Antimicrobial Resistance Mediated through Amp C β- Lactamases

Anjali Sunildatta Jog[1]

ABSTRACT

Antimicrobial resistance is a global problem. Developing country like India is facing it more where infectious disease burden is high.Penicillins and Cephalosporins are widely used antibiotics to deal with various infections. These antibiotics have four membered β-lactam ring in it's molecule which is essential for it's antibacterial activity.Bacteria produce Extended spectrum β-lactamase (ESBL) which made the newer generation of antibiotics ineffective. Amp C β-lactamases are important cephalosporinases encoded on chromosomes of many Enterobacteriaceae and they confer resistance to third generation cephalosporins and β-lactamase inhibitor/ β-lactam antibiotics. Amp C β-lactamases are either chromosomally or plasmid mediated. Various phenotypic tests are used by researchers like Amp C disk test, Modified Three-dimensional test, Boronic acid disk test method, Cefoxitin Agar Medium (CAM), Amp C E test, Cefoxitin Hodge test to detect Amp C β-lactamase producing organisms.Multiplex PCR is used for genotypic detection of Amp C β-lactamase. Different studies have shown Amp C β-lactamase production in *E.coli, K.pneumoniae, Proteus spp., Citrobacterspp., Pseudomonasspp, Providentiaspp., Acinetobacter*.Routine screening for detection of Amp C β-lactamase producing organisms should be done to avoid treatment failure and increased medical care cost.

Keywords: β-lactam Antibiotics, Amp C β-lactamase, Enterobacteriaceae, Amp C Disk Test, Modified Three Dimensional Test, Boronic Acid Disk Test, Cefoxitin Agar Medium, Amp C E test, PCR.

INTRODUCTION

Resistance to a variety of antimicrobial agents is emerging in bacterial pathogens throughout the world. Several resistance mechanisms have been emerged among many gramnegative bacteria especially related to β- lactam antibiotics is through production of β- lactamase enzyme that breaks down the structure of β- lactam ring of penicillin and other antimicrobial with a similar structure.

After penicillin, many newer β- lactam antibiotics which were resistant to hydrolytic action of β- lactamases were developed. However β- lactamase enzymes emerged which were capable of destroying these newer antimicrobial agents. Genetic control of β- lactamase production resides either on plasmid or chromosome.

Hundreds of β- lactamases have been described. β-lactamases are mostly classified accordingly to two general schemes:Ambler molecular classification and Bush-Jacoby-Medieros functional classification system. Ambler divides β- lactamases into four major classes(A-D). Class A, C, D are serine β- lactamases Class B comprises metallo β-lactamases.[1] Class B and class C enzymes though having broad spectrum of activity are being always encoded by chromosomes. They have been found to be carried by plasmids. Plasmids carrying genes for Amp C β-lactamases often carry multiple other resistances including genes for resistance to aminoglycosides, chloramphenicol, quinolones, sulfonamides, tetracycline.[2]

In 1980, Ambler classified β- lactamases on the basis of amino acid sequences which divides it into four groups (A, B, C, D). Molecular classification of β- lactamases is based on the nucleotide and amino-acid sequences in these enzymes.[3]

Class A – These are serine proteases and are inhibited by clavulanic acid. e.g. Extended spectrum β-lactamases (ESBL), penicillinase.

Class B – These are metallo β- lactamases which require bivalent transition metal Zn^{++} for their activity. These are inhibited by clavulanic acid.

Class C – Cephalosporinases, either inducible or constitutive found on chromosomes of gram negative bacteria. These are not inhibited by clavulanic acid. (Amp C class of enzymes).

Class D – Oxacillinases. These are moderately inhibited by clavulanic acid.

Although the structural approach is the easiest and least controversial way to classify such a diverse set of enzymes, a functional classification provides the opportunity to relate these various enzymes to their clinical role i.e. by providing selective resistance to different classes of β-lactam antibiotics.[4] Amp C β- lactamases are cephalosporinases that are poorly inhibited by clavulanic acid. They have been described in pathogens e.g. *Klebsiella pneumoniae, E.coli, Proteus mirabilis, Citrobacter freundii, Acinetobacter, and Pseudomonas aeruginosa.* Plasmid mediated Amp C β-lactamases have arisen through the transfer of chromosomal genes for inducible Amp C β- lactamase on to plasmids.

