International Journal of Life Sciences, Biotechnology and Pharma Research Vol. 14, No. 2, February 2025 Online ISSN: 2250-3137
Print ISSN: 2977-0122

DOI: 10.69605/ijlbpr_14.2.2025.164

ORIGINAL RESEARCH

Comparison of confirmatory phenotypic methods for amp c beta lactamases detection among clinical isolates of enterobacterales from a tertiary care hospital

[1]Dr. R Nagalakshmi, [2]Dr. L Gracia Paul, [3]Dr. J B Akbar Saleem

[1]Assistant Professor, [2]Tutor, [3]2nd Year Post graduate, Department of Microbiology, Tirunelveli Medical College, Tirunelveli, India

Corresponding Author
Dr. R Nagalakshmi
Assistant Professor, Department of Microbiology, Tirunelveli Medical College, Tirunelveli, India

Received: 28 December, 2024 Accepted: 24 February, 2025 Published: 28 February, 2025

ABSTRACT

Introduction: Enterobacterales have become increasingly resistant to antibiotics, especially beta (β)-lactam agents, the mainstay of treatment for infections caused by them. One among the major mechanisms causing resistance to β-lactam antibiotics are the AmpC β-lactamase production. Amp C b-lactamases are clinically significant because they confer resistance to penicillins, cephalosporins, oxyimino-cephalosporins. Detection of AmpC is important not only to improve the clinical management of patients suffering from infections but it would also provide us with sound epidemiological data. Hence, the present study aims to determine the prevalence of AmpC β- lactamase producing Enterobacterales among clinical isolates isolated in our hospital and compare the various phenotypic confirmatory methods for AmpC detection. **Methodology:** Screening For Ampc B- Lactamase Production was done using Cefoxitin (30-µg) disc was used for cefoxitin disc diffusion technique. Phenotypic Confirmatiory Methods for Ampc B- Lactamase Detection: was done using 1.Phenyl boronic acid method-PBA (Inhibitor based method) 2. Cefoxitin Cloxacillin-Double disc synergy test (CC-DDS). 3. Amp C TRIS EDTA disc test.4. Disk approximation test (Induction based method): **Results:** 90 (52%) of the 173 examined isolates demonstrated resistance to the initial cefoxitin screening test. Of these, 19 (21.12%) were classified as Klebsiella pneumoniae and 71 (78.88%) as Escherichia coli. Cefoxitin Cloxacillin Double Disc Synergy (CC-DDS) test, revealed a zone difference of more than 4mm in 46 (51.11%) of the isolates. 39 (43.33%) of the isolates tested positive for Amp C synthesis using the Disc Approximation Test. 37 (41.11%) of the isolates exhibited an indentation close to the EDTA disc, according to the TRIS EDTA technique. Finally the Phenylboronic Acid technique revealed a zone difference of greater than 5mm in 38 (42.22%) of the isolates. **Conclusion:** The observation that the Cefoxitin Cloxacillin Double Disc Synergy (CC-DDS) method exhibited a higher detection rate when compared to other phenotypic confirmatory methods in our study is of particular significance. It is advised that the CC-DDS approach be taken into consideration for routine Amp C detection when necessary in light of these findings. Its user-friendliness is one of its noteworthy benefits, which makes it a viable and affordable choice for labs and medical facilities where AmpC detection is required.

Keywords: Enterobacterales, AmpC beta lactamases, phenotype

INTRODUCTION

Enterobacterales have become increasingly resistant to antibiotics, especially beta (β)-lactam agents, the mainstay of treatment for infections caused by them. One among the major mechanisms causing resistance to β-lactam antibiotics are the AmpC β-lactamase production. AmpC β -lactamases confer resistance to penicillins, cephalosporins, oxyimino-cephalosporins, cephamycins (cefoxitin and cefotetan), and monobactams and their activity is not affected by the ESBL inhibitor clavulanic acid [(1)].

