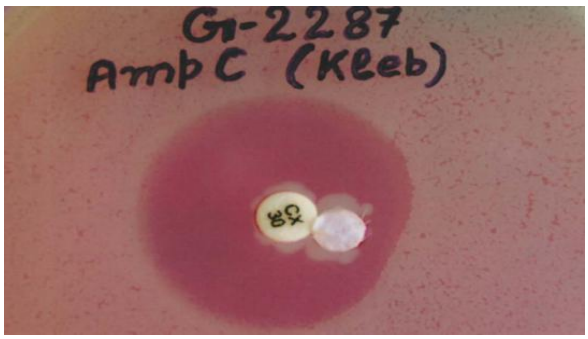


Figure.2 AmpC Disk Test



In most genera of the family *Enterobacteriaceae* AmpC is inducible. Plasmid-mediated inducible beta-lactamases are extremely rare. (Parveen *et al*., 2010) In this study 2.33% (28/1200) were chromosomally-mediated (inducible) while 4% (48/1200) plasmid-mediated (uninducible). In a study by Nasir *et al* at Jaipur 2.2 %(7/320) were chromosomally-mediated. (Nasir *et al*., 2015) In a study at Egypt by Wassef M and Behiry I 86.4% (19/22) were chromosomally-mediated and 13.6 %(3/22) plasmid-mediated AmpC. (Wassef *et al*., 2014) Amongst the 320

urinary *Enterobacteriaceae* isolates the inducible AmpC production was maximally detected by the ceftazidime-imipenem disk antagonism test (3.1%) and the non-inducible isolates by AmpC disk test (2.2%). (Shevade and Agarwal, 2013) Cefoxitin and imipenem are strong inducers of the AmpC β-lactamases, whereas cefotaxime and ceftazidime are weak inducers.

In Gram-negative bacteria belonging to family *Enterobacteriaceae,* AmpC beta-lactamase production is chromosomal or plasmid-mediated. In our study *E.coli, Klebsiella spp., Proteus spp, Salmonella Typhi* and *Citrobacter spp.* were chromosomal. *E.coli, Klebsiella spp, Citrobacter spp* and *Enterobacter spp*. were plasmid-mediated. Chromosomal AmpC is less in prevalence in comparison to plasmid mediated. (Barlow and Hall, 2002; Johnson, and O'Bryan, 2000; Thomson, 2010) In our study prevalence of chromosomal AmpC was 2.33% (28/1200) in comparison to 4.00 % (48/1200) plasmid mediated. This is statistically significant at $p<0.05$(0.019734). Various studies have shown different prevalence of AmpC producers among different organisms. This geographical difference may be due to different patterns of antibiotic use and difference in the selection of organisms for the study.

Given the need for a test for AmpC β-lactamases and the fact that many clinical laboratories are often short staffed and overworked, the AmpC disk test could fill a current gap in diagnostic microbiology. Adoption of this test would make it possible to learn more about the clinical implications of plasmid-mediated AmpC β-lactamases and to contain the spread of organisms possessing this resistance mechanism. The potential benefits would include better patient outcomes in terms of avoiding in appropriate therapy and a reduction in the escalation of antibiotic resistance through better infection control.

There was not gold standard test in our study. So the diagnostic accuracy and efficiency of combination of tests was not evaluated by sensitivity and specificity analysis. Phenotypic confirmation tests are inexpensive but nevertheless highly sensitive and specific (confirmed in another studies). (Peter-Getzlaff *et al*., 2011)

In this study, the number of AmpC producing *Enterobacteriaceae* isolates which were studied was less. Hence, further studies with more number of strains are needed. Also there is need of confirmatory phenotypic tests like Cloxacillin combined disk diffusion test (CCDDT) and molecular methods like PCR.

In conclusion, Phenotypic AmpC screening and confirmation tests are inexpensive but nevertheless highly sensitive and specific. Therefore, it can be performed in all types of clinical laboratories, whereas the implementation of molecular methods is often complex, requires specially trained personnel, and is associated with higher costs.

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