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Original Research Article

The incidence of AmpC β-lactamases producing *Klebsiella pneumoniae* subspecies *pneumoniae*

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ABSTRACT

Background: AmpC β- lactamases in the clinical isolates reduces the therapeutic value of β- lactam- β-lactam inhibitor combinations. if not detected can be disseminated in the hospital environment and pose a serious therapeutic challenge. Hence present study is undertaken to detect the incidence of AmpC β -lactamases producing Klebsiella pneumoniae subspecies pneumoniae out of total 300 *Klebsiella pneumoniae* subspecies pneumoniae isolated from different clinical samples of the patient attending Jawaharlal Nehru Medical College and its hospital in Dept of Microbiology.
Methods: Isolates are screened for presumptive AmpC production by testing their susceptibility to Cefoxitin using Kirby-Bauer disk diffusion method. Phenotypic confirmatory tests for detection of AmpC β- lactamases by Modified three dimentional test, Amp C disc test, Amp C disc test with Inhibitor (Boronic Acid) based disc potentiation test.
Results: In our present study 75 (25%) *Klebsiella pneumoniae* strains were positive for AmpC β lactamases production either alone or in combinations with other β- lactamases. 75 strains were positive for AmpC β-lactamase (25%). These 75 strains were further confirmed by E Test.
Conclusions: Overproduction of AmpC β- lactamases by mutation is responsible for resistance. if not detected can be pose a serious therapeutic challenge. So, its detection improves the therapeutic outcome in patient care.

Keywords: AmpC β- lactamases, DP-Test, ESBL, E-Test, *Klebsiella pneumonia*

INTRODUCTION

AmpC β-lactamase

AmpC β-lactamases of *Escherichia coli* was reported to be first bacterial enzyme to destroy Penicillin, although it had not been so named in 1940.[1] Mutation with stepwise-enhanced resistance were termed ampA and ampB and a mutation in an amp A strain that resulted in reduced resistance was designated as Amp C. Then it was suggested that AmpC was the structural gene for this enzyme.[2] The sequence of amp C gene from *Escherichia coli* was reported in 1981. The ampC β- lactamases differed from the penicillinase type β - lactamases such as TEM -1 but had serine at its active sites.[3] In Ambler classification AmpC beta lactamases belongs to class C while in functional classification of Bush Jacoby Mederois Amp C β-lactamases belongs to group 1.[4,5]

They are active on Penicillin but more active on Cephalosporins and can hydrolyse Cephamycins like Cefoxitin and Cefotetan. Oxyimino cephalosporins such as Ceftazidime, Cefotaxime and Ceftriaxone and monobactams such as Aztreonam and are not inhibited by clavulanic acid, sulbactum and tazobactam. Cloxacillin and Oxacillin are good inhibitors of AmpC β -lactamases. AmpC β-lactamase are found as chromosomally mediated

or plasmid mediated. The chromosomally mediated AmpC β -lactamase are inducible.

Chromosomally mediated AmpC β-lactamase are present in Gram negative bacilli e.g. *Pseudomonas aeruginosa*, Enterobacter spp, Acinetobacter spp. and Aeromonas spp.etc. Overproduction of their chromosomal AmpC β-lactamases by mutation is responsible for resistance.[6] Plasmid mediated AmpC enzyme e.g. CMY type β-lactamase found in bacterial spp., which naturally lacks chromosomal β- lactamases such as *Klebsiella pneumoniae*, *Proteus mirabilis* and Salmonella Spp. Plasmid mediated β- lactamases have arisen through transfer of chromosomal AmpC gene into plasmids.[7] Presence of ESBL may be masked by coincident AmpC β -lactamases enzyme.[8]

Aims and objective of the study was to study the incidence of AmpC β -lactamases producing *Klebsiella pneumoniae subspecies pneumoniae* out of total *Klebsiella pneumoniae subspecies pneumoniae* isolated from different clinical samples of the patient attending Jawaharlal Nehru Medical College and its hospital (Acharya Vinoba Bhave Rural Hospital), Wardha (MS).

METHODS

The present study was conducted in the Department of Microbiology, Jawaharlal Nehru Medical College, Wardha (M.S). The period of study was 1 years from 1.7.2014 to 30.6.2015

Screening test for AmpC β- lactamase

1. Isolates are screened for presumptive AmpC production by testing their susceptibility to Cefoxitin using Kirby-Bauer disk diffusion method. All the isolates with inhibition zone diameter of less than 18mm are labelled as screen positive.[9]
2. Phenotypic confirmatory tests for detection of AmpC β- lactamases

    - Modified three dimentional test (M3DT).[10]
    - Amp C disc test.[11]
    - Amp C disc test with Inhibitor (Boronic Acid) based disc potentiation test.[12]

3. Molecular methods for detection and confirmation of AmpC β-lactamase.

    - Polymerase chain reaction (PCR).[13]
    - Nucleotide sequencing analysis of AmpC encoding genes.

