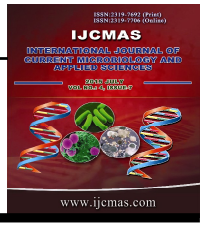
Int.J.Curr.Microbiol.App.Sci (2015) 4(8): 391-398

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 8 (2015) pp. 391-398**
http://www.ijcmas.com



Original Research Article

Plasmid Mediated AMP C β Lactamase Producing Gram Negative isolates in Neonatal Septicemia

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Keywords

Amp C β lactamase, neonatal Septicemia, Gram negative isolates

A B S T R A C T

Neonatal septicemia is a major cause of morbidity and mortality in the newborn. Emergence of plasmid mediated AmpC β lactamase producing strains of gram negative bacteria, as one of the leading causes of septicemia often complicates the clinical and therapeutic outcome. The present study was undertaken to investigate the prevalence of plasmid mediated AmpC β lactamase in bacteria isolated from neonatal septicemia cases along with their antimicrobial sensitivity pattern. Blood samples were collected from 273 suspected cases of neonatal septicemia. Apart from susceptibility testing, all the gram negative isolates resistant to Cefoxitin and third generation Cephalosporins were subjected to phenotypic tests for AmpC production. Among the positive test (n = 91) samples, 59 were gram negative isolates. Plasmid mediated AmpC β lactamase was detected in 10 isolates. Based on the species *Klebsiella pneumoniae* 9(25%) and *Escherichia coli* 1 (11%) harbored AmpC enzymes. Results indicate that routine plasmid mediated AmpC β lactamase detection should be made imperative and empirical use of third generation Cephalosporins must be discouraged.

Introduction

The most common causes of death in neonatal period is infection (32%) followed by birth asphyxia (29%) and prematurity (24%).Neonatal infections currently cause 1.6 million deaths in developing countries (Choudhury *et al.,* 2007). Multidrug resistant Gram negative bacilli belonging to the family Enterobacteriaceae have been increasingly responsible for infections among the neonates admitted to the Neonatal Intensive Care Unit in many countries including India and *Klebsiella pneumoniae* constitutes a majority of these pathogens (Krishna *et al.,* 2007). The most common mechanism of resistance in Gram negative bacteria is by the production of ß lactamases which inactivate β lactam antibiotics. Among the β lactamases, Extended Spectrum β lactamases (ESBL) and AmpC β-lactamases are most commonly produced. AmpC β lactamases are more important because they confer resistance to narrow, expanded, broad-spectrum cephalosporins, β lactam β lactamase inhibitor combinations and aztreonam, Earlier the AmpC β-lactamases were

presumed to be chromosomally encoded. Recently the plasmid mediated AmpC β lactamase has also arisen through the transfer of chromosomal genes for AmpC β lactamase on to plasmids. This transfer has resulted in plasmid-mediated AmpC beta lactamases in isolates of *E. coli*, *Klebsiella pneumoniae*, *Salmonella sp* and *Proteus mirabilis* thus providing a new mechanism of resistance for those originally AmpC deficient bacterial strains (Suranjana Arora and Manjusribal, 2005; Neelam Taneja *et al.*, 2008 and Shahid *et al.*, 2004). Prevalence of this resistance mechanism appears to be increasing and has been responsible for nosocomial outbreaks, avoidable therapeutic failures and outbreaks of multidrug resistant Gram negative pathogens that require expensive control efforts (Kenneth S Thomson, 2001). Hence this prospective study is conducted to evaluate the antibiotic resistance pattern and plasmid mediated AmpC ß lactamase production in gram negative isolates of neonatal septicemia.

Material and Methods

Blood samples were collected from 273 clinically suspected septicemia neonates admitted to Neonatal Intensive Care Unit, over a period of 14 months. Samples were inoculated in to brain heart infusion broth. The broth was incubated aerobically at 37°C. A subculture was done after 18 hours; if no growth was obtained, the bottles were tested for seven consecutive days. Any sign of growth was followed-up by subculture. Media used for sub culturing included chocolate agar, blood agar and MacConkey agar (HiMedia). Isolates were identified by using standard biochemical test. Antimicrobial susceptibility tests were performed using the Kirby Bauer disc diffusion method as per CLSI guidelines. Antimicrobials used were Amikacin (30μg), Cefotaxime (30 μg), Ceftazidime (30 μg), Ceftriaxone (30 μg) Cefuroxime (30μg), Ciprofloxacin (5μg), Gentamicin (10μg), Imipenem (10μg), Trimethoprim/ sulfamethoxazole (1.25/23.75μg), Cefoperazone/ sulbactam (75/30μg) and Piperacillin (100/μg). All disks were obtained from Hi - Media.

