Research Article

Phenotypic detection and associated factors of AmpC beta-lactamase among clinical isolates of *Escherichia coli*, *Klebsiella* species, and *Proteus* species at the National Hospital of Sri Lanka

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Abstract

Introduction: Bacteria that express AmpC beta-lactamases are important in clinical practice as they are resistant to most beta-lactam antibiotics. This study aimed to determine the rate of positivity of AmpC beta-lactamase-producing *Enterobacterales* isolated from blood and sterile fluids at the National Hospital of Sri Lanka. Further, we compared AmpC phenotypic detection methods and evaluated AmpC and extended-spectrum beta-lactamase (ESBL) co-existence.

Methods: A total of 141 samples of *Escherichia coli, Proteus* species, and *Klebsiella* species were tested for two screening tests and three confirmatory tests. The cefoxitin-cloxacillin double disc was used as the phenotypic reference method. For ESBL detection, the Clinical & Laboratory Standards Institute guidelines (CLSI) were followed.

Results: Out of 141 isolates, 34 (24.1%) were AmpC producers. AmpC test was positive in 26.3% of blood culture isolates (30/114) and 14.8% of other samples (4/27). AmpC-ESBL co-existence was observed in 20.6% (29/141) of total isolates.

All 34 AmpC-positive isolates were cefoxitin screening positive, while 31 were positive for cefotetan screening. Both screening methods produced five false positives. Among the 34 confirmed AmpC producers, all were positive with the cefoxitin–boronic acid test, 31 with the cefotetan–boronic acid test, and 31 for the EDTA test, with respective false positives of one, one, and none.

Conclusion: A relatively high prevalence of AmpC beta-lactamase production was observed in this clinical setting. AmpC and ESBL co-existed in approximately one-fifth of total isolates. Cefoxitin is a superior screening method, while the EDTA test demonstrated good confirmatory performance in addition to the cefoxitin-cloxacillin double disc test.

Keywords: *AmpC beta-lactamases, prevalence, Escherichia coli, Proteus*, *Klebsiella, Sri Lanka*

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Introduction

The emergence of antibiotic resistance is a serious problem and challenging when managing bacterial infections.

Bacteria that express AmpC beta-lactamase enzymes are considered important in clinical practice as they are resistant to most beta-lactam antibiotics, including penicillins, first-to third-generation cephalosporins, and beta-lactam/beta-lactamase inhibitor combinations. Beta-lactam inhibitors such as clavulanic acid do not inhibit these organisms, which express AmpC genes on chromosomes or plasmids.[1]

Currently, the Clinical and Laboratory Standards Institute (CLSI) does not have an approved method for detection of AmpC beta-lactamase. Various methods have been described in the literature, and genetic detection is considered the 'gold standard'. Although the CLSI screening test for detection of extended-spectrum beta-lactamases (ESBL) may be positive in bacteria that produce only AmpC, the confirmatory tests are usually negative.[2]

Knowledge of the occurrence of ESBL and AmpC beta-lactamase-producing organisms in a healthcare setting can help guide formulation of antibiotic policies and the effective implementation of infection control measures. Genotypic methods for AmpC detection may remain as the gold standard but are often not feasible in routine settings due to cost and resource constraints. Comparison of available phenotypic detection methods is important to select a low-cost, feasible, and reliable method for laboratories with limited resources.

We studied the positivity rate of AmpC beta-lactamase production in *Enterobacterales* isolates at the National Hospital of Sri Lanka. We also determined the factors associated with AmpC-positive bacterial infections, assessed the co-existence of AmpC and ESBL production among *Enterobacterales* isolates, and compared the phenotypic methods used for AmpC detection.

Methods

A descriptive cross-sectional study was carried out over a period of three months. The sample size was calculated using Cochran's sample size formula with a prevalence of 14.8 % adopted from an Indian study, as no local studies were available at the time the study was planned.[3]

Escherichia coli, Klebsiella spp., and *Proteus* spp., isolated from blood, sterile fluids, and pus samples and known to have plasmid-mediated AmpC production were included in the study. Species with known chromosomal AmpC production such as *Enterobacter cloacae*, *Citrobacter freundii* and *Serratia marcescens* were excluded.[1,4] Samples were collected from the National Hospital of Sri Lanka, which is the major tertiary care hospital in Sri Lanka which has a large bed capacity (approximately 3500 beds) and 18 intensive care units (ICUs), and caters to patients from all over the country.

