OCCURRENCE AND DETECTION OF AmpC BETA-LACTAMASES AMONG CLINICAL ISOLATES OF ENTEROBACTERIACEAE IN A TERTIARY CARE HOSPITAL, BAGALKOT; USING THREE PHENOTYPIC METHODS

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ABSTRACT

BACKGROUND

Antibiotic resistance has always been a major matter of concern and its amplification nowadays needs to be looked upon with utmost care and attention. Beta-lactam antibiotics are the most widely used antibiotics and resistance to this class of drugs will hamper all treatment options. AmpC beta-lactamase-mediated resistance is one of the emerging pattern. These are important as the substrate profile of this class of enzymes is very wide with special concern to beta-lactam antibiotics, especially cephalosporins.

MATERIALS AND METHODS

A total of 130 isolates of Enterobacteriaceae isolated from various samples from patients admitted to different wards in S. Nijalingappa Medical College and HSK Hospital, Bagalkot, Karnataka during the period from Jan 2013 – Dec 2013 were included in the study. All these were subjected to Cefoxitin (μg) screen test by disc diffusion method for AmpC detection. The screen positive (Cefoxitin zone size < 18 mm) isolates were further subjected to three phenotypic confirmatory methods namely TDET, PBA test and ACD test for confirmation of AmpC production. Antibiotic sensitivity for these AmpC positive isolates was also done.

RESULT

Of the total isolates of Enterobacteriaceae, 23.1% were cefoxitin screen test positive, which were subjected to three phenotypic confirmatory methods of which phenylboronic acid test detected 46.6%, AmpC disc test detected 36.6% and three dimensional extraction test detected 26.6% of the AmpC producers. *E. coli* (36.36%), *K. pneumonia* (36.36%) and *C. freundii* (27.27%) were the AmpC producers. The prevalence of AmpC beta-lactamases among isolates of Enterobacteriaceae is 16.9%. These were mainly sensitive to Imipenem (90.9%) followed by Piperacillin-Tazobactam (77.2%) and Ofloxacin (72.7%). For the urinary isolates, Nitrofurantoin showed 100% sensitivity followed by Norfloxacin (83.3%).

CONCLUSION

The prevalence of AmpC beta-lactamases may be low, but the antibiogram shows that they have a wide spectrum of drug resistance. Hence, proper methods using simple and less cumbersome techniques like PBA test and ACD test can be used to detect AmpC. Also, antibiogram of these isolates will help to formulate proper treatment options.

KEYWORDS

AmpC Beta-Lactamase, Cefoxitin Screen Test, TDET, PBA test, ACD test, ESAC (Extended Spectrum AmpC).

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BACKGROUND

Antibiotic resistance is emerging worldwide as a threat to the favourable outcome of common infections in community and hospital settings.¹

Among wide array of antibiotics, β -lactams are most varied and widely used agents accounting for over 50% of all systemic antibiotics in use.² Six decades ago, antibiotic era began with discovery of penicillin.¹

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Emergence of resistance to β -lactam antibiotics began even before Penicillin was developed.³ The most common cause of bacterial resistance is the production of β -lactamases,¹ began before Penicillin was developed.³

Over the last twenty years, many new β -lactam antibiotics have been developed that have been selectively and overused and has resulted into emergence of new variants of β -lactamase.³

Among emerging group of β -lactamases produced by gram negative bacteria are ESBLs, AmpC β -lactamases, metallo-beta-lactamases. High levels of AmpC production is typically associated to all β -lactam antibiotic resistance except for carbapenems and cefepime (4th Generation cephalosporins).⁴ They are clinically significant because many confer resistance to a wide variety of β -lactamase inhibitors, such as clavulanic acid, sulbactam and tazobactam.⁵ They may be plasmid-mediated/uninducible, which are typically associated with broad multidrug resistance or chromosomal-mediated/inducible AmpC wherein they are induced by β -lactam antibiotics such as cefoxitin and imipenem, but poorly

induced by 3rd and 4th generation cephalosporins.⁶ Cephamycin (Cefoxitin) resistance as non-AmpC producers may be due to porin-deficient mutants (As in non-AmpC producing *Klebsiella pneumoniae*)^{6,7} or species specific intrinsic resistance.⁸

