

# *Klebsiella Pneumoniae* in Septicemic Neonates with Special Reference to Extended Spectrum $\beta$ -lactamase, AmpC, Metallo $\beta$ -lactamase Production and Multiple Drug Resistance in Tertiary Care Hospital

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## ABSTRACT

**Background:**  $\beta$ -lactamases viz., extended spectrum  $\beta$ -lactamase (ESBL), AmpC, and metallo  $\beta$ -lactamase (MBL) production in *Klebsiella pneumoniae* has led to a serious concern about septicemic neonates in Neonatal Intensive Care Units due to high resistance against commonly used antimicrobials.

**Purpose:** To study the prevalence of ESBL, AmpC, and MBL production in *K. pneumoniae* isolates in neonatal septicemia, to check antimicrobial susceptibility to various drugs including tigecycline; and to assess burden of multiple drug resistance (MDR).

**Materials and Methods:** Total 24 clinical isolates of *K. pneumoniae* isolated from 318 blood samples of suspected cases of neonatal septicemia were studied. Isolates were screened for ESBL, AmpC, and MBL production by Clinical and Laboratory Standards Institute (CLSI) disk method, AmpC ceftazidime screen, and imipenem, meropenem, ceftazidime disk screen respectively; and confirmation was done by CLSI phenotypic disk confirmatory test, AmpC sterile disk method, and imipenem ethylenediamine tetracetic acid double disk synergy test respectively. Antimicrobial susceptibility was determined by Kirby–Bauer's disk diffusion method. Efficacy of tigecycline was evaluated using United States Food and Drug Administration guidelines.

**Results:** Of the 24 *K. pneumoniae* isolates, co-production of AmpC + MBL was found in more number of isolates (67%) ( $P < 0.0001$ ) compared to single enzyme production (ESBL and MBL 8% both, AmpC 12.5%). Rate of resistance for penicillins and cephalosporins was highest. Susceptibility was more for imipenem, co-trimoxazole, and meropenem. Nonsusceptibility to tigecycline was low (21%). A total of 23 (96%) isolates were MDR.

**Conclusions:** Routine detection of ESBL, AmpC, and MBL is required in laboratories. Carbapenems should be kept as a last resort drugs. Trend of tigecycline susceptibility has been noted in the study. Continued monitoring of susceptibility pattern is necessary to detect true burden of resistance for proper management.

**Key words:** AmpC, extended spectrum  $\beta$ -lactamase, *Klebsiella pneumoniae*, metallo  $\beta$ -lactamase, neonatal septicemia

## INTRODUCTION

Neonatal deaths account for over a one-third of the global burden of child mortality. Sepsis is the significant cause of morbidity and mortality in neonates. Prematurity, low birth weight and prolonged hospitalization are the predisposing factors for neonatal sepsis. Inadequate space, shortage of staff, high occupation rates, widespread use of

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	<b>DOI:</b> 10.4103/0974-2727.151689

antimicrobial agents and increased susceptibility of the population are responsible for early colonization and subsequent infection by virulent strains resulting in high morbidity and mortality.<sup>[1]</sup> Approximately one million deaths a year occurring in the neonatal period (0–28 days) are caused by infection, accounting for over 25% of global neonatal deaths and 99% of these deaths occur in developing countries.<sup>[2]</sup> The World Health Organization estimates that one million deaths per year are due to neonatal sepsis and that 42% of these deaths occur in the 1<sup>st</sup> week of life.<sup>[3]</sup>

Most common bacterial organisms responsible for neonatal septicemias in developed countries include coagulase-negative Staphylococcus and group B Streptococcus while in developing countries like Pakistan, India, Nigeria, Bangladesh, are *Escherichia coli*, *Klebsiella*, *Enterobacter* etc.<sup>[4]</sup> According to the data of National Neonatal Perinatal Database 2005, 40% of neonatal deaths were ascribed to sepsis in India.<sup>[5]</sup> *Klebsiella pneumoniae* was the most frequently isolated pathogen (32.5% and 30.1%) among both intramural and extramural admissions.<sup>[6]</sup>

In developing countries, multiple drug resistant (MDR) organisms causing neonatal sepsis are increasing, and *K. pneumoniae* is often reported in this context. *K. pneumoniae* is resistant to numerous antimicrobial agents by different mechanisms, including expression of extended spectrum  $\beta$ -lactamases (ESBLs), AmpC  $\beta$ -lactamases, 16S rRNA methylases, aminoglycoside modifying enzymes and carbapenemases.<sup>[7]</sup> Emergence of ESBLs is a vital factor in the treatment of infections associated with sepsis. ESBL producing isolates show resistance to  $\beta$ -lactam antibiotics, including third-generation cephalosporins, in addition; they often exhibit resistance to other classes of drugs such as aminoglycosides, co-trimoxazole, tetracycline and fluoroquinolones. Thus, they pose an intimidating challenge with limited therapeutic options, particularly in resource-challenged countries.<sup>[5]</sup>

AmpC  $\beta$ -lactamases are cephalosporinases that are poorly inhibited by clavulanic acid and can be differentiated from ESBLs by their ability to hydrolyze cephamycins. These days, AmpC is also being found in *K. pneumoniae* isolates. The increased prevalence of both ESBLs and AmpC in bacterial isolates creates prerequisite for laboratory testing methods that can accurately detect the presence of these enzymes.<sup>[8]</sup>

Recently, metallo  $\beta$ -lactamases (MBLs) have emerged as one of the most feared resistance mechanisms due to their ability to hydrolyze virtually all  $\beta$ -lactam agents including

carbapenems. Its spread on highly mobile gene elements in nosocomial pathogens limits the therapeutic options.<sup>[9]</sup> It has thus become essential to be alert about the trend in susceptibility patterns of organisms to save the therapies.