Amp C β- lactamases have gain importance since last 1970's, as one of the mediators of antimicrobial resistance in gram negative bacilli. Amp C β- lactamases are of two types- plasmid mediated and chromosomal or inducible Amp C.[5] Chromosomal Amp C enzymes are seen in organisms such as *Citrobacterfreundii, Enterobacter cloacae, Morgenellamorganii, Hafniaalvei and Serratia marcescens*

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and are typically inducible by antibiotics like cefoxitin but poorly induced (if at all) by third or fourth generation cephalosporins.[6]

In late 1980's, these inducible chromosomal genes were also detected on plasmids and were transferred to organisms which typically do not express chromosomal β- lactamases such as *Klebsiella spp., E.coli, Salmonella spp*.[7]

Amp C β- lactamases confer resistance to 7α methoxy cephalosporins such as cefoxitin or cefotetan and are usually not inhibited by commercial β- lactamase inhibitors. All plasmid mediated Amp C β- lactamases have similar substrate profile to parenteral enzymes from which they appear to be derived.[8]

In 1981, the sequence of Amp C gene from *E.coli* was reported.[9] Plasmid mediated Amp C β- lactamases were reported in 1988.[10]

Parez-Parez and Hanson(2002) developed a multiplex PCR assay for detection of plasmid encoded Amp C gene. This PCR based method, can distinguish hyper producing chromosomal Amp C *E.coli* isolates from *E.coli* isolates encoding an imported plasmid Amp C gene.[11]

Papanicolaou et al described novel plasmid mediated β- lactamase, MIR -1, with an isoelectric point 8.4, which was found in 11 clinical isolates of *K.pneumoniae* and subsequently cloned into *E.coli*. MIR-1 conferred resistance to penicillins and broad spectrum cephalosporins, including cefoxitin and ceftibuten but not to cefepime, meropenem and imipenem, cefiperome. The substrate and inhibition profiles of MIR-1 were compared with those of class A, TEM-1 and TEM-6 plasmid mediated β- lactamases and class C chromosomal β- lactamases from *E.coli* and *Enterobacter cloacae*.[12]

The first proof that Amp C β- lactamases had been captured on plasmid was provided by Papanicolaou et al, who described transmissible resistance to α- methoxy and oxyimino- β- lactams mediated by an enzyme (MIR-1) with biochemical properties of chromosomal β- lactamase and showed that cloned gene (MIR-1) was 90% identical to Amp C gene of *E, cloacae*.

Plasmid mediated Amp C β- lactamases including MIR/ ACT, DHA, MOX, CIT, ACC and FOX are derived from chromosomal Amp C genes of Enterobacteriaceae which display structural and functional similarity to their chromosomal origins.[13] The first isolation of bla_{DHA}(DHA7) gene from *E.cloacae* has been recently reported in Spain.[14]

The increase in antibiotic resistance among gram negative bacilli mediated through Amp C β- lactamases shows that their laboratory detection and characterization are important for clinical, epidemiological and infection control point of view.

MATERIAL AND METHODS

Researchers have used different methods to detect Amp Cβ- lactamase production in bacteria.

Amp C β- lactamase detection methods**:** There are screening methods and confirmatory methods for it'sdetection.

a) Amp C screening tests-

1) **Disk approximation technique**[15]- Lawn culture of test isolate (0.5 McFarland) is done over Mueller- Hinton agar plate (MHA). Antibiotic disks used are Imipenem (10µg), Cefoxitin (30µg) Amoxycillin- clavulanate disk (20/10µg) as inducing substrates and Ceftazidime disk (30µg) as reporter substrate. Ceftazidime disk is placed at the center of MHA plate. Other disks are applied at a distance of 20 mm from ceftazidime disk. Any blunting or flattening of zone of inhibition between Ceftazidime disk and the inducing substrate is interpreted as positive result for Amp C β- lactamase.

2) Modified double disk approximation method[16]**–** Alawnculture of test strain (0.5 McFarland) is done over Mueller- Hinton agar plate. Disk of Ceftazidime (30µg) and Cefotaxime (30µg) are placed adjacent to clavulanic acid (10µg) and Cefoxitin (30µg) at a distance of 20 mm from each other. Incubate at 37^0C for 18-24 hours. Isolates showing blunting of Ceftazidime or cefotaxime zone of inhibition adjacent to Cefoxitin disk or showing reduced susceptibility to either of above test drugs (Ceftazidime or Cefotaxime) are considered as '**screen positive'.**

3)Disk antagonism test[16,17] **–** A lawnculture of test isolate (0.5 McFarland) is put on Mueller- Hinton agar plate. Ceftazidime (30µg) diskand Cefoxitin (30µg) disk are placed 20 mm apart from centre to centre. Incubate at 37^0C for 18-24 hours. Isolates showing blunting of Ceftazidime zone of inhibition adjacent to Cefoxitin disk are considered as screen positive.