Detecting AmpC is clinically important not only because of their broader cephalosporin resistance, but also because carbapenem resistance can arise in such strains by further mutation, resulting in reduced porin expression.⁹ Whereas, standardised screening and confirmatory methods for ESBL identification are agreed upon (CLSI2010), no such methods for AmpC detection exist.¹⁰ Although AmpC β -lactamases are less prevalent than ESBLs,¹⁰ enterobacteriaceae producing both ESBL and AmpC have been increasing worldwide.¹¹

This study was taken up at this setup to detect occurrence of isolates of enterobacteriaceae producing AmpC β -lactamases, furthermore to device effective treatment guidelines for the same.

Antibiotic Sensitivity Testing^{11,12}

Using the Kirby Bauer disc diffusion method, the diameter of clear zone around the disc was measured under transmitted light and results interpreted as susceptible, intermediate or resistant as per CLSI 2012 guidelines.

Enterobacteriaceae isolates were tested against following antibiotics - Ceftriaxone (30 μ g), cefotaxime (30 μ g), ceftazidime (30 μ g), ofloxacin (30 μ g), amoxicillin-clavulanic acid (20/10 μ g), imipenem (10 μ g), nitrofurantoin (300 μ g), norfloxacin (10 μ g), cefotaxime (30 μ g), piperacillin+tazobactam (100/10 μ g). All antibiotic discs were obtained from HiMedia Laboratories Pvt. Limited, Mumbai, India.

Quality Control

For routine antibiotic susceptibility test for gram negative bacilli, *Escherichia coli* ATCC 25922 was used.

AMPC Beta-Lactamase Screening Test

The isolates of Enterobacteriaceae from clinical samples were screened for AmpC β -lactamase enzyme using standard Kirby-Bauer disc diffusion method.^{CLSI2012} Cefoxitin 30 μ g

MATERIALS AND METHODS

The present study was carried out in the Department of Microbiology, S. Nijalingappa Medical College, Bagalkot. Clinical isolates of Enterobacteriaceae from patients admitted to H. S. Kumareshwar Hospital and Research Centre, Bagalkot, during a period from January 2013 to December 2013 were included for the study.

Inclusion Criteria

Gram-negative organisms belonging to the family of Enterobacteriaceae were isolated from various clinical materials like Sputum, Urine, Blood, Exudates/Pus, Ascitic fluid, Cerebrospinal Fluid (CSF) from patients admitted to the hospital.

(HiMedia Labs Pvt. Ltd., Mumbai) was used as an indicator disc and zone of inhibition of < 18 mm was considered as screen positive isolates for AmpC β -lactamase.^{13,14,15,16}

AMPC β -Lactamase Phenotypic Confirmatory Tests

The isolates which were screen test positive were further subjected to three phenotypic confirmatory tests for detecting AmpC β -lactamases.

1. **Three Dimensional Extraction Test (TDET)** - Test organism was suspended in peptone water and incubated at 37°C for about 4 hours. The turbidity was adjusted to 0.5 McFarland. This suspension was then concentrated by centrifugation. The pellet was then subjected to repeated cycles of freeze-thawing. Around 5 rounds gave satisfactory results. This enzyme extraction about 20 μ L was then dispensed in slits, which were cut on a Mueller-Hinton agar plate. It was then lawn cultured with *E. coli* ATCC25922 and cefoxitin 30 μ g (HiMedia Labs, Mumbai) placed at the centre. Slits were cut around 5 mm away from cefoxitin disc outwards.

Overflowing of the slit is avoided. This media is incubated at 37°C overnight.

Enhanced growth of surface organism at the point where slit and zone of inhibition of cefoxitin disc intersected was considered a positive TDET result.^{4,14,16,17,18}

2. AmpC Disc Test (ACD) - A lawn culture of *E. coli* ATCC 25922 was prepared on Mueller-Hinton agar plate. Sterile discs (6 mm) of Whatman's filter paper no. 1 were prepared and moistened with sterile saline. Several colonies of test organisms were incubated on this disc. This disc was placed adjacent to cefoxitin (30 µg) (HiMedia Labs, Mumbai), almost touching it and the plate was then incubated overnight.