Identification to species level was carried out using the Remel Rapid Identification® – ONE system (USA).

For AmpC production, all isolates were tested using two screening tests and three confirmatory methods. The cefoxitin-cloxacillin double-disc test was used as the reference method because it has been reported to have the highest specificity and sensitivity among confirmatory methods for AmpC phenotypic detection.[5]

All screening and confirmatory tests were quality controlled using a known AmpC beta-lactamase positive control (*Enterobacter cloacae* subsp. *cloacae* ATCC BAA-1143) and negative control (*E.coli* ATCC 25922) in accordance with the 'Rosco Diagnostica', ESBL and AmpC screening recommendations (2011) and Jacoby (2009).[2] The Kirby-Bauer disk diffusion method was performed according to the CLSI guidelines.[6] Thermo Scientific Oxoid antimicrobial discs and media bases from Himedia Laboratories were used for laboratory testing.

Phenotypic methods for detection of AmpC

Screening tests

All isolates were screened separately using cefoxitin (30 µg) and cefotetan (30 µg) discs. Organisms resistant to either antibiotic were considered screening-positive for AmpC beta-lactamase production.[6]

Confirmatory tests

Cefoxitin-cloxacillin double-disc synergistic test

Cefoxitin discs (30 µg) and cefoxitin-cloxacillin discs (30 µg cefoxitin + 200 µg cloxacillin) were placed on a lawn of the test organism.

A positive test was defined as a difference of ≥4 mm between the zone diameters of the two paired discs. This method is based on the inhibitory effect of cloxacillin on AmpC.[7,8] The zone size of cefoxitin increases when it is combined with cloxacillin in the presence of AmpC due to the inhibitory effect.

Cefoxitin + boronic acid disc test / Cefotetan + boronic acid disc test

Phenylboronic acid (120 mg, SRL – Sisco Research Laboratories, catalogue code - 92890) was dissolved in 3 mL of dimethylsulphoxide (Deajung-Korea, catalogue code – 3047 - 4105) and diluted 1:1 with 3 mL sterile distilled water to make the stock solution. From this solution, 20µL was added to each cefoxitin 30 µg and cefotetan 30 µg disc. Each disc contained 400 µg of boronic acid. The discs were air-dried and stored in an airtight container at 4 °C.[7,9]

The organisms were tested using cefoxitin and cefotetan discs, each paired with a corresponding boronic acid disc. An increase in zone diameter of 5mm in the presence of boronic acid was considered positive for AmpC production.[7,9] (Figure 1)

Boronic acid, being an inhibitor of AmpC, increases the zone diameter of cefoxitin or cefotetan when combined with boronic acid.[7,9]

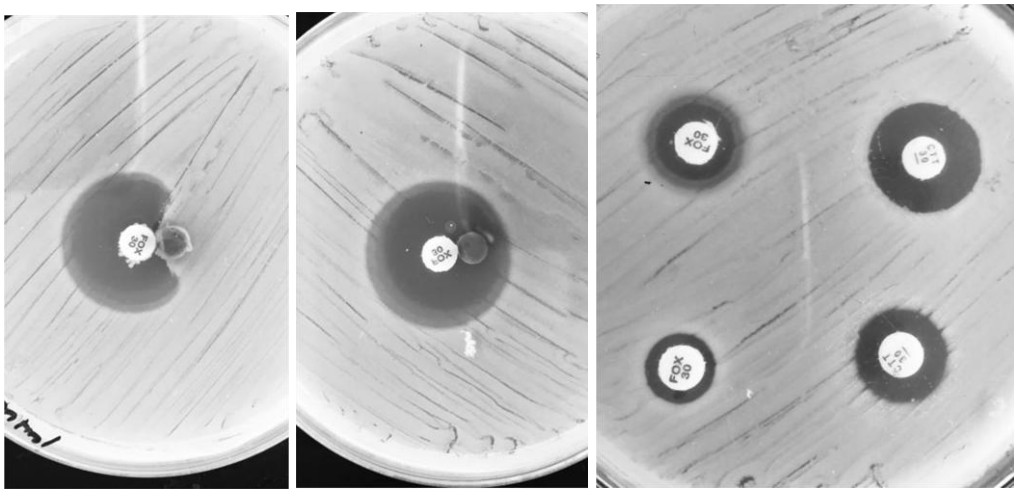
EDTA disc test

Discs were prepared in-house as given by Black et al.*(*2005).[10] Tris-EDTA (Ethylene diamine tetra acetic acid) buffer solution, 100×concentrate (catalogue code T - 9285; Sigma-Aldrich) was mixed with normal saline in a 1:1 ratio, and 20 µL of this solution was applied to sterile filter paper discs, which were then air-dried and stored at 2-8 °C. Discs were hydrated with 20 µL of normal saline immediately before use.[10]

