Indian J Med Res 136, August 2012, pp 237-241

AmpC β-lactamases in nosocomial isolates of *Klebsiella pneumoniae* from India

Varsha Gupta, Karthikeyan Kumarasamy*, Neelam Gulati, Ritu Garg, Padma Krishnan* & Jagdish Chander

*Department of Microbiology, Government Medical College & Hospital, Chandigarh & *Department of Microbiology, Dr ALM PG IBMS, University of Madras, Chennai, India*

Received June 2, 2010

***Background & objectives*: AmpC β-lactamases are clinically significant since these confer resistance to cephalosporins in the oxyimino group, 7-α methoxycephalosporins and are not affected by available β-lactamase inhibitors. In this study we looked for both extended spectrum β-lactamases (ESBL) and AmpC β-lactamases in *Klebsiella pneumoniae* clinical isolates.**

***Methods*: One hundred consecutive, non-duplicate clinical isolates of *K. pneumoniae* collected over a period of one year (June 2008 - June 2009) were included in the study. An antibiotic susceptibility method was used with 10 antibiotics for Gram-negative infections which helped in screening for ESBL and AmpC β-lactamases and also in confirmation of ESBL production. The detection of AmpC β-lactamases was done based on screening and confirmatory tests. For screening, disc diffusion zones of cefoxitin <18 mm was taken as cefoxitin resistant. All cefoxitin resistant isolates were tested further by AmpC disk test and modified three dimensional test. Multiplex-PCR was performed for screening the presence of plasmid-mediated AmpC genes.**

***Results*: Of the 100 isolates of *K. pneumoniae* studied, 48 were resistant to cefoxitin on screening. AmpC disk test was positive in 32 (32%) isolates. This was also confirmed with modified three dimensional test. Indentation indicating strong AmpC producer was observed in 25 isolates whereas little distortion (weak AmpC) was observed in 7 isolates. ESBL detection was confirmed by a modification of double disk synergy test in 56 isolates. Cefepime was the best cephalosporin in synergy with tazobactam for detecting ESBL production in isolates co-producing AmpC β-lactamases. The subsets of isolates phenotypically AmpC β-lactamase positive were subjected to amplification of six different families of AmpC gene using multiplex PCR. The sequence analysis revealed 12 CMY-2 and eight DHA-1 types.**

***Interpretation & conclusions*: Tazobactam was the best β-lactamase inhibitor for detecting ESBL in presence of AmpC β-lactamase as this is a very poor inducer of AmpC gene. Amongst cephalosporins, cefepime was the best cephalosporin in detecting ESBL in presence of AmpC β-lactamase as it is least hydrolyzed by AmpC enzymes. Cefepime-tazobactam combination disk test would be a simple and best method in detection of ESBLs in *Enterobacteriaceae* co-producing AmpC β-lactamase in the routine diagnostic microbiology laboratories.**

Key words AmpC enzyme - β-lactamases - ESBL - *Klebsiella pneumoniae* - plasmid mediated - resistance

In the Ambler structural classification of β-lactamases, AmpC enzymes belong to Class C, while in the functional classification scheme of Bush these are assigned to group 3[1,2]. These enzymes can be chromosomal or plasmid encoded. AmpC β-lactamases are clinically significant[3], since these confer resistance to cephalosporins in the oxyimino group (cefotaxime, ceftazidime, ceftriaxone), 7-α methoxy cephalosporins (cefoxitin or cefotetan) and are not affected by available β-lactamase inhibitors (clavulanate, sulbactam, tazobactam)[4]. Plasmid mediated AmpC β-lactamases differ from chromosomal AmpCs in being uninducible and are typically associated with broad multidrug resistance[5]. Plasmid mediated AmpC β-lactamases are present in isolates of *Klebsiella pneumoniae, K. oxytoca, Salmonella* spp., *Proteus mirabilis, Escherichia coli, Citrobacter freundii* and *Enterobacter aerogenes*[6]. In *E. coli,* high level production of chromosomally mediated AmpC β-lactamases is also present[7]. Most of the clinical laboratories are not looking for plasmid mediated AmpC β-lactamases routinely. The treatment options for infections caused by organisms expressing AmpC β-lactamases are limited. Thus there is a need for detecting AmpC β-lactamases so as to avoid therapeutic failures. The reports of AmpC β-lactamases from India are still limited. We, therefore, undertook this study to look for both extended spectrum β-lactamase (ESBL) and AmpC β-lactamases in *K. pneumoniae* clinical isolates in which chromosomal AmpC enzymes are conspicuously absent.