The prevalence of AmpC producing isolates reported in various Indian studies ranges from 8% to 47% [(2)(3)(4)]. Even though many phenotypic methods are available, the Clinical and Laboratory Standards Institute (CLSI) guidelines recommended criteria for AmpC resistance detection do not exist. In our country where most of the infections are empirically

DOI: 10.69605/ijlbpr_14.2.2025.164

treated with third-generation cephalosporins, failure to detect AmpC β- lactamase-related resistance may lead to treatment failure [5]. Therefore, screening and confirming the AmpC producing Enterobacterales isolates is crucial for effective treatment, prevention and control of these resistant bacteria.

Amp C, the first bacterial enzyme found to break down penicillin was beta lactamase, which was found in Escherichia coli. Beta lactamases with progressively increased resistance as a result of mutations were referred to as amp A and amp B. Amp C is a mutation in an amp A strain that results in decreased resistance. Although Amp C expression is low in many Enterobacteriaceae, it can be increased in response to exposure to beta lactam antibiotics. The process of induction is extremely intricate. Mutations in amp D that result in constitutive hyperproduction or Amp C hyperinducibility are the most frequent cause of Amp C overexpression in the majority of clinical isolates.

Amp C b-lactamases are clinically significant because they confer resistance to penicillins, cephalosporins, oxyimino-cephalosporins (e.g., ceftriaxone, cefotaxime, and ceftazidime), cephamycins (e.g., cefoxitin and cefotetan) and monobactams. Amp C b-lactamase activity is not affected by the beta lactamase inhibitor like clavulanic acid.

Amp C b-lactamase in Gram-negative bacteria can be either chromosomal or plasmid mediated. Low levels of constitutive expression are seen for chromosomal Amp C genes. An inducible Amp C gene is present in a small number of Enterobacteriaceae, including Enterobacter species, Citrobacter species, and Serratia species. Amp C genes based on plasmids are often constitutively expressed. Since plasmids transfer both Amp C and ESBL enzymes inside the same plasmid, all plasmid-carried Amp C genes have therapeutic value. Due to challenges with laboratory testing procedures, the actual incidence of plasmid-mediated Amp C b-lactamases in Klebsiella pneumoniae, E. coli, and Proteus mirabilis is unknown, despite their growing frequency in case isolates.

Additionally, there are recognized criteria for ESBL detection but none for Amp C detection, according to the Clinical Laboratory Standards Institute (CLSI). Usually, organisms that make Amp C b-lactamase will test positive for ESBL screening, although there is no evidence of enhanced sensitivity to clavulanic acid, and cloxacillin and phenyl boronic acid block Amp C manufacturers. 7. In organisms that produce an inducible chromosomal Amp C β-lactamase, such as 100% isolates of Enterobacter cloacae, Enterobacter aerogenes, and Citrobacter freundii, testing is not thought to be necessary. However, since these organisms typically lack chromosomal Amp C β-lactamases, the detection of an Amp C β-lactamase in Escherichia coli, Klebsiella sp., and Citrobacter koseri is confirmatory for plasmid-mediated Amp C production.

Detection of AmpC is important not only to improve the clinical management of patients suffering from infections but it would also provide us with sound epidemiological data. Hence, the present study aims to determine the prevalence of AmpC β- lactamase producing Enterobacterales among clinical isolates isolated in our hospital and compare the various phenotypic confirmatory methods for AmpC detection.

METHODOLOGY

This study was done as a Cross sectional study for 6 months at department of Microbiology, Tirunelveli Medical College, Tirunelveli. All isolates of Enterobacterales obtained during the study duration irrespective of the specimen from which it is isolated, were included in this study. Gram negative isolates other than Enterobacterales and all gram-positive bacteria were excluded from the study. Ethical clearance was obtained from the Institutional Ethical committee.

Isolates were initially identified using the biochemical processes. Phenotypic confirmation tests on 90 AmpC b-lactamase from a total of 173 Enterobacteriaceae clinical isolates were carried out since they were positive by Cefoxitin screening. After screening with cefoxitin disc, isolates were subjected to the phenyl boronic acid method (PBA), Cefoxitin Cloxacillin-Double disc synergy test (CC-DDS), TRIS EDTA method, and Disc approximation test for Amp C confirmation.