Mueller-Hinton agar plates were inoculated with *E. coli* ATCC 25922. A few colonies of the test organism were applied to the ETDA discs, which were then placed on the lawn culture

with the inoculated surface facing the agar, touching the lawn and close to the cefoxitin disc. (Figure 1) The plates were incubated aerobically at 35–37 °C for 16–18 hours.

Flattening or indentation of the cefoxitin inhibition zone adjacent to the EDTA disc was interpreted as positive (Figure 1). This method is based on the ability of Tris-EDTA to permeabilise the bacterial cell wall, allowing beta-lactamase to diffuse into the surrounding medium and reduce the zone diameter on that side.[10]



(A) **(B)** **(C)**

Figure 1

(A) Positive result of AmpC EDTA disc test: An indentation of the zone was taken as positive
(B) Negative result of AmpC EDTA disc test: No indentation of the zone was taken as negative
(C) Positive Boronic Acid Disc test Cefoxitin (Left side) and Cefotetan (Right Side): Top two discs are added with phenyl boronic acid. Zone size has increased compared to the lower two discs without phenyl boronic acid

ESBL Screening and Confirmatory Tests

ESBL screening was performed according to CLSI guidelines using cefpodoxime 10 µg, ceftazidime 30 µg and cefotaxime 30 µg discs.[6] Confirmation was carried out by comparing zones of inhibition of ceftazidime-clavulanic acid with ceftazidime alone and cefotaxime-clavulanic acid with cefotaxime alone.[6]

Demographic and clinical data, including the diagnosis, antibiotic treatment, associated factors, comorbidities, and patient outcomes, were collected from bed head tickets and medical officers' records using a structured data extraction sheet.

Statistical Analysis

Statistical analysis was done using SPSS software. Associations between categorical variables were assessed using the chi-square test. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated to compare the screening and confirmatory tests.

Results

We analysed 141 isolates of *Klebsiella* spp., *Proteus* spp., and *E coli* obtained from blood, sterile fluid and pus samples. The majority (n=114; 80.9%) were blood culture isolates, while the remaining 27 isolates (19.1%) were obtained from pus (n=21), bile (n=4) and tissue (n=2) cultures. The patients' ages ranged from 15 to 88 years, with a mean age of 54 years. Males accounted for 52.5% (n=74) and females for 47.5% (n=67) of the study. Of the total samples, 43% were from medical units, 33% from surgical units, and 24% from intensive care units (ICUs). The isolates were identified as *E coli* (n=68; 48%), *Klebsiella pneumoniae* (n=59; 42%), *Proteus mirabilis* (n=12; 8.5%), and other *Klebsiella* species (n=2; 1.5%).

The positivity rate of AmpC beta-lactamase production by the reference method, cefoxitin-cloxacillin double-disc synergistic test, was 24.1% (34/141). Among blood culture isolates, 26.3% (30/114) were AmpC producers, compared with 14.8% (4/27) isolates from other specimen types.

The majority of AmpC-positive isolates were *K. pneumoniae* (79%; n=27), followed by *E. coli* (18%; n=6) and *Klebsiella oxytoca* (3%; n=1). All *Proteus mirabilis* isolates were negative for AmpC production.

Cefoxitin disc screening test demonstrated higher sensitivity compared to the cefotetan disc method. (Table 1)

Table 1: Comparison of screening tests with the cefoxitin-cloxacillin double disk test (reference method)

Test method	Results	Confirmatory test cefoxitin-cloxacillin double disc test		Total	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
		positive	negative					
Cefoxitin disc Test	positive	34	5	39	100 %	95.33 %	87.17 %	100 %
	negative	0	102	102				
Cefotetan disc test	positive	31	5	36	91.18 %	95.33 %	86.11 %	97.14 %
	negative	3	102	105				

Among the confirmatory tests, the EDTA disc test showed the highest specificity (100%) with a sensitivity of 91.2%. Both the cefoxitin–boronic acid and cefotetan–boronic acid disc tests demonstrated equal specificity (99.1%), while the cefoxitin-boronic acid method gave a higher positive predictive value. (Table 2).