Material & Methods

One hundred consecutive, non-duplicate isolates of *K. pneumoniae* obtained from blood, urine and pus samples received in the microbiology department of the Government Medical College and Hospital, Chandigarh, over a period of one year (June 2008 - June 2009) were included in the study. The identification of the isolates was done by standard biochemical methods[8]. An antibiotic susceptibility method was devised using 10 antibiotics covering most Gram-negative infections and helped in screening for ESBL and AmpC β-lactamases and also confirmation of ESBL production. The antibiotics used were ceftazidime, cefotaxime, cefepime, cefoxitin, amikacin, ciprofloxacin, imipenem, amoxicillin-clavulanic acid, cefoperazone-sulbactam and piperacillin-tazobactam (Hi-Media Ltd., Mumbai). The discs were placed at a distance of 2 cm from each other. This arrangement of discs provided a modification of double disc approximation test for ESBL detection[9]. Also cefepime was included as one of the cephalosporins for ESBL detection as high level AmpC production has minimal effect on the activity of cefepime, making this drug as a more reliable detection agent in the organisms co-producing AmpC and ESBLs[10]. The three β-lactamase inhibitors used helped in comparing the detection of ESBL production, and also in studying the effect of detection of ESBL in presence of AmpC production. The organisms were considered to be producing ESBL when the zone of inhibition around any of the extended spectrum cephalosporin or cefepime discs showed a clear cut increase towards the piperacillin- tazobactam, cefoperazone-sulbactam or amoxicillin-clavulanate discs[11]. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 (supplied by Hi-Media Ltd., Mumbai, India) were used as control strains.

The sensitivity pattern of these isolates to various antibiotics was studied by the Kirby-Bauer disk diffusion method[12] according to Clinical Laboratory Standards Institute (CLSI) guidelines[13]. The detection of AmpC β-lactamases was done based on screening tests and confirmatory tests. For screening, disc diffusion zones of cefoxitin <18 mm was taken as cefoxitin resistant[14]. All cefoxitin resistant isolates were tested further by AmpC disk test and modified three dimensional test[7,14]. Plates were examined for either an indentation or flattening of the zone of inhibition in the disc test. In the modified three dimensional tests, three different kinds of results were recorded. Isolates that showed clear distortion of zone of inhibition of cefoxitin were taken as AmpC producers. Isolates with no distortion were taken as AmpC non-producers, and isolates with minimal distortion were taken as intermediate producers[13].

In 20 of the total 32 phenotypically AmpC positive isolates, AmpC genes were also looked for using multiplex-PCR[15].

PCR screening: A multiplex-PCR was performed to screen the presence of six families of plasmid mediated AmpC β-lactamases[15]. DNA was prepared by emulsifying 2-5 colonies in 100 µl of molecular-grade water; 1 µl of the DNA template was added to 20 µl of the PCR reaction mixture. The cycling conditions were: initial DNA release and denaturation at 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 64°C for 30 sec and 72°C for 30 sec, followed by a final elongation step at 72° C for 5 min. The PCR products were analyzed by gel electrophoresis with 2 per cent agarose in TBE buffer with ethidium bromide (5 µg/

ml) and visualized by UV-transillumination. A 100 bp DNA ladder (Invitrogen, USA) was used as a marker. Multiplex-PCR yielded the products with sizes of 462 and 405 bp which corresponded to CMY-Z and DHA-1 group enzymes, respectively. PCR-products were purified and sequenced by Big-dye chain-termination method[15].