Screening For Ampc B- Lactamase Production was done using Cefoxitin (30-µg) disc was used for cefoxitin disc diffusion technique. AmpC producers were isolated with zone widths of less than 18 mm. They were subsequently confirmed using a variety of phenotypic techniques.

Phenotypic Confirmatiory Methods for Ampc B-Lactamase Detection: was done using

Phenyl boronic acid method-PBA (Inhibitor based method)

To 3ml of distilled water, 120 µg of phenyl boronic acid was added together with 3ml of dimethyl sulfoxide (DMSO). Then, 20µL of this liquid were applied to each cefoxitin (CX) disc. Cefoxitin (30 µg) disc was placed on the agar at a distance of 30 mm from another Cefoxitin (30 µg) disc with PBA. Inoculated plates were kept at 37°C overnight. An AmpC producer was defined as an organism with a zone difference of greater than 5 mm [8],[12]

Cefoxitin Cloxacillin-Double disc synergy test (CC-DDS)

This test was relied on cloxacillin's ability to inhibit AmpC production. The isolates were inoculated on Mueller Hinton agar. Both cefoxitin discs (30 µg) and cefoxitin/cloxacillin discs (200 µg/30 µg) were placed. A 4 mm zone difference between the two discs was a sign that AmpC was being produced [9][12].

DOI: 10.69605/ijlbpr_14.2.2025.164

Amp C TRIS EDTA disc test

The test depends on the release of b-lactamases into its external environment after permeabilizing a bacterial cell with Tris-EDTA. In order to prepare AmpC discs, sterile filter paper discs were coated with 20 ml of a 1:1 solution of saline and Tris-EDTA, dried, and then refrigerated. Amp C discs were rehydrated with 20 ml of saline before use, and several colonies of each test organism were then applied to a disc. At the inoculated surface of the Mueller-Hinton agar containing the ATCC E coli strain, a 30 µg cefoxitin disc was applied. Then, with the inoculated disc face in contact with the agar surface, the inoculated Amp C disc was positioned in proximity to the antibiotic disc [(6)(7)]. The plate was then turned over and incubated for the next day at 35°C. Any indentation or flattening of inhibition zone showing enzyme inactivation of cefoxitin is considered as positive [(8)(10)].

Disk approximation test (Induction based method)

A 0.5 McFarland bacterial suspension was made, and it was inoculated onto the MHA plate's surface. A Ceftazidime (30µg) disc was placed in the middle of the plate, followed by discs containing imipenem (10 µg), cefoxitin (30 µg), and amoxicillin/clavulanate (20/10 µg), all of which were placed 20 mm apart from ceftazidime. The plate was incubated at 37°C inverted for the entire night. After overnight incubation, any visible blunting or flattening of the zone of inhibition between the ceftazidime disc and the inducing substrates (imipenem, cefoxitin, and amoxicillin/clavulanate disc) was considered as AmpC production [(11)(12)].

RESULT

As indicated in Table 1, 90 (52%) of the 173 examined isolates demonstrated resistance to the initial cefoxitin screening test. Of these, 19 (21.12%) were classified as Klebsiella pneumoniae and 71 (78.88%) as Escherichia coli, as shown in Table 2.

Table 1: Screening Test (Total Isolates: 173)		
n=173	Sensitive to Cefoxitin (CX)	Resistant to Cefoxitin (CX)
	83	90

Table 2: Distribution of isolates (Amp C – Positive) among screening	
Escherichia coli	71 (78.88%)
Klebsiella pneumoniae	19 (21.12%)

As shown in Figure 1, phenotypic confirmation techniques, such as the Cefoxitin Cloxacillin Double Disc Synergy (CC-DDS) test, revealed a zone difference of more than 4mm in 46 (51.11%) of the isolates. As shown in Figure 2, 39 (43.33%) of the isolates tested positive for Amp C synthesis using the Disc Approximation Test. As illustrated in Figure 3, 37 (41.11%) of the isolates exhibited an indentation close to the EDTA disc, according to the TRIS EDTA technique. Finally, as shown in Figure 4, the Phenylboronic Acid technique revealed a zone difference of greater than 5mm in 38 (42.22%) of the isolates.