ESBL production in clinical isolates

We found that 64.5% (91/141) of isolates were ESBL producers. Among these, 31.9% (29/91) were also AmpC producers. Co-existence of AmpC and ESBL production was observed in 20.6% (29/141) of all isolates.

Statistically significant factors associated with AmpC positivity included the hospital unit to which the patient belonged ($p = 0.0001$), hospital-acquired infection ($p = 0.010$), ICU stay ($p = 0.004$), and history of recurrent infections or hospitalisation ($p = 0.003$). Gender, prior antibiotic use, comorbidities, and post-surgical status were not significantly associated with AmpC positivity. (Table 3)

Table 2: Evaluation of three AmpC confirmatory methods by cefoxitin-cloxacillin disk test (reference method)

Test method		Confirmatory cefoxitin-cloxacillin double disc reference method		Total	Sensitivity %	Specificity %	Positive Predictive Value %	Negative Predictive Value %
		Positive	Negative					
Cefoxitin–boronic Acid disc test	Positive	34	1	35	100	99.07	97.14	100
	Negative	0	106	106				
Cefotetan–boronic Acid disc test	Positive	31	1	32	91.18	99.07	96.87	97.24
	Negative	3	106	109				
Tris-EDTA test	Positive	31	0	31	91.18	100	100	97.17
	Negative	3	107	110				

Table 3: Factors associated with AmpC positivity in patients with AmpC-positive organisms

Factor		Total number	Positive for AmpC in each group		P value
		n	n	%	
Gender	Male	74	20	27	0. 257
	Female	67	14	21	
Location	Medical	61	11	18	**0.0001**
	Surgical	46	4	8.7	
	ICUs	34	19	55.9	
Type of infection	Community acquired	51	7	13.7	**0.010**
	Hospital acquired	39	16	41	
Prior use of antibiotics	Used	64	20	31	0.199
	Not used	40	8	19.6	
Presence of comorbid Illnesses	Present	85	23	27	0.211
	Absent	56	11	20	
Intensive care unit (ICU) stay	Yes	52	23	44	**0.004**
	No	89	11	12.3	
Recurrent infections or recurrent hospitalisation	Yes	60	22	36.7	**0.003**
	No	81	12	14.8	
Post-surgical patients	Yes	32	5	15.6	0.148
	No	109	29	26.6	

Infections with AmpC producers were more common in patients with neutropaenic sepsis (3/4; 75%), followed by respiratory tract infections (10/16; 62%), line-associated infections (2/5; 40%), and deep-seated abscesses (3/8; 38%). (Table 4)

Table 4: Comparison of patients' diagnosis and infections with AmpC-positive organisms

Type of infection	AmpC negative		AmpC positive		Total
	n	%	n	%	
Urinary tract infection/ Pyelonephritis	45	90	5	10	50
Respiratory tract infection	6	38	10	62	16
Skin/soft tissue infection	12	80	3	20	15
Intra-abdominal/ hepato-billiary infection	20	95	1	5	21
Post-surgical sepsis	5	71	2	29	7
Central nervous system infection	6	86	1	14	7
Neutropenic sepsis	1	25	3	75	4
Line associated infection	3	60	2	40	5
Leptospirosis/ dengue	2	100	0	0	2
Deep abscesses	5	62	3	38	8
Total	105		30		135

Discussion

Prevalence rates of antimicrobial resistance vary between countries and even between regions within the same country, and these rates continue to rise over time.[11] In our study, 24.1% of *Enterobacterales* isolates produced AmpC beta-lactamase, and 64.5% produced ESBLs. These findings are consistent with other reports from Sri Lanka; notably, AmpC beta-lactamase production in uropathogenic *Enterobacterales* in the Western Province has been reported at 19%, while ESBL production was 50%.[12] In India, ESBL prevalence among *Enterobacterales* increased from approximately 40% in 2013 to over 70% by 2019, while AmpC prevalence rose from 14.8% to 42% over the same period.[3,11,13] In contrast, ESBL prevalence remains relatively low in high-income settings such as the United Kingdom, Europe and the USA (2–6%), and <15% in countries such as Australia, Japan, Korea and Singapore. AmpC prevalence in Europe and North America is estimated at around 10%.[11,14,15] This stark difference may be attributed to widespread over-the-counter availability of antibiotics, insufficient regulatory control, overuse of antimicrobial medications in both human health and animal husbandry, and the high population density in South Asia, which facilitates rapid transmission of resistant bacteria.[16]