Results & Discussion

Of the 100 isolates of *K. pneumoniae* studied, 48 were resistant to cefoxitin on screening. These 48 isolates when further taken up for AmpC disk test showed positive results for 32 (32%) isolates. This was also confirmed with modified three dimensional test. Indentation indicating strong AmpC producer was observed in 25 isolates whereas little distortion (weak AmpC) was observed in seven isolates. The negative isolates were confirmed as negative by three dimensional test for AmpC production.

ESBL detection was confirmed by a modification of double disc synergy test in 56 isolates. Cefepime was the best cephalosporin in synergy with tazobactam for detecting ESBL production in isolates co-producing AmpC β-lactamases. All 56 isolates were detected with cefepime-tazobactam.

The sensitivity pattern of these isolates showed 100 per cent sensitivity to imipenem and 70 per cent to amikacin. Cefoperazone-sulbactam (80%) and piperacillin-tazobactam (63%) showed a good sensitivity *in vitro* but were not prescribed as the isolate was an AmpC producer. Ciprofloxacin gave a sensitivity of 37 per cent, followed by cefepime (32%), cefotaxime (13%), ceftazidime (11%) and amoxicillin-clavulanic acid (9%). The subsets of isolates phenotypically AmpC β-lactamase positive were subjected to amplification of six different families of AmpC gene using multiplex PCR. The sequence analysis revealed 12 CMY-2 and eight DHA-1 types.

Despite the discovery of ESBLs and AmpC β-lactamases more than a decade ago, still many clinical laboratories have problems in detecting ESBLs and AmpC β-lactamases. Currently, CLSI documents do not indicate the screening and confirmatory tests that are optimal for detection of AmpC β-lactamases; although for ESBL detection, CLSI has laid down certain guidelines for *E. coli* and *Klebsiella* spp. and in 2003, *Proteus* species was added[13]. Several methods are available to test for AmpC[16-20]. In spite of many phenotypic tests, isoelectric focusing[21] and genotypic characterization based on multiplex PCR[15] are considered gold standard as the results with the phenotypic tests can be ambiguous and unreliable. Attempts are being made to standardize some phenotypic method, as for most of the diagnostic laboratories it is difficult to do molecular techniques on a routine basis.

In our study, tazobactam proved to be the best β-lactamase inhibitor in detecting ESBL production followed by sulbactam, clavulanic acid being the poorest. As it has been reported that in organisms producing both ESBL and AmpC together, clavulanic acid may induce expression of high level AmpC production, and may then antagonize rather than protect the antibacterial activity of the partner β-lactam[22,23], thus masking any synergy arising from inhibition of an ESBL. Much better inhibition is achieved with the sulphones, such as tazobactam and sulbactam which are preferable inhibitors for ESBL detection tests in AmpC producing organisms[24]. In this study we were not able to get good results with amoxicillin-clavulanic acid, so piperacillin-tazobactam combination was used. Cefepime was the best cephalosporin in detecting ESBL in presence of AmpC producer as it is less affected by AmpC β-lactamases. Cefotaxime and ceftazidime were not able to detect ESBL because even if the isolates produced ESBL β-lactamase, it was not able to show any potentiation of zones in presence of β lactamase inhibitor as the AmpC β-lactamase gave a resistant zone to the cephalosporin.

The studies from India showed a wide range of figures of AmpC production. In 2003, the reports from Karnataka[25] and Delhi[14] showed 3.3 and 20.7 per cent of Gram-negative bacteria (GNB) to be AmpC producers based on phenotypic tests. In 2005, the figures from Delhi[26] and Kolkata[27] were 36.06 and 6.7 per cent, respectively for GNB showing AmpC production. Further, based on molecular techniques, a report from Delhi[28] showed the figures to be as high as 50 per cent. In 2007, from Chennai[29] the figures were 47.3 per cent. Recently a study showed high level of AmpC β-lactamases in cases with complicated urinary tract infection (UTI)[30]. Black *et al* showed 31 per cent of AmpC production in cefoxitin nonsusceptible strains by AmpC disc test[7]. Our study showed a high rate of AmpC β-lactamases production. The drug of choice remains carbapenems in AmpC producers as the β-lactam - β-lactamases inhibitor combinations fail even if these show sensitivity *in vitro*. Our study showed 100 per cent sensitivity to imipenem.

