Indian J Med Res 127, January 2008, pp 85-88

Occurrence of ESBL & Amp-C β-lactamases & susceptibility to newer antimicrobial agents in complicated UTI

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Received November 16, 2006

***Background & objectives*: Production of extended spectrum β-lactamases (ESBLs) and AmpC β-lactamases are the most common mechanisms of antimicrobial resistance in Gram negative bacilli. A prospective study was undertaken to know the occurrence of ESBL and AmpC producing strains and their antibiotic susceptibilities to newer agents to guide empirical therapy for complicated urinary tract infections.**

***Methods*: Over a period of five months (January to May 2003), organisms grown in pure culture and in significant numbers from urine sample were identified by standard biochemical tests and antibiotic susceptibility determined by disc diffusion method. Gram-negative bacilli that were resistant to third generation cephalosporins, ciprofloxacin and gentamicin/amikacin were defined as highly drug resistant uropathogens (HDRU). HDRU were further tested for ESBL and AmpC phenotypes.**

***Results*: Uropathogens were isolated in significant numbers in 1979 (21.8%) of the total 9072 samples, of which 438(22.1%) were HDRU. Two hundred and five consecutive HDRU isolates were tested for ESBL production and 36.5 per cent were found to be ESBL producers. The highest positivity was found to be in *Klebsiella* spp. (51.2%), followed by *Escherichia coli* (40.2%), *Enterobacter aerogenes* (33.4%) and *Pseudomonas aeruginosa* (27.9%). Both ESBL producers and non producers showed a high degree resistance to piperacillin (93.1 and 90.9%), amoxycillin-clavulanic acid (93.4 and 90.9%), aztreonam (79.4 and 78%), cefepime (76.7 and 78%), and ampicillin-sulbactam (76.7 and 70.4%). The most effective antibiotics for ESBL producers were imipenem (8.2% resistance), piperacillin-tazobactam (9.5%) and ceftazidime-clavulanic acid (23.2%). Among ESBL non-producers, piperacillin-tazobactam (31.06%), ceftazidime-clavulanic acid (49.2%) and imipenem (11%) were less effective when compared to ESBL producers. Fifty three piperacillin and piperacillin-tazobactam positive and 20 negative isolates were further tested for AmpC production and found that all 53 positive isolates were also positive by for AmpC β-lactamase.**

***Interpretation & conclusions*: Overall, 22.1 per cent of our isolates were highly drug resistant, and ESBL producers could explain only 36.5 per cent of HDRU in our study. Therefore, we assume that AmpC β–lactamases are more important in our setting. Based on our finding a test using discs containing piperacillin and piperacillin-tazobactam (PtPc) disc at a distance of 20 mm would act as a useful screening procedure for AmpC production as AmpC β-lactamase producers are more susceptible to tazobactam as compared to clavulanic acid.**

Key words AmpC β-lactamases - drug resistance - ESBL - uropathogens

Antibiotic resistance in uropathogens is increasing worldwide in both outpatients as well as hospitalized patients. It varies according to geographic locales and is directly proportional to the use and misuse of antibiotics. Understanding the impact of drug resistance is of critical importance as the changing rate of antibiotic resistance has a large impact on the empirical therapy of urinary tract infections[1,2]. The various mechanisms of drug resistance in Gram-negative bacilli include extended spectrum beta lactamase (ESBL) production[3], AmpC lactamase production[4], efflux mechanisms[5] and porin deficiency[6]. Amongst the mechanisms of resistance to third generation cephalosporins, production of ESBLs and AmpC β-lactamases are the most common[7]. AmpC β-lactamases are clinically important because they confer resistance to narrow-, expanded-, and broad-spectrum cephalosporins, β-lactam-β-lactamase inhibitor combinations and aztreonam. Group1 AmpC β-lactamases are poorly inhibited by clavulanic acid; however, they are inhibited by cloxacillin[8]. Many clinical laboratories currently test *Escherichia coli* and *Klebsiella* spp. for production of ESBLs but do not attempt to detect plasmid mediated AmpC β-lactamases (also known as imported, transmissible, foreign, or mobile AmpC β-lactamases). These enzymes are typically associated with multiple antibiotic resistances, leaving a few therapeutic options[7].

Since both ESBL and AmpC β lactamase are encoded on plasmids and confer a selective advantage to strains harbouring these in a hospital setting. It is important to know the occurrence of ESBL and AmpC producing strains as well as their antibiotic susceptibilities to newer agents to guide empirical therapy for various infections. In an earlier study conducted in 2002 at our centre, overall 26.6 per cent of urinary isolates were ESBL producers[9]. Keeping in view the high-level drug resistance in our setting, we conducted this study to determine the occurrence of ESBL and AmpC producing strains among the highly drug resistant uropathogens at our center.

