

Detection of Amp C beta lactamases production in *Escherichia coli* & *Klebsiella* by an inhibitor based method

V. Hemalatha, M. Padma, Uma Sekar, T. M. Vinodh* & A.S. Arunkumar*

Departments of Microbiology, *Anaesthesia & Critical Care, Sri Ramachandra Medical College & Research Institute [Deemed University] Chennai, India

Received September 25, 2006

Background & objectives: Detection of AmpC-mediated resistance in Gram-negative organisms poses a problem due to misleading results in phenotypic tests. There are no recommended guidelines for detection of this resistance mechanism and there is a need to address this issue as much as the detection of extended spectrum beta lactamases (ESBLs) since both may co-exist and mask each other. Though resistance to ceftiofuran is used as a screening test, it does not reliably indicate Amp C production. This study was undertaken to detect Amp C beta lactamases in certain Gram-negative bacteria employing an inhibitor base test using boronic acid.

Methods: A total number of 76 consecutive non repetitive clinical isolates of *Escherichia coli* (n=67) and 9 *Klebsiella pneumoniae* (n=9) obtained over a period of two months, were screened for amp C production by disc diffusion method using ceftiofuran (30 µg) discs and confirmed by inhibitor based test using boronic acid as inhibitor.

Results: A total of 36 of 76 isolates (47.3%) screened harboured amp C enzymes, of which a majority 31 (86.1%) co-produced ESBL enzymes. Pure ampC production was seen in 7 (9.2%) of isolates only.

Interpretation & conclusion: Most of the amp C producers also produced ESBL enzymes. The inhibitor based test was useful in identifying ceftiofuran susceptible amp C producers and could also effectively differentiate ESBL from amp C producing isolates.

Key words Amp C beta lactamases - extended spectrum beta lactamases - inhibitor based test

Amp C β lactamases have gained importance since the late 1970s as one of the mediators of antimicrobial resistance in Gram negative bacilli. These enzymes are cephalosporinases capable of hydrolyzing all β -lactams to some extent¹. Amp C β lactamases are of two types- plasmid-mediated and chromosomal or inducible ampC. Chromosomal ampC enzymes are seen in organisms such as *Citrobacter freundii*, *Enterobacter cloacae*,

Morganella morganii, *Hafnia alvei* and *Serratia marcescens* and are typically inducible by β -lactam antibiotics such as ceftiofuran and imipenem but poorly induced (if at all) by the third- or fourth-generation cephalosporins². In the late 1980s, these inducible chromosomal genes were also detected on plasmids (most without induction capabilities) and were transferred to organisms, which typically do not express chromosomal

β -lactamases such as *Klebsiella* spp., *Escherichia coli*, or *Salmonella* spp.¹. For clinical microbiologists, detection of AmpC-mediated resistance in Gram-negative organisms poses a problem because the phenotypic tests may be misleading resulting in misreporting and treatment failures. There are no recommended guidelines for detection of this resistance mechanism and clinical laboratories need to address this issue as much as the detection of extended spectrum β -lactamases (ESBLs) since both may co-exist and mask each other³. Screening with cefoxitin disc is recommended for initial detection. However, it does not reliably indicate AmpC production. Some of the phenotypic tests include the three-dimensional test⁴, AmpC disc test⁵ and modified disc diffusion test⁶. None of these tests are standardized and can be time consuming when screening large numbers of isolates.

We therefore undertook this study to estimate the presence of Amp C beta lactamases in certain Gram-negative bacteria by inhibitor based method using boronic acid (BA)⁷ and also assess if this test could be used to differentiate between ESBLs and amp C producers.