Table 3: Phenotypic methods	
Methods	n=90 (Confirmatory tests positive)
CC-DDS(Cloxacillin – Double Disc Synergy test)	46 (51.11%)
Phenyl Boronic Acid Method	38 (42.22%)
Disc Approximation Test	39 (43.33%)
TRIS EDTA Method	37 (41.11%)

Escherichia coli was the most common isolate exhibiting Amp C synthesis across all four techniques, followed by Klebsiella pneumoniae, according to the data shown in Table 4.

Table 4: Distribution of organisms based on various phenotypic methods			
		Escherichia coli	**Klebsiella pneumoniae**
Phenyl boronic acid	(n=38)	27 (71.07 %)	11 (28.93 %)
CC- DDS	(n=46)	30 (65.21 %)	16 (34.79 %)
Disc Approximation test	(n=39)	25 (64.10 %)	14 (35.90 %)
TRIS EDTA method	(n=37)	27 (72.97 %)	10 (27.03 %)

DOI: 10.69605/ijlbpr_14.2.2025.164

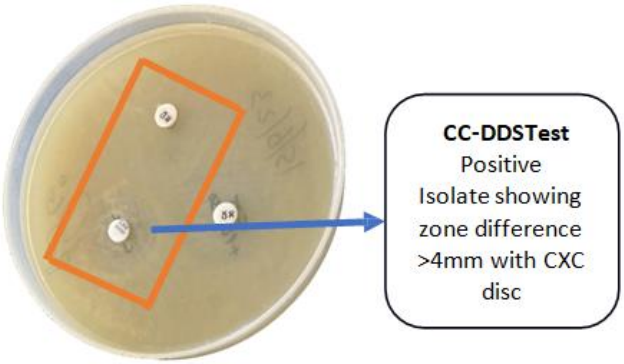

Figure 1. Cefoxitin Cloxacillin – Double Disc Synergy Test

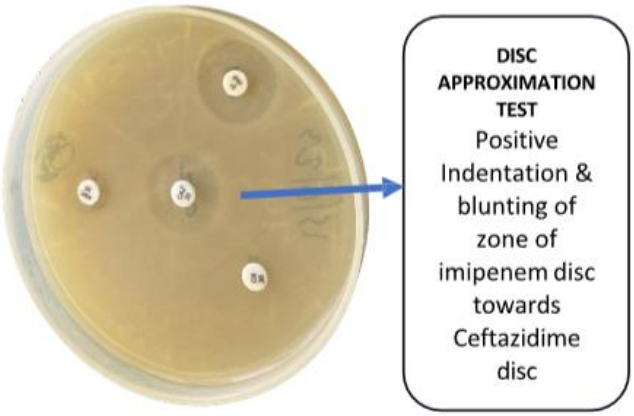

Figure 2. Disc Approximation Test

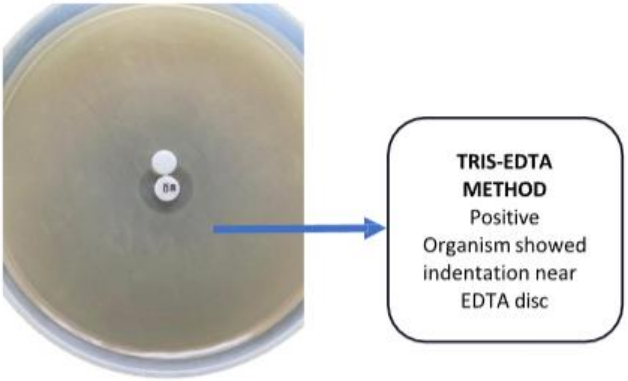

Figure 3. TRIS-EDTA Method

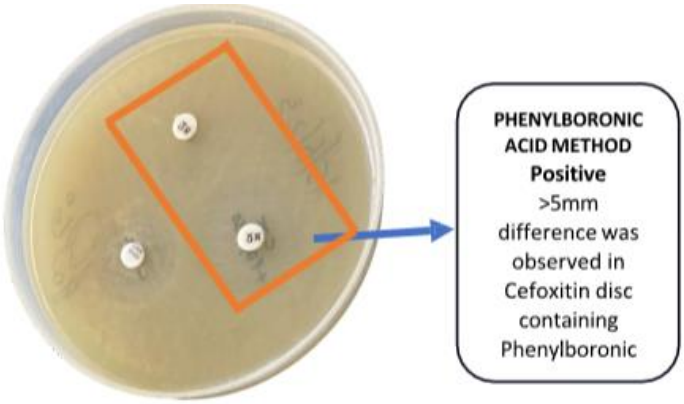

Figure 4. Phenylboronic acid Method

DOI: 10.69605/ijlbpr_14.2.2025.164

DISCUSSION

Ninety (52%) of the 173 isolates tested in our study showed cefoxitin resistance after testing. This conclusion is consistent with a research by Dhanashree et al., (2013), which found that 57% of isolates tested positive throughout the screening process. Notably, Klebsiella pneumoniae was the second most common isolate in our investigation, after Escherichia coli. This pattern can be explained by the fact that urine samples were the source of the bulk of our clinical isolates, followed by pus samples. It's interesting to note that our investigation confirms the 2011 study by Polfuss et al.(14), which likewise found Escherichia coli to be the most common isolate. It's crucial to remember, too, that our findings differ with those of a study by Soha et al. in 2015(11), in which Klebsiella pneumoniae was the most common isolate. These differences demonstrate the range of microbiological patterns that can be found in diverse investigations, which may be impacted by a number of variables such geographic location, patient demographics, and specimen sources.

With 46 (about 51.11%) isolates showing positive, the Cefoxitin Cloxacillin Double Disc Synergy (CC-DDS) test showed the highest level of detection according to the phenotypic confirmatory assays. This result is in line with a study by Polsfuss et al. (2011)(14), which mirrored the results of the current investigation and emphasized the excellent specificity of the cefoxitin-cloxacillin CC-DDS confirmation test in identifying and characterizing AmpC beta-lactamases.