Detection of AmpC β- Lactamases

For Detection of AmpC β- Lactamases, no satisfactory technique has been established till date as per CLSI guideline. As AmpC β- Lactamases production is mostly plasmid mediated in Enterobacteriaceae and not chromosomally induced, D-zone test cannot be done in case of *Klebsiella pneumoniae.*

Screening Test

The isolates were screened for presumptive AmpC production by testing their susceptibility to Cefoxitin and Cefotetan using Kirby- Bauer disk diffusion method.[14] The inhibition zone sizes were interpreted as per CLSI guideline. All the isolates with an inhibition zone diameter of ≤18mm for Cefoxitin and ≤16mm diameter for Cefotetan respectively were labeled as screen positive.

Confirmatory Test

All 300 *Klebsiella pneumoniae* strains were studied for AmpC β- lactamases production and were confirmed by confirmatory test using Cefoxitin (CX) and Cefoxitin-Cloxacillin (CXX) discs.[9] This test is based on the inhibitory effect of Cloxacillin on AmpC-β lactamases. The discs containing Cefoxitin and Cefoxitin plus Cloxacillin were put on MH agar plate and the plates were incubated at 35^0C for 16-18 hours. An increase in zone size of ≥4mm around the disc Cefoxitin plus Cloxacillin compared to Cefoxitin disc only was considered positive for AmpC production.

AmpC β- Lactamases production was further confirmed by disk potentiation test using Ceftazidime / Ceftazidime plus 3-aminophenyboronic acid.[12]

Disk potentiation test

All 300 *Klebsiella pneumoniae* strains were tested by disc potentiation test for AmpC β-lactamases production. An overnight broth culture of test strain was inoculated on Mueller Hinton agar plate with sterile swab. Two Ceftazidime disc with center to center distance of 30mm were placed on that inoculated plate. 3- APB solution was added to one of the Ceftazidime disc. After overnight incubation at 37^0C, an increase in zone size of ≥5mm around the Ceftazidime plus 3-APB disc compared to Ceftazidime alone was considered as positive for AmpC β-lactamase production.

Confirmation of AmpC β- lactamase producing strains by E test

AmpC β-lactamases producing *Klebsiella pneumoniae* strains were futher confirmed by E test (bioMerieux). The E test strip has concentration gradient of Cefoxitin (CN .5-32µg/ml) on one half and Cefoxitin-Cloxacillin (CNI .5-32µg/ml) on the other half. The AmpC β lactamase E test was done and interpreted as per manufacturer's instructions manual. In this method lawn culture of test strain (turbidity adjusted to 0.5 Mc Farland Standard) was done on a Mueller Hinton agar plate. With a sterile forceps the E-test strip was placed onto the inoculated plate. After overnight incubation at 37^0C, the zone of

inhibition was read from two halves of the strip. MIC ratio of Cefoxitin/ Cefoxitin-Cloxacillin) ≥8 or deformation of ellipse or phantom zone present was considered as positive for AmpC β-lactamase production.

RESULTS

Table 1 shows that 75 (25%) *Klebsiella pneumoniae* strains were positive for AmpC β lactamases production either alone or in combinations with other β- lactamases. All 300 *Klebsiella pneumoniae* strains were tested by screening tests and confirmatory tests (CX /CXX and CAZ /CAZ+ 3-APB). The screening tests were done by Kirby- Bauer disc diffusion method using Cefoxitin (CX) and Cefotetan (CTN). Out of 300 *Klebsiella pneumoniae* strains 91(30.3%) strains were screen positive by Cefoxitin disc and 101 (33.7%) strains were screen positive by Cefotetan. The Confirmatory tests were done by combined disc method using and Ceftazidime (CAZ) / Ceftazidime plus 3- Aminophenyl boronic acid (CAZ+ 3-APB) Cefoxitin (CX) / Cefoxitin plus Cloxacillin (CXX) and 75 strains were positive for AmpC β-lactamase (25%). These 75 strains were futher confirmed by E Test (CN/ CNI).

Table 1: Detection of AmpC β- lactamases producing *Klebsiella pneumoniae* strains by different methods (n=300).