The limitation of our study was that cefoxitin resistance in *K. pneumoniae* might not only be due to AmpC production, it could be due to certain carbapenemases, a few Class A β-lactamases and by decreased levels of production of outer membrane porins. Although all our isolates were carbapenem susceptible but other modes remain a possibility.

The various families of plasmid mediated AmpC enzymes cannot be distinguished based on phenotypic tests. Thus gold standard for detection of plasmid mediated AmpC β-lactamase enzymes was multiplex PCR utilizing six primer pairs.

In conclusion, by using ten antibiotics on a single plate, we could test for ESBL and screen for AmpC at the same time. Tazobactam was the best β-lactamase inhibitor in detecting ESBL production followed by sulbactam, clavulanic acid being the poorest. Cefepime was the best cephalosporin in detecting ESBL in presence of AmpC producer as it was less affected by AmpC β-lactamases. Our study showed a high rate (32%) of AmpC β-lactamases production in *K. pneumoniae*.

References

1. Ambler RP. The structure of β-lactamases. *Philos Trans R Soc Lond B* 1980; *289* : 321-31.
2. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for β-lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother* 1995; *39* : 1211-33.
3. Cordron PE, Moland ES, Thomson KS. Occurrence and detection of AmpC β-lactamases among *E. coli, Klebsiella pneumoniae* and *Proteus mirabilis* isolates at Veterans medial centre. *J Clin Microbiol* 2000; *38* : 1791-6.
4. Phillipon A, Arlet G, Jacoby GA. Plasmid determined AmpC type β-lactamases. *Antimicrob Agents Chemother* 2002; *46* : 1-11.
5. Barnaud G, Arlet G, Verdet C, Gaillot O, Langrange PH, Philippon A. *Salmonella enteritidis* AmpC plasmid mediated inducible - β- lactamases (DHA-1) with an ampR gene from *Morganella morganii*. *Antimicrob Agents Chemother* 1998; *42* : 2352-8.
6. Thomson KS. Controversies about extended spectrum and AmpC β-lactamases. *Emerg Infect Dis* 2001; *7* : 333-6.
7. Black JA, Moland ES, Thomson KS. AmpC disk test for detection of plasmid mediated AmpC β-lactamases in *Enterobacteriaceae* lacking chromosomal AmpC β-lactamases. *J Clin Microbiol* 2005; *43* : 3110-3.
8. Colle JG, Miles RS, Watt B. Tests for identification of bacteria. In: Colle JG, Fraser AG, Marimon BP, Simmon A, editors. *Mackie & MacCartney practical medical microbiology*, 14th ed. Edinburgh: Churchill Livingstone; 1996. p. 151-79.
9. Sturenberg E, Mack D. Extended spectrum β-lactamases: implications for the clinical microbiology laboratory, therapy, and infection control. *J Infect* 2003; *47* : 273-95.
10. Tzouvelekis LS, Vatopoulos AC, Katsanis G, Tzelepi E. Rare case of failure by an automated system to detect extended-spectrum β lactamase in a cephalosporin-resistant *Klebsiella pneumoniae* isolate. *J Clin Microbiol* 1999; *37* : 2388.
11. Khan MKR, Thukral SS, Gaind R. Evaluation of a modified double-disc synergy test for detection of extended spectrum β-lactamases in AmpC β-lactamases producing *Proteus mirabilis*. *Indian J Med Microbiol* 2008; *26* : 58-61.
12. Bauer AW, Kirby WMM, Sherris JC, Jurck M. Antibiotic susceptibility testing by a standardized single disc method. *Am J Clin Pathol* 1966; *45* : 493-6.
13. Clinical Laboratory Standards Institute. *Performance standards for antimicrobial and susceptibility testing,* 8th ed. Approved standards M2-A8. Wayne, Pa: Clinical Laboratory Standards; 2003.
14. Manchanda V, Singh NP. Occurrence and detection of AmpC β-lactamases among Gram-negative clinical isolates using a modified three dimensional test at Guru Teg Bahadur Hospital, Delhi, India. *J Antimicrob Chemother* 2003; *51* : 415-8.
15. Perez-Perez FJ, Hanson ND. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* 2002; *40* : 2153-62.
16. Thompson KS, Sanders CC. Detection of extended spectrum β-lactamases in members of the family *Enterobacteriaceae*: comparison of the double disk and three dimensional tests. *Antimicrob Agents Chemother* 1992; *36* : 1877-82.
17. Pitout JD, Riesbig MD, Venter EC, Church DL, Hanson ND. Modification of the double disk test for detection of *Enterobacteriaceae* producing extended spectrum and AmpC β-lactamases. *J Clin Microbiol* 2003; *41* : 3933-5.
18. Black JA, 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.
19. Cordron PE. Inhibitor-based methods for detection of plasmid-mediated AmpC β-lactamases in *Klebsiella* spp. *Escherichia coli* and *Proteus mirabilis*. *J Clin Microbiol* 2005; *43* : 4163-7.
20. Nasim K, Elsayed S, Pitout JDD, Conly J, Church DL, Gregson DB. New method for laboratory detection of AmpC β-lactamases in *Escherichia coli* and *Klebsiella pneumoniae.* *J Clin Microbiol* 2004; *42* : 4799-802.
21. Mathew M, Harris AM, Marshall MJ, Ross GW. The use of analytical isoelectric focusing for detection and identification of β-lactamases. *Gen Microbiol* 1975; *88* : 169-78.
22. Akova M, Yang Y, Livermore DM. Interactions of tazobactam and clavulanate with inducibley and constitutively expressed class 1 β-lactamases. *J Antimicrob Chemother* 1990; *25* : 199-208.
23. Lister PD, Gardner VM, Sanders CC. Clavulanate induces expression of the *Pseudomonas aeruginosa* AmpC cephalosporinase at physiologically relevant concentrations and antagonizes the antibacterial activity of ticarcillin. *Antimicrob Agents Chemother* 1999; *43* : 882-9.
24. Thomson KS, Sanders CC, Moland ES. Use of microdilution panels with and without β-lactamase inhibitors as a phenotypic