The Phenyl Boronic Acid method, an inhibitor-based detection technique, showed 100% sensitivity and 96% specificity in identifying AmpC manufacturers in a 2013 study by Tanushree Baru (15). Because of its high sensitivity and ease of usage, they consequently suggested this approach. In contrast to the current study's findings, only 38 (or roughly 42.22%) of the isolates in our investigation proved positive using the phenolboronic acid technique.

Similarly, 37 (about 41.11%) isolates with an indentation close to the EDTA disc were found using the TRIS EDTA technique in our investigation. This result is different from that of the 2011 study by Ingram et al. (16), who suggested the TRIS EDTA technique for confirmation, claiming 95% sensitivity and 98% specificity.

Because Escherichia coli was the most common isolate in our samples—a large percentage of which came from urine samples—it was the strain that produced Amp C the most frequently across all of these approaches. These differences in detection techniques and results highlight how crucial it is to take into account a number of variables when evaluating AmpC beta-lactamase synthesis across various investigations.

LIMITATIONS

AmpC identification by phenotypic approach is limited in our investigation since genotypic methods, which are thought to be the gold standard, were not available in our setting and were not economical to use. Furthermore, due to a number of limitations, we were not always able to incorporate the goal of clinical correlation and patient follow-up following AmpC identification. It's critical to recognize that practical constraints, such the availability of resources and financial considerations, frequently impact research investigations. Phenotypic approaches can still yield important information and advance our knowledge of AmpC beta-lactamase synthesis, even though genotypic approaches can have certain advantages in terms of precision and specificity.

CONCLUSION

The observation that the Cefoxitin Cloxacillin Double Disc Synergy (CC-DDS) method exhibited a higher detection rate when compared to other phenotypic confirmatory methods in our study is of particular significance. It is advised that the CC-DDS approach be taken into consideration for routine Amp C detection when necessary in light of these findings. Its user-friendliness is one of its noteworthy benefits, which makes it a viable and affordable choice for labs and medical facilities where AmpC detection is required.