A positive test appeared as flattening or indentation of cefoxitin inhibition zone in the vicinity of the test disc.^{19,20}

3. Inhibitor based Method using Phenylboronic Acid (PBA) - In this method, discs containing phenylboronic acid (400 µg) were prepared. Cefotetan 30 µg (HiMedia Labs, Mumbai) was used, onto which 400 µg phenylboronic acid (Sigma - Aldrich) solution was added. To prepare this strength of solution, 240 mg of phenylboronic acid was dissolved in 3 mL distilled water + 3 mL Dimethyl sulfoxide; 10 µL of this solution was dispensed on cefotetan disc. Discs were allowed to dry for 30 mins. and used immediately or stored in airtight vials at -70°C. MHA was inoculated with test organism (Lawn culture), Cefotetan and Cefotetan + phenylboronic acid discs were placed on it. Plated were incubated at 37°C overnight.

If zone of inhibition around the cefotetan + phenylboronic acid disc was 5 mm or more than zone of inhibition around cefotetan disc alone, the organism was considered as AmpC producer.^{10,16,17}

Quality Control

Negative control - *E. coli* ATCC 25922.

Positive control - No positive control as standard strains for AmpC was available.

RESULTS

Sex	Number	%
Female	48	36.9
Male	82	63.1
Total	130	100

Sex Wise Distribution of Isolates of Enterobacteriaceae

Ward Wise Distribution of Isolates of Enterobacteriaceae

Sample Wise Distribution of Isolates of Enterobacteriaceae

Distribution of Enterobacteriaceae Isolates

Sample Wise Distribution of AmpC Producers

Cefoxitin Screen Test Positive Isolates among all the Isolates of Enterobacteriaceae

All the isolates of Enterobacteriaceae were subjected to Cefoxitin screen test, of which 30 (23.1%) were screen test positive and were considered potential AmpC producers and were further subjected to phenotypic confirmatory test.

Results of Cefoxitin screen test for presumptive detection of AmpC producers

AmpC Screen Test Positive Organisms among Isolates of Enterobacteriaceae

Of the total (30) screen positive isolates only 23.9% *K. pneumoniae*, 26.1% *E. coli* and 35% *C. freundii* were found to be potential AmpC producers.

Distribution of AmpC Screen test positive organisms among isolates of Enterobacteriaceae

Performance of Phenotypic Confirmatory Tests Performed on the Screen Test Positive Isolates

Performance of phenotypic confirmatory methods

Organism Wise Performance of Phenotypic Confirmatory Tests

Proportion of AmpC β -Lactamases

AmpC producers among 30 screen test positive isolates

AmpC producers among 130 TOTAL Enterobacteriaceae isolates

AmpC Screen Test

In our study of the total isolates of Enterobacteriaceae (130) 30 (23.1%) were screen test positive, which were further subjected to phenotypic confirmatory methods following which 22 (77.33%) showed AmpC production.

However, resistance to cefoxitin does not imply potential AmpC.

Organismwise distribution of 22 ISOLATES OF AmpC producers

Organism Wise Distribution of AmpC Producers

Antibiotic sensitivity pattern of isolates of Enterobacteriaceae

Antibiogram of the Isolates of Enterobacteriaceae Isolated

Antibiotic sensitivity for urinary isolates with specific drugs apart from regular antibiotics

Antibiotic sensitivity pattern of AmpC producers

Antibiogram of the AmpC Producing Isolates

Figure 1. Cefoxitin screen test

Figure 4. AmpC Disk test

Figure 2. Three dimensional extract test (Positive)

Figure 5. Phenylboronic acid test

Figure 3. Three dimensional extraction test (Negative)

Figure 6. Antibiotic sensitivity testing

DISCUSSION

The present study was carried out in the Department of Microbiology, S. Nijalingappa Medical College, Bagalkot (Karnataka) during period from January 2013 to December 2013.

In this study, isolates of Enterobacteriaceae from clinical samples coming to the Microbiology Laboratory constituted the study material.