Modified three dimensional test

AMPC enzyme production was tested by a modified three-dimensional test as described by Manchanda and Singh (2003). 10 to 15 mg fresh overnight growth from MHA was taken in a centrifuge tube. Peptone water was added and centrifuged 3000 rpm 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 Muller Hinton Agar plates and Cefoxitin (30 μg) discs were placed on the plate. Slits were cut using a sterile surgical blade 3 mm away from the Cefoxitin disc. 10μg enzyme extract was added to a well made at the outer edge of the slit. The plates were incubated at 37°C overnight. 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).

AMPC disk test

Plasmid mediated AmpC beta lactamases production was further tested by the AmpC disk test as described by Jennifer A Black and Ellen Smith Moland (2005). AmpC disks were prepared by applying 20ml of a 1:1 mixture of saline and 100×tris-EDTA to sterile filter paper disks. Several colonies of each test organism were applied to a disk. 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. A 30 mcg cefoxitin disc was placed on the inoculated surface of the Mueller-Hinton agar. The AmpC disk was then placed almost touching the antibiotic disc with the inoculated disc face in contact with the agar surface. The plate was then inverted and incubated overnight at 35°C. 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).

Confirmation of plasmid mediation in AmpC β lactamases production

Conjugation assay

Transfer of cefoxitin resistance and AmpC β lactamase production

Transconjugation experiments were done as per the procedure by Subha *et al.* (2003) with few modifications. Mating was performed with *Escherichia coli* K12 J53 Azi^r (kindly provided by Professor Dr Raju B Appala, PSG Medical College & Hospital, Coimbatore) as the recipient strain. Overnight brain heart infusion (BHI) broth cultures (0.5 Mac Farland turbidity matched) of the donor and the recipient strains were mixed in the ratio of 1:10…200 µl of the mixed culture after overnight incubation was spread on the trypticase soy agar plates containing 250 µg/ml of sodium azide and 10 µg/ml of cefotaxime with the help of the L-rod. The plates were incubated overnight at 37^{o}C.

The plates were then observed for the presence of the transconjugants, which were then biochemically identified. The transconjugants were then tested for their antibiotic sensitivity pattern by the disc diffusion technique.

Ethical consideration

Approval was obtained from the ethical committee prior to conduct of this study. Informed consent was obtained from the parents of neonates.

Results and Discussion

Out of 273 suspected cases of neonatal septicaemia, 91(33%) were positive in culture for pathogenic organisms. Gram negative organisms constituted 64 % (n=59) of the total 91 bacterial isolates whereas Gram positive organisms constituted 36 % (n =32). *Klebsiella pneumoniae* 36(40%) was the most common organism among the GNB isolates followed by *Escherichia coli* 9(10%), *Enterobacter spp* 7(7%), *Pseudomonas aeruginosa* 5 (5%), and *Acinetobacter spp* 2 (2%). Antibiotic susceptibility testing revealed Gram negative organisms were 100% sensitive to Imepenam followed by 96% to Cefoperazone - sulbactum, 83% to Cefepime and 61% to Amikacin (Table 1).

Of the 59 gram negative rods, 15(25%) were found to be resistant to Cefoxitin and third generation Cephalosporins. Gram negative isolates (n=15) resistant to 3rd Generation Cephalosporins and Cefoxitin were tested for AmpC β lactamase production by the Modified Three Dimensional Extract Method and AmpC disk method. AmpC β lactamase production was observed in 25% and 22% of *K. pneumoniae* and *E. coli* respectively (Table 2).

The AmpC β lactamase producing 9 *Klebsiella* isolates and 2 *Escherichia coli* isolates were subjected to transconjugation assay to demonstrate whether the AmpC β lactamase is plasmid mediated one by using *Escherichia coli* K12 J53 Azi^r (Recipient strain) as per Abigail et al with few

modifications. The results showed transfer of cefoxitin resistance (marker of AmpC) to the recipient strain from all the 9 AmpC β lactamase producing 9 *Klebsiella pneumoniae* strains. In case of *Escherichia coli,* only one strain showed the transfer of cefoxitin resistance in the transconjugation assay (Table 2).