REFERENCES

1. Jacoby, G. A. 2009. AmpC _-lactamases. Clin. Microbiol. Rev. 22:161–182.
2. Mohamudha Parveen R, Harish BN, Parija SC. AmpC Beta lactamases among gram negative clinical isolates from a tertiary hospital, South India. Braz J Microbiol. 2010;41(3):596-602.
3. Sinha P, Sharma R, Rishi S, Sharma R, Sood S, Pathak D. Prevalence of extended spectrum beta lactamase and AmpC beta lactamase producers among Escherichia coli isolates in a tertiary care hospital in Jaipur. Indian J PatholMicrobiol2008;51:367-9.
4. Halstead FD, Vanstone GL, Balakrishnan I. An evaluation of the MAST D69C AmpC Detection Disc Set for the detection of inducible and derepressed AmpC beta-lactamases. J AntimicrobChemother. 2012;67(9):2303–4.
5. Handa D, Pandey A, Asthana AK, Rawat A, Handa S, Thakuria B. Evaluation of phenotypic tests for the detection of AmpC beta-lactamase in clinical isolates of Escherichia coli.Indian J PatholMicrobiol 2013;56(2):135–138.
6. Pal N, Hooja S, Sharma R, Maheshwari RK. Phenotypic detection and antibiogram of β-lactamase-producing Proteus species in a Tertiary Care Hospital, India. Ann Med Health Sci Res 2016;6(5):267– 273.
7. CLSI. Performance standards for Antimicrobial Susceptibility testing. 30th ed. CLSI supplement M100.Wayne, PA: Clinical and Laboratory Standards Institute;2021.
8. Hemalatha V, Padma M, Sekar U, Vinodh TM, Arunkumar AS. Detection of Amp C beta lactamases production in Escherichia coli & Klebsiella by an

DOI: 10.69605/ijlbpr_14.2.2025.164

inhibitor-based method. Indian J Med Res. 2007;126(3):220-223.
9. Mohd Khari FI, Karunakaran R, Rosli R, Tee Tay S. Genotypic and Phenotypic Detection of AmpC β-lactamases in Enterobacter spp. Isolated from a Teaching Hospital in Malaysia. PLoS One. 2016;11(3):e0150643.
10. Black JA, Moland ES, Thomson KS. AmpC Disk Test for Detection of Plasmid-Mediated AmpC -Lactamases in Enterobacteriaceae Lacking Chromosomal AmpC -Lactamases. J Clin Microbiol. 2005;43(7):3110–3.
11. El-Hady Soha A, Adel LA. Occurrence and detection of AmpC b - lactamases among Enterobacteriaceae isolates from patients at Ain Shams University Hospital. Egypt J Med Hum Genet. 2015;16(3):239–44.
12. Inamdar DP, Anuradha B. Phenotypic methods for detection of Amp C β lactamases in gram negative clinical isolates of a tertiary care hospital. Indian J Microbiol Res. 2020;7(2):125–9.
13. Dhanashree P Inamdar, Anuradha B et.al. Phenotypic methods for detection of Amp C b lactamases in Gram negative clinical isolates of a tertiary care hospital. Indian J Microbiol. Res.h 2020;7(2):125–129
14. Polsfuss S, Bloemberg GV, Giger J, Meyer V, ttger ECB, Hombach M. Practical Approach for Reliable Detection of AmpC Beta-Lactamase-Producing Enterobacteriaceae. J Clin Microbiol. 2011;p. 2798–2803.
15. Barua T, Shariff M, Thukral SS. Detection and Characterization of AmpC B-Lactamases in Indian Clinical Isolates of Escherichia coli, Klebsiella pneumoniae and Klebsiella oxytoca. Univ J Microbiol Res. 2013;1(2):15–21.
16. Ingram PR, Inglis TJJ, Vanzetti TR, Henderson BA, Harnett GB, Murray RJ. Comparison of methods for AmpC b -lactamase detection in Enterobacteriaceae. J Med Microbiol. 2011;60(6):715–21.