This study showed the prevalence of AmpC producers in isolates of Enterobacteriaceae is 16.9%. Comparison of % AmpC productions in various studies -

Coudron PE ⁵ et al	2000	1.2%
Ratna K ²¹ et al	2003	3.31%
Rodrigues ¹³ et al	2004	7.0%
Neelam ²² et al	2008	25.8%
Sinha P ²³ et al	2008	24%
Present Study	2013	16.9%

Sample Wise Distribution of AmpC Isolates	
Present Study	Pus (18.18%) Urine (27.27%) Blood (18.18%) Sputum (9.9%)
Ingram P R ¹⁰ et al	Urine (76%) Blood (8%) Wound swabs (6%) Sputum (4%)
Ratna K ²² et al	Urine (63%) Pus (25%) Sputum (18.7%)
Fem N ²⁵ et al	Urine (63%) Pus (17%) Sputum (12%)
Subha A ⁷ et al	Sputum (42.8%) Urine (28.6%) Blood (28.6%)

Present Study	2013	Escherichia coli 36.36% K. pneumoniae 36.36% C. freundii 27.27%
Subha A ⁷ et al	2001	Klebsiella pneumoniae - 24.1%, Escherichia coli - 37.5%
Ratna K ²¹ et al	2003	Citrobacter freundii - 5%, E. coli - 3.3%.
Singhal ¹⁹ et al	2004	Acinetobacter - 28.57%, Escherichia coli - 6.97%, Klebsiella spp. - 6.18%
Marasolkhe ²⁴ et al	2007	Escherichia coli - 4.44% Klebsiella pneumoniae - 5.19%
Fam N ²⁵ et al	2008	Escherichia coli - 52% Klebsiella spp. - 38%
Distribution of AmpC in Different Species of Enterobacteriaceae		

Comparing with all these, our study shows similarity in species distribution production. A study by Ingram PR¹⁰ et al in 2006 showed that for cefoxitin screen test, sensitivity was 92%, but specificity was 70%.

However, a study by Subha A⁷ in 2001, it was found that of the total 104 isolates which were cefoxitin resistant 38.46% showed AmpC production and moreover all cefoxitin resistant isolates from stool were non-AmpC producers.

Maraskolhe²⁴ et al in 2007 found that all cefoxitin resistant strains included in study showed AmpC production (100%). However, of the 30 cefoxitin sensitive strains, only 5 (16.67%) showed AmpC production.

	TDET	PBA	ACD
Vandana KE and Honnvar P ⁹	75% of isolates (Escherichia coli (70.7%) and Klebsiella pneumonia (91%))	63.5% of isolates	
Coudron P E ⁵	TDET 80% of Escherichia coli, Klebsiella pneumoniae (13.1%)	42.4% were detected by PBA test of which PBA test picked up 65% E. coli and Klebsiella pneumonia (9.8%)	
Singhal ¹⁹ et al	Detected 36.06% of AmpC producers Escherichia coli and Klebsiella pneumoniae (39.13%)		Same as TDET
Maraskolhe ²⁴ et al	TDET detected 100% cefoxitin resistant AmpC beta-lactamases. Modified TDET also detected 16.67% isolates, which were cefoxitin sensitive and missed by PBA test	The PBA test detected 43.7% of AmpC producers of which 30% Escherichia coli and 85.71% Klebsiella pneumoniae	
Subha A ⁷ et al	Klebsiella pneumonia (20.8%) Escherichia coli (37.5%)		
Fam N ²⁵ et al		64.7% sensitivity and 88.4% specificity	76.5% sensitivity and 86% specificity
Ingram A ¹⁰ et al		Less sensitivity (66%), more specific (78%)	
Manoharan A ²⁶ et al		Sensitivity of 72.9% and specificity of 45.4% when compared to PCR	

Manchanda V and Singh N P¹⁵ found that of the total 28 AmpC producers only 17 were cefoxitin resistant, i.e. 60.7%

and of the 102 non-AmpC producers 9 (8%) were cefoxitin resistant.

Hence, cefoxitin screen test cannot be totally designated as a specific screening test for AmpC detection, as it may be mediated by certain class A β -lactamases, carbapenemases and decreased production of outer membrane porins.²⁷

Moreover, in our study only cefoxitin-resistant isolates were further processed, so there are chances that AmpC producers which are cefoxitin sensitive have been missed upon.