Material & Methods

A prospective study was conducted over a period of five months (January to May 2003) at the Enteric Laboratory of the Department of Medical Microbiology, Postgraduate Institute of Medical Education & Research (PGIMER), Chandigarh. Urine samples were collected from patients suspected to have urinary tract infection at the discretion of the provider. These included clean catch midstream urine, catheter, suprapubic and nephrostomy samples. Urine (1 µl) was inoculated onto cysteine lactose electrolyte deficient (CLED, Hi-Media Laboratories, Mumbai, India) medium. Organisms grown in pure culture and in significant numbers ($>10^5$ cfu/ml for midstream urine samples and $>10^3$ for other types of samples) were identified by standard biochemical tests[10] and antibiotic susceptibility by disc diffusion method[11]. Gram-negative bacilli that were resistant to third-generation cephalosporins (cefotaxime/ceftazidime), ciprofloxacin and gentamicin/amikacin were defined as highly drug resistant uropathogens (HDRU). These isolates were further tested for ESBL and AmpC phenotype. Susceptibilities of these HDRUs to newer antibiotics and β-lactam-β-lactamase combinations were also determined. Only the first isolate from each patient was included.

ESBLs were detected by the confirmatory method of National Committee for Clinical Laboratory Standards (NCCLS)[11] now known as Clinical and Laboratory Standards Institute (CLSI) using cefotaxime (30 µg) and ceftazidime (30 µg) and a disc of cefotaxime plus clavulanic acid (30 and 10 µg) and ceftazidime and clavulanic acid (30/10 µg) (Oxoid, UK) placed at a distance of 20 mm on a lawn culture (0.5 McFarland inoculum size) of suspected ESBL producing clinical isolate on Mueller-Hinton Agar (MHA, Hi-Media, Mumbai). *Escherichia coli* ATCC 25922 was used as the negative control and *Klebsiella pneumoniae* ATCC 700603 was used as the ESBL positive control (kindly provided by Dr M. K. Lalitha, Christian Medical College, Vellore). ESBL production was inferred if the inhibition zone increased by 5 mm towards the cefotaxime plus clavulanic acid disc or ceftazidime plus clavulanic acid disc in comparison to the third generation cephalosporin disc alone[11].

Screening for the inducible AmpC β-lactamase was done by the disc antagonism test[12] by placing cefoxitin disc (30 µg, Oxoid, UK) at a distance of 20 mm from ceftazidime (30 µg) on the surface of MHA. β-lactamase inducibility was recognized by blunting of the ceftazidime zone adjacent to cefoxitin disc.

AmpC enzyme production was tested by a modified three-dimensional test as described by Manchanda & Singh[13]. Briefly, 10-15 mg fresh overnight growth from MHA was taken in a microcentrifuge tube. Peptone water was added and centrifuged at 800 g for 15 min. Crude enzyme extract was prepared by repeated freeze thawing for five to seven times. Lawn cultures of *E. coli* ATCC 25922 were prepared on MHA plates and cefoxitin (30 µg) discs were placed on the plate. Linear

slits were cut using a sterile surgical blade 3 mm away from the cefoxitin disc; 10 µg enzyme extract was added to a well made at the outer edge of the slit. The plates were incubated at 37°C overnight. Quality control was achieved by using known AmpC positive isolate of *K. pneumoniae* ATCC 700603.

Plasmid mediated AmpC beta lactamases production was further confirmed by the AmpC disk test as described by Black *et al*[7]. The test is based on use of tris-EDTA to permeabilize a bacterial cell and release β-lactamases into the external environment. Amp C disks were prepared in-house by applying 20 µl of a 1:1 mixture of saline and 100×tris-EDTA (Sigma-Aldrich Corp., USA) to sterile filter paper disks. The surface of a Mueller-Hinton agar plate was inoculated with a lawn of 0.5 McFarland suspension of cefoxitin susceptible *E. coli* ATCC 25922. Several colonies of each test organism were applied to a disk. A 30 µg cefoxitin disk (Oxoid, UK) was placed on the inoculated surface of the Mueller-Hinton agar. The inoculated AmpC disk was then placed almost touching the antibiotic disk with the inoculated disk face in contact with the agar surface. The plate was then inverted and incubated overnight at 35°C in ambient air. After incubation, plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (positive result), or the absence of a distortion, indicating no significant inactivation of cefoxitin (negative result).

Antibiotic susceptibility to newer antibiotics (µg) like piperacillin (100), cefepime (30), imipenem (10 µg), aztreonam (30 µg) and β-lactam β-lactamase inhibitor combinations such as amoxycillin-clavulanic acid (30/10), ampicillin-sulbactam (10/10), piperacillin-tazobactam (100/10) and ceftazidime-clavulanic acid (30/10) was performed by the NCCLS method[11]. *E. coli* ATCC 25922 was used as the susceptible control strain.