Klebsiella pneumonia	Screening Test		Confirmatory Test		
Total AmpC β- lactamases Producers No.	Cefoxitin (CX) No.	Cefotetan (CTN) No.	Cefoxitin/ Cefoxitin + Cloxacillin (CX/CXX) No.	Ceftazidime / Ceftazidime + 3-Aminophenylboronic acid (CAZ / CAZ + 3-APB) No.	E -Test No.
75 (25%)	91 (30.3%)	101 (33.7%)	75	75	75

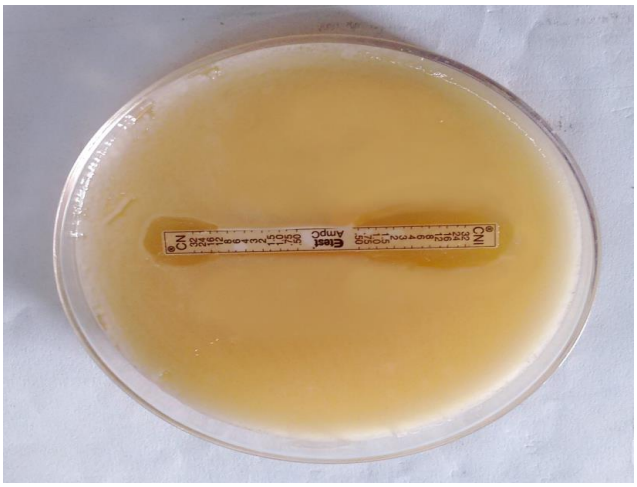

Figure 1: Strain confirmation by E Test (CN/ CNI).

DISCUSSION

Table 2 shows the incidence of AmpC β-lactamase producing *Klebsiella pneumoniae* strains reported by various workers. In 2005 Suranjana et al, had reported 13% AmpC β-lactamase producing *Klebsiella pneumoniae* strains. In the present study total 25% AmpC β-lactamase producing *Klebsiella pneumoniae* strains were isolated.[15]

In present study, 22.2% strains were ESBL and AmpC β -lactamase Co producers. Only few studies have reported both ESBL and AmpC β -lactamase producing *Klebsiella pneumoniae* strains.[16] In 2010 Mohanty et al, have reported as high as 58.4% ESBL and AmpC β -lactamase producing strains and Kaur J et al, have reported that only 4% of *Klebsiella pneumoniae* strains in their study were both ESBL and AmpC β -lactamase producers. Actually, it is difficult to detect ESBL if strain is Co-producers of ESBL and AmpC β -lactamase as AmpC β -lactamase mask the ESBL production.[17] In that case if just combined disc CAZ/CAC is used for ESBL detection, ESBL may be missed. In the present study it was found that in ESBL and AmpC β- lactamase Co-producers strains a thin rim of growth around the edge of zone of inhibition was found in CAC or zone diameter of CAC was just >5mm more when compared to CAZ alone.[18]

Table 2: Incidence of AmpC β-lactamase producing *Klebsiella pneumoniae* strains reported by various workers.

Author	Years	Place of study	AmpC β-lactamase %
Suranjana A, et al	2005	Kolkata, India	13
Gupta V, et al	2012	Chennai, India	32
Barua T, et al	2013	Muscat, Oman	34.3
Ranjini CY, et al	2015	Karnataka, India	35.2
Present study	2016	Wardha, India	25

In the present study, in screening test, Cefotaxime (CTX), Ceftazidime (CAZ) and Cefpodoxime (CPD) were resistant to 142 (47.3%), 139 (46.3% and 148 (49.3%) strains respectively though the total ESBL producing strains were 136 (45.3%). The reason for more resistant strains in screening test may be due to the mechanism other than ESBL production such as porin loss, efflux pump etc. The same may be the reason for more resistance to Cefoxitin (CX) and Cefotetan (CTN) i.e. 91 (30.3%) and 101 (33.7%) respectively in screening test of AmpC β- lactamases, as confirmatory tests detected only 75 (25%) strains. In the present study also, Cefoxitin (CX) was a better indicator than Cefotetan (CTN) as all 75 strains positive by confirmatory tests were resistant to Cefoxitin (CX). Four strains i.e. (4/75) were false negative by Cefotetan (CTN).[19] Polsfuss et al, have also reported that Cefoxitin (CX) was a better indicator of AmpC β -lactamase production than Cefotetan.The studies from India showed a wide range of incidence of AmpC production by Enterobacteriaceae. Gupta et.al in 2012 reported that 32% of their *Klebsiella pneumoniae* strains were AmpC producers. Ranjini et al, have reported 30.8% of *Klebsiella* spp isolated from diabetic foot were ESBL producers and 56.3% were AmpC β-lactamase producers. The studies from India showed a wide range of incidence of AmpC production by Enterobacteriaceae.[20] Gupta et al, reported that 32% of their *Klebsiella pneumoniae* strains were AmpC producers. Ranjini et al, have reported 30.8% of *Klebsiella* spp. isolated from diabetic foot were ESBL producers and 56.3% were AmpC β-lactamase producers.

CONCLUSION

A total number of 300 *Klebsiella pneumoniae* strains were isolated from different clinical specimens like urine, blood, pus and wound swab etc. and were identified by conventional methods. It shows that 75 (25%) *Klebsiella pneumoniae* strains were positive for AmpC β lactamases production either alone or in combinations with other β-lactamases. 75 strains were positive for AmpC β-lactamase (25%). These 75 strains were further confirmed by E Test (CN/ CNI).

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