Phenotypic Confirmatory Methods

In our study, all the screen test positive isolates were subjected to three phenotypic confirmatory tests. The TDE test (TDET) detected 26.6%, AmpC disc test (ACD) detected 36.6% and Phenylboronic acid test detected 43.3% AmpC producers. *Klebsiella pneumoniae* were mostly picked up by Phenylboronic acid test (30.7%), *Escherichia coli* by TDE test (50%) and *Citrobacter freundii* by AmpC disc test (36.4%).

Hence, on an average Phenylboronic Acid (PBA) test performed better followed by ACD test and TDET in detecting AmpC producers.

PCR is considered as gold standard for AmpC detection.¹⁷ In our study, the phenotypic test results were not compared to PCR, this being a major limitation of our study.

In all of the above mentioned, TDET gave better results followed by PBA and ACD test in contrast to our study which showed PBA performed better followed by ACD test and least performance by TDET.

However, PBA test and AmpC disc test are less cumbersome and easy to perform as compared to TDET. So, laboratories can take up these methods for AmpC detection even if their performance is less as compared to TDET as in other studies.

Antibiotic Sensitivity Pattern

Present Study	Sensitivity- Imipenem (90.9%), PIT (77.2%) and ofloxacin (72.7%). Urinary isolates, Nitrofurantoin showed 100% sensitivity followed by norfloxacin 83.3%. Resistance- Ceftriaxone-77.2%, Cefotaxime-68.2%, Cefuroxime-60%, Ceftazidime-50%
Manoharan A ²⁶ et al	Sensitivity - Imipenem (98%), Amikacin (83.3%) and PIT (79.2%)
Fam N ²⁵ et al	Resistant Amoxicillin-Clavulanic Acid (AMC) Sensitivity - PIT (59%) Fluoroquinolones (CIP) 94.1% All susceptible to Imipenem
Subha A ⁷	(77.6%) showed resistance to at least one 3 rd generation cephalosporin and all strains sensitive to imipenem
Patel M ²⁸ et al	100% sensitivity to imipenem and 18.29% resistance to cefepime, and least to cefuroxime and ceftriaxone
Ratna A K ²¹ et al	100% sensitivity to imipenem and a good amount of sensitivity to 4 th generation cephalosporin-cefepime

As compared to above studies, our study shows similarities with respect to very low amount of resistance to imipenem, PIT and nitrofurantoin, moderate amount of resistance to fluoroquinolones and high resistance to 3rd generation cephalosporins.

Many studies showed 100% susceptibility to imipenem; however, our study showed few resistant isolates. This may be due to additional carbapenemase resistance mechanism or due to porin deficiency.^{17,29,30}

Fluoroquinolones can be used for non-life-threatening cases. Cefepime is poor inducer of AmpC and many AmpC organisms show susceptibility to cefepime,^{17,31,32} so it can be used. However, Imipenem remains drug of choice for life-threatening conditions.

Another issue remains is the co-production of ESBL and AmpC. High level of AmpC expression prevents recognition of ESBL, which is a more serious concern now-a-days. CLSI guidelines are present for ESBL detection^{CLSI2012}, but no such guidelines exist for AmpC detection. Antibiotic resistance is high among ESBL, AmpC and co-producers. This is due to the fact that plasmids carrying these enzymes may carry co-resistance genes for other antibiotics. If one fails in detecting ESBL and AmpC, a risk of emergence of ESAC arises.³³

Laboratory phenotypic methods cannot distinguish between plasmid-mediated and chromosomal-mediated enzymes of *Escherichia coli* but as a guideline, lack of multidrug resistance is suggestive of chromosomal-mediated AmpC, whereas multidrug resistance may be due to plasmid mediated/chromosomal-mediated AmpC production. However, in other organisms like *Klebsiella* spp. and *Citrobacter* spp., these methods are confirmatory of plasmid-mediated AmpC production.¹⁷

Plasmid-mediated AmpC beta-lactamases have now arisen through transfer of chromosomal genes onto plasmids³⁴ and these become highly mobile.³⁵ Plasmid-mediated genes can be transferred horizontally due to antibiotic pressure, whereas chromosomal-mediated can be transferred vertically from generation to generation. The original plasmid-mediated and the ones transferred from chromosomes show similar substrate profiles from which they appear, except that plasmid-mediated differ from chromosomal in being non-inducible.³⁴ PCR remains method of choice for detection of both plasmid-mediated and chromosomal-mediated genes.