Material & Methods

This study was restricted to *Escherichia coli* and *Klebsiella* isolates only as Clinical Laboratory Standards Institute (CLSI) guidelines for ESBL screening and confirmation are available only for these species⁸. A total number of 76 consecutive non repetitive clinical isolates *E. coli* (N=67) and *Klebsiella pneumoniae* (N=9) were obtained in microbiology laboratory of Sri Ramachandra Medical College & Research Institute, Chennai, over a period of two months (January-February 2006) for the study. The isolates were obtained from the cultures of urine (65), wound (8), blood (2) and bronchoalveolar lavage (10). Significant proportions of the strains were from the hospitalized patients (64) and seven among them were from patients in the intensive care unit (ICU).

Antibiogram: Antibiotic susceptibility testing was performed by the Kirby Bauer method on Mueller Hinton agar according to CLSI protocols⁹. The drugs tested (in μ g) were ampicillin (10), cefazolin (30), ceftazidime (30), cefotaxime (30), cefepime (30), ciprofloxacin (5), gentamicin (5), amikacin (30) (Hi-media Laboratories, Mumbai), piperacillin-tazobactam (100/10), cefoxitin (30) and imipenem (10) (BD Diagnostics, Bawal, District Rewari, Haryana, India). *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as control strains.

ESBL screen: Isolates were also tested for ESBL production by the CLSI confirmatory method⁸ using ceftazidime and cefotaxime disks with and without clavulanic acid (10 μ g). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603, were used as negative and positive controls. All the isolates were tested for susceptibility to imipenem with E test strips (AB Biodisk, Solna Sweden).

Inhibitor based test: All isolates were tested for amp C beta lactamase production on disks containing boronic acid⁷. A disk containing 30 μ g of cefoxitin and another containing 30 μ g of cefoxitin with 400 μ g of boronic acid were placed on the agar. Similarly, discs of ceftazidime (30 μ g) and ceftazidime-clavulanic acid (30/10 μ g) were placed on the medium at a distance of 30 mm. Inoculated plates were incubated overnight at 35°C. An organism demonstrating a zone diameter around the disk containing cefoxitin and boronic acid ≥ 5 mm than the zone diameter around the disk containing cefoxitin alone was considered an amp C producer. Likewise, an organism exhibiting 5 mm or greater zone size increase around the ceftazidime-clavulanic acid disk compared to the ceftazidime disk was considered indicative of ESBL production.

Results & Discussion

Of the 76 isolates tested, 28 were susceptible to all the antibiotics tested including the third generation cephalosporins (3GC). Of the remaining 48 isolates which exhibited 3GC resistance, 14 were resistant to cefoxitin and the remaining 34 were susceptible to it. All these 34 isolates exhibited a zone enhancement with clavulanic acid confirming their ESBL production. In addition, 12 of 14 isolates which exhibited cefoxitin resistance also had zone enhancement with clavulanic acid, thereby indicating both ESBL and amp C production. Hence, only two of 14 isolates were presumed pure amp C producers.

Of the 76 isolates 36, (47.3%) showed zone enhancement of > 5 mm with boronic acid impregnated disk and hence confirmed as amp C producers. This included 12 isolates positive for both ESBL and amp C; 19 of 34 ESBL positive cefoxitin susceptible isolates and 5/28 3GC sensitive isolates screened negative for both enzymes. All the isolates were susceptible to imipenem with the E-test method (MIC 0.125 to 0.38 μ g/ml).

Currently, CLSI documents do not indicate the screening and confirmatory tests that are optimal for detection of these beta lactamases⁸. However, several

studies have been done on various test methods namely, the three dimensional test⁴, modified double disk test⁶, amp C disk test⁵, inhibitor based method employing inhibitors like boronic acids⁷, broth micro dilution method⁷ and cefoxitin agar method¹⁰. In spite of many phenotypic tests, isoelectric focusing¹¹ and genotypic characterization¹² are considered gold standard as the results with the phenotypic tests can be ambiguous and unreliable.

In our study, we screened *E. coli* and *Klebsiella pneumoniae* as these were the most common isolates in our clinical laboratory.