4) Amp C screening using dilution method[18]**-** MICs of Cefoxitin are determined for all study isolates by agar dilution following CLSI methods. Doubling dilutions of Cefoxitin are prepared in Mueller Hinton agar, with or without addition of a fixed concentration of Cloxacillin (100 µg/ml) to provide two fold concentration ranging from 0.125 µg/ml to 256 µg/ml. Isolate with four fold reduction in Cefoxitin MIC in presence of Cloxacillinis considered to be positive for Amp C β-lactamase.

b) Confirmatory test for Amp C β-lactamase:

1) Amp C disk test[16,19,20] **-** A lawn culture of standard strain of *E.coli* ATCC 25922 is prepared on Mueller Hinton Agar plate. Blank filter paper disk (6mm in diameter, usingWhatmann filter paper no. 2) are made sterile and moistened with sterile saline (20µl). This disk is inoculated with several colonies of test organism. Place the inoculated disk beside Cefoxitin disk (30µgm) almost touching on inoculated plate. Incubate overnight at 35^0C. Interpretation – Positive test appear as flattening or indentation of Cefoxitin inhibition zone in the vicinity of test disk. Undistorted zone is taken as negative.

2) Modified three dimensional test[21] **–** Fresh overnight growth from Mueller Hinton Agar plate is transferred to a preweighed sterile micro centrifuge tube. The bacterial mass is suspended in peptone water and pelleted by centrifugation at 3000 rpm for 15 min. Crude enzyme extract is prepared by repeated freeze thawing of bacterial pellet (approximately 10

cycles). A Lawn culture of *E.coli* ATCC 25922 is prepared on MHA plate and Cefoxitin disk is placed on the plate. Linear slits (3cm) are cut using sterile surgical blade, 3mm away from Cefoxitin disk. At the other end of the slit a circular well is made and the enzyme extract is loaded. A total of 30-40µl of extract is loaded in the well at a 10µl increment. The plates are kept upright for 5-10 min. until the liquid dries and are incubated at 37^0C for 24 hrs. Enhanced growth of surface organism at a point where slit is inserted at the zone of inhibition of Cefoxitin is considered positive three dimensional test. It is taken as evidence for presence of Amp C β-lactamase.

3) Boronic acid disk test method[5] **–** Prepare 0.5 Mcfarland bacterial suspension from an overnight blood agar plate. Inoculate surface of MHA plate using this suspension. Place Cefoxitin disk (30µgm)on inoculated surface of MHA. Using sterile tips, dispense 20 µl of 15 µl /ml phenyl boronic acid onto disk. Let the disk absorb it. Incubate overnight 35^0C. Interpretation -After overnight incubation, compare the zone diameter around antibiotic disk with added boronic acid and the antibiotic disk alone. An organism that demonstrates a defined increase (≥5mm) in zone diameter around the antibiotic disk containing added boronicacid, is considered to be Amp C producer.

4) Cefoxitin Agar Medium (CAM)[15] **–** For CAM assay, crude enzyme extracts are prepared by freezing and thawing cell pellets from centrifuged trypticase soy broth culture. Mueller Hinton agar with Cefoxitin concentration 2, 4, 8, 16 µgm/ml is used. Plates are inoculated with *E.coli* ATCC 25922 (std. strain) to cover entire surface. Circular wells with diameter of 5mm are made in agar and filled with 30µl of extract from individual strains. Positive and negative control strains are inoculated on each plate. Incubate at 35^0C aerobically overnight. A zone growth around the periphery of a well is positive CAM assay and evidence for Amp C enzyme. Advantages – easier to perform, interprete and allows for testing multiple isolates on single plate.

5) AmpC E test[22] - Double sided E strips containing cefotetan (0.5-32 µgm/ml) with or without cloxacillin are applied on Mueller Hinton agar plate which is evenly inoculated with 0.5 McFarland suspension of the test inoculate according to manufacturer's instructions. After overnight incubation an eight fold reduction in cefotetan MIC (minimum inhibitory concentration) in presence of cloxacillin, the presence 'phantom' zone of inhibition or deformation of inhibition ellipse is interpreted as presence of $Bla_{Amp\ c}$

6) Cefoxitin Hodge Test[22,23] - The surface of Mueller Hinton agar plate is inoculated with a lawn of the indicator strain *E.coli* ATCC 25922. After drying of lawn culture, a test strain is heavily streaked from the center of plate to the periphery and a Cefoxitin disk is placed at the center. The plate is incubated overnight at 37^0C. The presence of explicit growth of the indicator strain in the inhibition zone along with the test strain is interpreted as positive.

7)Molecular detection method[11] **-** Multiplex PCR for detection of family specific plasmid mediated Amp C β-lactamase.