Overexpression of chromosomal genes confers resistance to amino-penicillins and early generation cephalosporins and reduced susceptibility to 3rd generation cephalosporins³⁶; 4th generation cephalosporins and carbapenems are poor substrates.³⁷

However, if proper detection and differentiation of ESBL, AmpC, co-producers is not done, ESAC beta-lactamases arises which show additional resistance to carbapenems and 4th generation cephalosporins. This will lead to major therapeutic treatment failure and also outbreaks of multi-drug resistance. Our study however had some limitations -

1. Cefoxitin susceptible isolates, which may have AmpC enzymes are missed.
2. PCR was not done for comparison of methods.
3. It is however advisable to find out co-production of ESBL and AmpC, which was not included in this study.
4. Inducible AmpC using imipenem disc³⁸ not detected.

In spite of these limitations this is the first study of this study; is a small effort to find the prevalence of AmpC beta-

lactamases and their antibiotic sensitivity pattern. The proportion was less as compared to other parts of South India, because this Institute being in a semi-urban area, use of higher antibiotics is not at par with those as in metropolitan cities.

The study will help in detecting prevalence of AmpC and create awareness about AmpC among clinicians. The awareness of ESBL is well known; however, it is not same with AmpC. This study will help to create awareness of AmpC type of resistance. Treatment with 3rd generation cephalosporins and with inhibitors may be the reason for appearance of AmpC type of resistance. Hence, this study will help in sensitising all the clinicians regarding the prevalence of AmpC beta-lactamases. ESBL detection is being done as standard guidelines³⁹ are laid down; however, if ESBL is associated with AmpC production, AmpC enzymes will hamper the detection of ESBL. Inhibitor discs, i.e. Clavulanic acid which are used for detection of ESBL tend to induce AmpC production, which in turn will attack the indicator cephalosporin and mask ESBL detection.⁸ This will however lead to treatment failures.

Therefore, appropriate laboratory methods for detection of AmpC, ESBL + AmpC co-producers using combination of inhibitor discs should be routinely done.^{38,40}

Our study showed that PBA performed better followed by ACD test and TDET. PBA and ACD test are less cumbersome. So, these can be used for AmpC detection routinely along with ESBL detection.

Also Imipenem, PIT, Nitrofurantoin, Cefepime, Ofloxacin have shown acceptable susceptibility for AmpC isolates, which can be used depending upon the need for treatment.

CONCLUSION

Enterobacteriaceae have been one of the major chunk of organisms causing a wide array of infections. AmpC β -lactamase producing Enterobacteriaceae have added to this chunk. As per our study, AmpC β -lactamases, though showing low prevalence, harbours in a variety of organisms like *E. coli*, *K. pneumoniae* and *C. freundii* isolated from patients.

Routinely, no screening or phenotypic tests are done for AmpC β -lactamases at this setup. Also, antibiotic sensitivity pattern of these isolates stands important for treatment formulation. Moreover, no standard guidelines for AmpC β -lactamase detection are laid and also PCR though being considered as a gold standard, cannot be applied everywhere because of its high cost. Taking into consideration all this and the outcome of our study, routinely screening and phenotypic confirmation of AmpC β -lactamase with PBA test or AmpC disc test should be done. They are easy to perform and less cumbersome. It is advisable to look into the antibiotic sensitivity pattern and thereby followed to avoid treatment failures and further amplification of this type of resistance pattern.

For overcoming the limitations of the study, further studies should be carried out using molecular methods using specific primers⁴¹ - bla_{DHA}, bla_{ACC}, bla_{MOX}, bla_{CIT}, bla_{FOX}, bla_{EBC} to detect exact prevalence of AmpC β -lactamases and also their antibiotic sensitivity pattern for treatment formulation. Also, molecular methods should be developed for proper detection. Lastly, this protocol should be devised at par with the higher centres, where routinely these procedures are carried out.

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