Results & Discussion

A total of 9072 urine samples (midstream, catheter, suprapubic, nephrostomy) were collected from the same number of patients during the period of study. Among these, uropathogens were isolated in significant numbers in 1979 (21.8%) samples, of which 438 (22.1%) were highly drug resistant uropathogens. Two hundred and five consecutive HDRU isolates were tested for ESBL production and 75 (36.5%) were found to be ESBL producers. The highest positivity was found to be in *Klebsiella* 20/39, (51.2%), followed by *E. coli* 31/77 (40.2%), *Enterobacter* 7/21 (33.4%) and *Pseudomonas aeruginosa* 12/43 (27.9%). These 205 HDRU isolates (both ESBL producers and non-producers) were tested for susceptibility to newer antibiotics and β lactam-β lactamase inhibitor combinations. ESBL producing isolates showed a high degree resistance to piperacillin (93.1%), amoxycillin-clavulanic acid (93.4%), aztreonam (79.4%), cefepime (76.7%), ampicillin-sulbactam (76.7%) and ticarcillin (60.2%). The most effective antibiotics were imipenem (8.2% resistance), piperacillin-tazobactam (9.5%) and ceftazidime-clavulanic acid (23.2%). Similarly ESBL non-producers also showed a high degree of resistance to piperacillin and amoxycillin-clavulanic acid (90.9% each), followed by cefepime (78%), aztreonam (78%), and ticarcillin (75.7%). Among ESBL non-producers, piperacillin-tazobactam (31.06%), ceftazidime-clavulanic acid (49.2%) and imipenem (11%) were less effective when compared to ESBL producers.

Of the 205 isolates that were tested for ESBL production, 140 were inhibited by piperacillin-tazobactam. These isolates were tested by placing the piperacillin and piperacillin-tazobactam discs at a distance of 20 mm and a > 5 mm increase in the zone towards piperacillin-tazobactam was observed. Of these 140 isolated, 66 were also inhibited by clavulanic acid *i.e.*, they were ESBL positive by our test.

Fifty three piperacillin and piperacillin-tazobactam positive and 20 negative isolates were further tested for AmpC production. It was found that all the 53 positive isolates were also positive by modified 3-D test for AmpC β-lactamase and the disk test using tris-EDTA. Only one isolate of *Enterobacter cloacae* produced inducible β-lactamase as tested by cefoxitin disc antagonism test.

In this study we focused on the multi- drug resistant uropathogens and their sensitivity pattern to newer antibiotics. Overall, 22.1 per cent of our isolates were highly drug resistant and *E. coli (*32.6%), *Klebsiella* spp (16.6%) and *P. aeruginosa* (28.5%) accounted for the most resistant isolates. The ability to detect and distinguish between AmpC and ESBL-producing organisms has epidemiological significance and may have therapeutic importance as well[7]. ESBL producers could explain only 36.5 per cent of HDRU in our study. Therefore, we assume that AmpC lactamases are more important in our setting. Only one isolate was positive for inducible AmpC beta lactamases. This shows that the chromosomally encoded AmpC beta-lactamases are rare in our setting.

NCCLS has issued guidelines for testing and interpretation of ESBLs in routine laboratory[11] whereas no such guidelines are currently available for AmpC lactamases. Modified three-dimensional test has been described for testing AmpC enzymes in Gram-negative bacterial isolates[13,14]. In routine laboratory, three-dimensional test for detection of AmpC is not feasible as it is cumbersome and time consuming. What is required is a simple screening method like a combined disc method for ESBL. Black *et al*[7] have recently described a disc test using 100×tris -EDTA to detect plasmid mediated AmpC beta-lactamases. Based on our finding we presume that a test using discs containing piperacillin and piperacillin-tazobactam (PtPc) disc at a distance of 20 mm would act as a useful screening procedure for AmpC production.

In our study both ESBL producers and non producers showed similar level of resistance to the antibiotic tested, except to piperacillin and piperacillin-tazobactum. This can be explained by the fact that AmpC β-lactamase producers are more susceptible to tazobactam as compared to clavulanic acid[4]. Both ESBL producers and non-producers showed high level resistance to cefepime. AmpC producers are susceptible to fourth generation cephalosporins like cefepime while ESBL producers are variably resistant to fourth-generation cephalosporins[15]. A high inoculum effect has been reported with cefepime for ESBL-producing and AmpC-producing isolates of *Enterobacteriaceae*[16]. The available data suggest that carbapenems are more effective than cefepime in treating serious infections that involve large numbers of AmpC-producing organisms[17]. In conclusion, routine screening for ESBL and AmpC production need to be done for all uropathogens causing complicated urinary tract infection.

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