Amp C beta lactamase was detected in 47.3 per cent isolates, four-fifths of which occurred in combination with ESBLs. Pure amp C beta lactamases were detected only in 9.2 per cent of the isolates. This prevalence was higher when compared to the reports from other parts of the world^{13,14}. Two Indian studies^{15,16} reported 8 and 43 per cent prevalence of amp C beta lactamases.

It has been stated that the amp C beta lactamases when present along with ESBLs can mask the phenotype of the latter³. In this study, we found that both these enzymes were equally expressed suggesting a possible low level expression of amp C enzymes. However, in all these amp C producers, we were not able to distinguish between the chromosomal derepressed and plasmid mediated enzymes as this requires genotypic confirmatory tests. Three of the nine *Klebsiella* isolates screened were amp C producers suggesting the presence of plasmid mediated mechanism as these species do not harbour chromosomal amp C genes³. The susceptibility to ceftazidime and cefotaxime serves as a poor marker for the identification of amp C enzymes⁷. It is known that plasmid mediated amp C enzymes can sometimes appear falsely susceptible to these drugs¹⁷. There are newer Ambler class C (ACC) type of enzymes which have relatively a lower activity to cefoxitin¹⁸ and hence appear susceptible. In our study, 5 of the 7 amp C producing isolates were susceptible to the third generation cephalosporins and cefoxitin by the disc diffusion method. This indicate the probable presence of such ACC enzymes. This needs to be confirmed by molecular methods. All isolates producing amp C beta lactamases including *K. pneumoniae* were susceptible to imipenem. This suggests the absence of outer membrane porin defect frequently reported in such species⁷.

The inhibitor based confirmatory method appears promising for amp C detection as it increased the

sensitivity of the test by picking up additional amp C producers. Boronic acid has been reported to be an effective inhibitor of class C beta lactamases¹⁹⁻²¹. In addition, it can also differentiate ESBL enzymes from the amp C enzymes.

To conclude, a mixed type of drug resistance mechanisms seem to operate in the isolates tested. There is a need for a correct and reliable phenotypic test to identify amp C beta lactamases and to discriminate between pure amp C and ESBL producers. Inhibitor based method using boronic acid appears to be effective in discriminating this type of resistant isolates.

References

1. Nancy D. Hanson. Amp C beta-lactamases: what do we need to know for the future? *J Antimicrob Chemother* 2003; 52 : 2-42.
2. Philippon A, Arlet G, Jacoby GA. Plasmid-determined Amp C-type beta lactamases. *Antimicrob Agents Chemother* 2002; 46 : 1-11.
3. Thomson KS. Controversies about extended-spectrum and AmpC b-lactamases. *Emerg Infect Dis* 2001; 7 : 333-6.
4. Thomson KS, Sanders CC. Detection of extended spectrum beta-lactamases in members of the family Enterobacteriaceae: comparison of the double-disk and three-dimensional tests. *Antimicrob Agents Chemother* 1992; 36 : 1877-82.
5. Black J, Moland ES, Thomson KS. A simple disk test for detection of plasmid-mediated AmpC production in *Klebsiella*. In: *Program and abstracts of the forty-second interscience conference on antimicrobial agents and chemotherapy*; Abstract D-534, Washington, DC, USA: American Society for Microbiology; 2002 p. 140.
6. Pitout JD, Reisbig MD, Venter EC, Church DL, Hanson ND. Modification of the double-disk test for detection of enterobacteriaceae producing extended-spectrum and AmpC beta-lactamases. *J Clin Microbiol* 2003; 41 : 3933-5.
7. Coudron PE. Inhibitor-based methods for detection of plasmid-mediated AmpC beta-lactamases in *Klebsiella* spp., *Escherichia coli* and *Proteus mirabilis*. *J Clin Microbiol* 2005; 43 : 4163-7.
8. Clinical Laboratory Standards Institute. *Performance standards for antimicrobial and susceptibility testing: 14th informational supplement (M100-S14)*. Wayne, Pa: Clinical Laboratory Standards Institute; 2004.
9. Clinical Laboratory Standards Institute. *Performance standards for antimicrobial disk susceptibility tests*, 8th ed. Approved standard M2-A8. Wayne, Pa: Clinical Laboratory Standards; 2003.
10. Nasim K, Elsayed S, Pitout JDD, Conly J, Church DL, Gregson DB. New method for laboratory detection of AmpC beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae*. *J Clin Microbiol* 2004; 42 : 4799-802.
11. Mathew M, Harris AM, Marshall MJ, Ross GW. The use of analytical isoelectric focusing for detection and identification of betalactamases. *Gen Microbiol* 1975; 88 :169-78.