test for β-lactamases production among *Escherichia coli, Klebsiella* spp, *Enterobacter* spp, *Citrobacter frundii*, and *Serratia marcescens*. *Antimicrob Agents Chemoth* 1999; *43* : 1393-400.

25. Ratna AK, Menon I, Kapur I, Kulkarni R. Occurrence and detection of AmpC β-lactamases at a referral hospital in Karnataka. *Indian J Med Res* 2003; *118* : 29-32.

26. Singhal S, Mathur T, Khan S, Upadhyay DJ, Chugh S, Gaind R, *et al*. Evaluation of methods for AmpC β-lactamase in Gram-negative clinical isolates from tertiary care hospitals. *Indian J Med Microbiol* 2005; *23* : 120-4.

27. Arora S, Bal M. AmpC β-lactamase producing bacterial isolates from Kolkata hospital. *Indian J Med Res* 2005; *122* : 224-33.

28. 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.

29. Hemalatha, Padma M, Sekar U, Vinodh TM, Arunkumar AS. Detection of AmpC β-lactamases production in *Escherichia coli* and *Klebsiella* by an inhibitor based method. *Indian J Med Res* 2007; *126* : 220-3.

30. Taneja N, Rao P, Jitender A, Dogra A. Occurrence of ESBL and AmpC β-lactamases and susceptibility to newer antimicrobial agents in complicated UTI. *Indian J Med Res* 2008; *127* : 85-8.

Reprint requests: Dr Varsha Gupta, Professor, Department of Microbiology, Government Medical College & Hospital
Sector-32, Chandigarh 160 030, India
e-mail: varshagupta_99@yahoo.com