Septicemia is still a major cause of mortality and morbidity in the neonates. Nowadays Gram negative microorganisms are increasingly reported as the major cause of neonatal septicemia particularly in Asian countries. The emergence of multidrug resistant Gram-negative bacteria is mainly due to inadvertent use of broad-spectrum antibiotics. Majority of the Gram negative and Gram positive organisms isolated in the present study were resistant to one or more antibiotics. This is in concurrence with other studies by Tallur *et al.* (2000) and Kurien Anil Kuruvilla *et al.* (1998). Present study revealed a very high degree of resistance of Gram negative bacilli not only to commonly used antibiotics, but also predominantly to broad spectrum Cephalosporins. These findings were compatible with other studies by Joshi *et al.* (2000) and Movahedian *et al.* (2006). This is probably due to emergence of new variant of the existing strain as a result of mutations or may be plasmid borne.

In the present study, resistance rate of *Klebsiella pneumoniae, E. coli, pseudomonas* and *Enterobacter* to third generation cephalosporins were 67%, 56%, 20% and 14%, respectively. *Acinetobacter* spp were 100% sensitive to third generation Cephalosporins. These findings were similar to the study by Ziba *et al.* (2003) reported that resistance rate of *Klebsiella pneumoniae*, *E. coli, Enterobacter* to third generation Cephalosporins were 60%, 30% and 20%. *Pseudomonas* and *Acinetobacter* were 100% sensitive to third generation Cephalosporins.

Amongst the mechanisms of resistance to third generation Cephalosporins, production of ESBLs and AmpC ß lactamases are the most common. 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 (Neelam Taneja *et al.,* 2008).

Currently, CLSI documents do not indicate the screening and confirmatory tests that are optimal for detection of AmpC β lactamases. However, several studies have been done on various test methods namely, the Three Dimensional test, Modified Double Disk test, AmpC disk test (Nigel *et al.,* 2005). Inhibitor based method employing inhibitors like boronic acids, Broth micro dilution method and Cefoxitin Agar method. In spite of many phenotypic tests, isoelectric focusing and genotypic characterization by various molecular methods are considered gold standard as the results with the phenotypic tests can be ambiguous and unreliable (Hemalatha *et al.,* 2007). However these techniques are expensive and the requiring reagents are costly and not easily available. Hence simple, reliable and inexpensive phenotypic methods like Modified three dimensional test method and AmpC disk test method were used in this study. Singal S Mathur (2005) and Parul Sinha *et al.* (2008) also reported that AmpC disc test was easier, reliable and rapid method of detection of isolates that harboring AmpC β lactamases.

AmpC β lactamases production was tested by the modified three dimensional extract method and AmpC disc method. Isolates harboring AmpC β lactamases were tested for plasmid mediation by conjugation assay.

In present study, 9 (25 %) *Klebsiella i*solates and 2 (22 %) *Escherichia coli* isolates were positive for AmpC β lactamase production.

In *K. pneumoniae* AmpC β lactamase production is only plasmid mediated but in *E. coli* hyper production of chromosomal mediated AmpC and plasmid mediated. Transfer of cefoxitin resistance to recipient strain was observed in all AmpC producing *Klebsiella* isolates and 1 of 2 AmpC producing *Escherichia coli* isolate.

Cefoxitin resistance in non AmpC producing *Klebsiella pneumoniae* is often due to porin deficient mutants. The interruption of a porin gene by insertion sequences is a common type of mutation that causes the loss of porin expression and increased Cefoxitin resistance in *Klebsiella pneumoniae*. In *E. coli* hyper production of chromosomal AmpC with OMP F porin loss can produce similar resistance phenotype (Ratna *et al.,* 2003; Ananthan and Subha, 2005).