12. Perez-Perez FJ, Hanson ND. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* 2002; 40 : 2153-62.
13. Liu PYF, Gur D, Hall LMC, Livermore DM. Survey of the prevalence of β -lactamases amongst 1000 Gram-negative bacilli isolated consecutively at the Royal London Hospital. *J Antimicrob Chemother* 1992; 30 : 429-47.
14. Coudron PE, Moland ES, Thomson KS. Occurrence and detection of AmpC betalactamases among *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* isolates at a veterans medical center. *J Clin Microbiol* 2000; 38 : 1791-6.
15. Singhal S, Mathur T, Khan S, Upadhyay DJ, Chugh S, Gained R, *et al.* Evaluation of methods for AmpC beta-lactamase in Gram negative clinical isolates from tertiary care hospitals. *Indian J Med Microbiol* 2005; 23 : 120-4.
16. Manchanda V, Singh NP, Shamweel A, Eideh HK, Thukral SS. Molecular epidemiology of clinical isolates of AmpC producing *Klebsiella pneumoniae*. *Indian J Med Microbiol* 2006; 24 : 177-81.
17. Black JA, Thomson KS, Buynak JD, Pitout JDD. Evaluation of beta-lactamase inhibitors in disk tests for detection of plasmid-mediated AmpC β -lactamases in well-characterized clinical strains of *Klebsiella* spp. *J Clin Microbiol* 2005; 43 : 4168-71.
18. Bauernfeind A, Schneider I, Jungwirth R, Sahly H, Ullmann U. A novel type of AmpC beta-lactamase, ACC-1, produced by a *Klebsiella pneumoniae* strain causing nosocomial pneumonia. *Antimicrob Agents Chemother* 1999; 43 : 1924-31.
19. Beesley T, Gascoyne N, Knott-Hunziker V, Petursson S, Waley SG, Jaurin B. *et al.* The inhibition of class C beta-lactamases by boronic acids. *Biochem J* 1982; 209 : 229-33.
20. Liebana E, Gibbs M, Clouting C, Barker L, Crifton-Hardley FA, Pleydell E, *et al.* Characterization of beta-lactamases responsible for resistance to extended-spectrum cephalosporins in *Escherichia coli* and *Salmonella enterica* strains from food-producing animals in the United Kingdom. *Microb Drug Resist* 2004; 10 : 1-9.
21. Powers RA, Blazquez J, Scott Weston G, Morosini M, Baquero F, Shoichet BK. The complexed structure and antimicrobial activity of a non-beta-lactam inhibitor of AmpC β -lactamase. *Protein Sci* 1999; 8 : 2330-7.
22. Steward CD, Rasheed JK, Hubert SK, Biddle JW, Raney PM, Anderson GJ, *et al.* Characterization of clinical isolates of *Klebsiella pneumoniae* from 19 laboratories using the National Committee for Clinical Laboratory Standards extended-spectrum betalactamase detection methods. *J Clin Microbiol* 2001; 39 : 2864-72.

Reprint requests: Prof. Uma Sekar, Department of Microbiology, Sri Ramachandra Medical College & Research Institute (Deemed University), Porur, Chennai 600116, India
e- mail: umasekar02@yahoo.co.in