The AmpC and ESBL co-existence was 20.6% in our study, and comparable to Indian data, 9.9% reported in 2013 and 21.7% in 2019.[3,13] Co-production poses significant clinical challenges because concurrent beta-lactamase activity markedly limits treatment options.[3]

When organism-specific rates were examined, *K. pneumoniae* had the highest rate of AmpC positivity, followed by *E. coli*. This differs from findings in several other countries where *E. coli* generally shows higher AmpC production than *Klebsiella* species.[3,5,15] Our analysis of factors associated with AmpC positivity also mirrored the results of previous studies. Prolonged hospital stays, ICU admission, prior broad-spectrum antibiotic use, chronic illnesses and presence of invasive devices such as arterial or urinary catheterisation are associated with infections by AmpC and other beta-lactamase-producing pathogens.[12,17] Having a hospital-acquired infection, ICU stay, and recurrent infections or hospitalisation was significantly associated with AmpC positivity in our study. Prior antibiotic usage did not reach statistical significance, but under-reporting is likely in Sri Lanka where records of antibiotic prescriptions from general practitioners are not always traceable and patient records are often incomplete. The actual exposure to antibiotics may therefore be higher than documented.

Polsfuss *et al.* (2011) proposed an algorithm for phenotypic detection of AmpC. Isolates with cefoxitin resistance or isolates with a positive ESBL screening test but negative ESBL confirmatory test are tested with a cefoxitin-cloxacillin double disc test to confirm presence of AmpC. Inconclusive results must be followed by multiplex PCR.[5]

The cefoxitin disc test is a good screening test for AmpC as it gives a sensitivity of 100% with 95.33% specificity. Cefoxitin has been recognised as a reliable screening marker in previous studies.[5,18] Among confirmatory tests, the EDTA-disc method shows the highest specificity (100%) with 91% sensitivity. In previous studies, the Tris-EDTA method had demonstrated specificity of 98%, and sensitivity of 97–100%.[10,18] However, this test needs complex laboratory work, and readings may be subjective in certain instances. Cefoxitin-boronic acid and cefotetan-boronic acid combination discs tests also yielded a high specificity of 99%.[10,18] In our study, cefoxitin-boronic acid and Tris-EDTA tests showed better results for AmpC detection than the cefotetan-boronic acid test. However, boronic acid can inhibit some ESBLs, KPC and OXA-12-type enzymes, leading to false positive results.[19]

Our phenotypic approach, while practical and reliable locally, may still miss certain genotypes. Genotypic methods remain the gold standard but are often not feasible in routine settings due to cost and resource constraints.

Conclusion

A relatively high prevalence of AmpC beta-lactamase production was observed in our clinical setting of which the majority were *K. pneumoniae*. AmpC and ESBL co-existence was found approximately in one-fifth of the isolates. Cefoxitin is a superior screening method, while the EDTA test demonstrated good confirmatory performance in addition to the cefoxitin-cloxacillin double disc test.

Declaration

Conflict of interest: The authors declare that they have no conflicts of interest
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Ethics Statement: The study was approved by the Ethics Review committees of Medical Research Institute, Colombo (Project No.16/2016) and National Hospital of Sri Lanka (No. ETH/COM/2016). Procedures followed were in accordance with the ethical principles of the Helsinki Declaration of 1975, as revised in 2000.
Author contributions:
1: Ranasinghe RATK: Conceptualised and designed the study, carried out laboratory investigations, analysed and interpreted data, drafted the manuscript
2: Karunanayake L: Supervised the study and contributed to the drafting of the manuscript.
3 Patabendige CGUA: Supervised the study and contributed to the drafting of the manuscript.
All authors reviewed the final version of the manuscript and are accountable for all aspects of the work.

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