Table.1 Antibiotic susceptibility pattern of Gram negative isolates

Drugs	*Klebsiella*	*E. coli*	*Entero bacter*	*Pseudom onas*	*Acineto bacter*
Gentamycin	14%	33%	28%	20%	50%
Amikacin	44%	78%	43%	80%	100%
Cephalexin	33%	44%	72%	80%	100%
Cefotaxime	33%	44%	86%	80%	100%
Ceftazidime	44 %	56%	86%	60%	100%
Ceftriaxone	31%	44%	72%	80%	100%
Cefoxitin	67%	78%	86%	80%	100%
Cefepime	67%	66%	100%	80%	100%
Aztreonam	44%	44%	86%	80%	100%
Ciprofloxacin	64%	66%	86%	80%	100%
Ofloxacin	72%	56%	86%	80%	100%
Imepenam	1oo%	100%	100%	100%	100%
Piperaziline	33%	56%	-	80%	-
Cs+sulbactum	91%	100%	-	-	-
Amox-Cal	67%	78%	86%	80%	100%

Table.2 Distribution of AmpC β lactamase producers

Bacterial isolates	Cefoxitin resistance	Modified three dimensional test	AmpC disk test	Conjugation assay
Klebsiella pneumoniae (36)	12(33%)	9 (25%)	9(25%)	9(25%)
Escherichia coli (9)	2 (22%)	2 (22%)	2(22%)	1 (11%)
Enterobacter (7)	1 (14%)	-	-	-

Figure.1 Modified three dimensional test

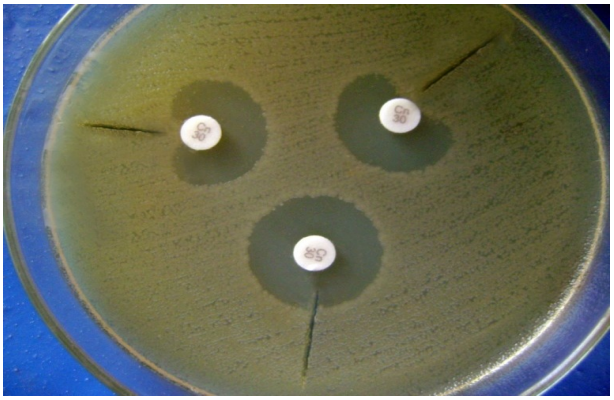
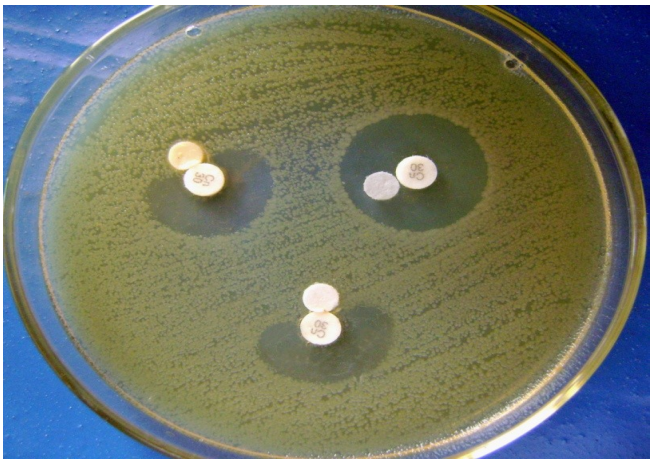


Figure.2 AmpC disk test



In the present study, Plasmid mediated AmpC β lactamase production was observed in 25% of *Klebsiella* isolates and 11% of *Escherichia coli* isolates. Similar findings were observed by Subha *et al.* (2003) from Chennai reported that, twenty eight isolates (24.1%) of *Klebsiella* spp. and 12 (37.5%) of *E. coli* were plasmid mediated AmpC β lactamase producers. Manchanda and Singh (2003) from Delhi also reported 20.7 per cent of the clinical isolates were harboring AmpC β lactamases.

In contrast Ratna *et al.* (2003) from Karnataka reported sixteen (3.3%) isolates were positive for plasmid mediated AmpC β lactamases. Based on the species 9 (3.3%) *Escherichia coli*, 4 (2.2%) *Klebsiella pneumoniae*, 2 (5%) *Citrobacter freundii* and 1 (5.5%) isolate of *Enterobacter aerogenes* harbored AmpC enzymes.

Plasmid mediated AmpC β lactamase production observed in 22% of Gram negative isolates from neonatal septicemia. AmpC detection has epidemiological significance and as well as therapeutic importance. Since AmpC β lactamase production is frequently accompanied by multidrug resistance, therapeutic options become limited. Also failure to identify AmpC β lactamase producers may lead to inappropriate antimicrobial treatment and may result in increased mortality.

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