DISCUSSION

Amp C β-lactamases are clinically important cephalosporinases encoded on the chromosome of many *Enterobacteriaceae* and a few other organisms where they mediate resistance to cephalothin, cephazolin, cefoxitin, most penicillins and β-lactamase inhibitor / β-lactam combinations.[2] Enzyme expression confers resistance to third generation cephalosporins and is particularly problematic as isolates may initially appear susceptible to these agents but become resistant during therapy.[2] Accurate identification of Amp C β-lactamase production is significant in order to establish adequate antibiotic therapy.[24]

Various studies are done by researchers to detect Amp C β-lactamases in gram negative bacilli by using different phenotypic methods. Here are few studies showing prevalence of Amp C β-lactamases in gram negative bacilli. A study in 2008, done by ParulSinha, Rajanisharma et al, showed 24% Amp C β-lactamase producers in *E.coli* isolates. In their study, Amp C Disc test was used to detect Amp C β-lactamase producers. It is simple, easy and rapid screening test for detection of Amp C β-lactamase producers.[20] V. Hemlatha, M. Padma et al conducted a study in 2007 in Chennai, for detection of Amp C β-lactamase production in *Escherichia coli* and *Klebsiella* by using Inhibitor based method. They reported 47.3% isolates of *Escherichia coli* and *Klebsiella* showing Amp C β-lactamase production, four fifth of which occurred in in combination with ESBL. All these isolates were susceptible to imipenem.[5] A study in Tertiary care hospital, Rawalpindi, Pakistan reported 52.43% of Amp C β-lactamase producing isolates of *E.coli and K.pneumoniae* in UTI by Disc Approximation Test (DAT) method. The sensitivity of DAT was 88% with specificity 92%. They reported implementation of simple, accurate and cost effective diagnostic techniques like DAT in routine laboratory investigations in developing countries, which can help to eradicate and control antimicrobial resistance due to Amp C β-lactamases.[25]

A study in Canada done in 2004, by K.Nasim, S.Elsayed used a new Cefoxitin Agar Medium (CAM) based assay to compare previously published modified 3-dimensional (M3D)assay for detection of Amp C production in *E.coli and K.pneumoniae*.[15] They showed that 54 out of 55 *E.coli* strains and 1 in 6 *K. pneumoniae* strains were positive by M3D method. The results of CAM with 4 µgm cefoxitin / ml was equivalent to M3D method for detecting Amp C production in *E.coli and K. pneumoniae*. This method is easier to perform, interprete and allows for testing multiple isolates on a single plate. A study of Amp C β-lactamases among Pseudomonas and Acinetobacter by Sana Jamali et al in 2015, reported *Pseudomonas spp*. showed high resistance to cefoxitin (91.78%) while in case of *Acinetobacter spp.* maximum resistance was 94.12%. Out of total isolates showing cefoxitin resistance 79.52% were positive for Amp C production by modified 3 dimensional extract method.[26]

A research done in PRIST University, Tanjavar, Tamilnadu, India in 2012 by J. Vinoth et al revealed the distribution of Amp C β-lactamases in *Proteus spp.* and *Providentia spp.* in hospital. Amp C β-lactamase production was seen in *P. vulgaris* 52%, *P.mirabilis* 35%, *P.stuartii* 11%, *P rettgeri* 3.5% by using phenotypic methods like Amp C disk test and Cefoxitin Hodge Test. The strains were highly sensitive to carbapenems, namely imipenem and meropenem.[23]

Manchanda Singh in 2003, reported Amp C production in *K. pneumoniae* 33.3%, *E.coli* 28%, *P.mirabilis* 33.3%.[21] Multiplex PCR test remains Gold Standard test for identification of Amp C producers using six primer pairs.[11]

A study conducted by Priyadarshini et al, at Tertiary Care Hospital, Banglore showed that *Citrobacterkoseri* and *Citrobacterfreundii* were the commonest species isolated and Amp C mediated resistance in these isolates was reported as 76%.[27]

Thus various studies are conducted to detect Amp C β-lactamase production in gram negative bacilli by using different tests. Overall, Amp C β-lactamase producing strains are resistant to β-lactamase inhibitors like clavulanic acid but show 100% sensitivity to imipenem, meropenem. So carbapenem is the drug of choice.

CONCLUSION

Resistance of gram negative bacilli to 3rd generation cephalosporins mediated through Extended spectrum β-lactamases (ESBL) and Amp C β-lactamases is increasing. Detection of Amp C β-lactamase producers is important to select appropriate antibiotic at the earliest in management of patient. However there are no Clinical and Laboratory Standards Institute (CLSI) guidelines for detection of Amp C mediated resistance in gram negative bacilli and hence it poses a problem.[28] Regular reporting of Amp C β-lactamase producing organisms will help to start proper antibiotic at the earliest and will reduce health care cost and treatment failure.

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