To combat the escalating rate of drug resistance, newer drugs like tigecycline are being discovered. Tigecycline, an expanded broad-spectrum antibiotic; is a semi-synthetic glycylicycline derived from minocycline and has activity against Gram-negative pathogens that are refractory as a result of both efflux and ribosomal protection mechanisms. In addition, organisms that are resistant to other antimicrobial classes do not exhibit cross-resistance to tigecycline, affirming its potential therapeutic use for ESBL and carbapenemase producing *Enterobacteriaceae* infections.<sup>[10]</sup> Due to the promising microbiological, pharmacodynamic and pharmacokinetic profile against significant pathogens, its role is being evaluated for empirical therapy in seriously ill-patients.

The present study was conducted (1) To know the incidence of ESBL, AmpC, and MBL production among clinical isolates of *K. pneumoniae* in neonatal sepsis, (2) To identify the susceptibility pattern to the number of antimicrobials including carbapenems and especially tigecycline, (3) To assess the burden of MDR. To the best of our knowledge, this is the first comprehensive study of three different  $\beta$ -lactamases producing *K. pneumoniae* implicated in neonatal sepsis in a developing country setting, with special reference to MDR and tigecycline susceptibility.

## MATERIALS AND METHODS

The present study was carried out from January 2010 to February 2012 in Microbiology Department of BLDEU's Shri B. M. Patil Medical College, Hospital and Research Center, Bijapur. A total of 318 blood samples from cases of neonatal septicemia sent for blood culture were included in the study. Briefly, 1–2 ml of blood was collected for culture into 10 ml of soybean casein digest broth. Cultures were processed in BacT/ALERT 3D system (bioMérieux, Marcy l'Etoile, France). These broths were incubated in system's incubator at 37°C under aerobic conditions for 7 days and observed for the growth of organisms. Any sign of growth was followed by sub-culture on MacConkey's agar and blood agar plates (HiMedia Laboratories, Mumbai) and identified using standard microbiological techniques.

### Detection of extended spectrum $\beta$ -lactamase

The screening for ESBL production was done as per Clinical, and Laboratory Standards Institute (CLSI)

recommended method.<sup>[11]</sup> Isolate showing inhibition zone of  $\leq 17$  mm for cefpodoxime (10  $\mu\text{g}$ ),  $\leq 22$  mm for ceftazidime (30  $\mu\text{g}$ ),  $\leq 27$  mm for aztreonam (30  $\mu\text{g}$ ),  $\leq 27$  mm for cefotaxime (30  $\mu\text{g}$ ),  $\leq 25$  mm for ceftriaxone (30  $\mu\text{g}$ ) was taken for ESBL confirmation. The confirmation was done by CLSI phenotypic disk confirmatory test using disks of ceftazidime (30  $\mu\text{g}$ ) and ceftazidime-clavulanic acid (30  $\mu\text{g}/10$   $\mu\text{g}$ ). Both the disks were placed at least 20 mm apart, centre to centre on Mueller-Hinton agar plate and incubated over night at 37°C. A zone difference of  $\geq 5$  mm around ceftazidime and ceftazidime-clavulanic acid was taken as ESBL positive. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as positive and negative control respectively.<sup>[11]</sup>

### Detection of AmpC $\beta$ -lactamase

All the isolates were screened for AmpC  $\beta$ -lactamase by Kirby–Bauer's disk diffusion method using cefoxitin (30  $\mu\text{g}$ ) disk. An isolate demonstrating reduced susceptibility ( $< 18$  mm zone of inhibition) to cefoxitin was taken as screen positive. Screen positive isolates were further confirmed by AmpC sterile disk test. *E. coli* ATCC 25922 was used as control.<sup>[12]</sup>

### Detection of metallo $\beta$ -lactamase

Organisms showing resistance to any of the three antimicrobials viz., imipenem (10  $\mu\text{g}$ ), meropenem (10  $\mu\text{g}$ ), and ceftazidime (30  $\mu\text{g}$ ) was considered as a screen test positive. Screen positive isolates were confirmed by imipenem ethylenediamine tetracetic acid double disk synergy test *Pseudomonas aeruginosa* ATCC 27853 was used as control.<sup>[13]</sup>

### Antimicrobial susceptibility testing

Antibiotic susceptibility testing was done by Kirby–Bauer's disk diffusion method as per CLSI guidelines.<sup>[11]</sup> Antimicrobial disks used in the study were purchased from HiMedia Laboratories, Mumbai. Efficacy of tigecycline was evaluated by using United States Food and Drug Administration (US FDA) guidelines since, interpretive criteria for this drug was not proposed by CLSI (susceptible  $\geq 19$  mm, nonsusceptible  $\leq 14$  mm).<sup>[14]</sup> Quality control was achieved by using standard strain of *E. coli* ATCC 25922.

## RESULTS

During the study period, a total of 318 neonates were suspected to have sepsis. Of these, 114 (36%) cases were culture positive. Among these culture positives, 24 (21%)

isolates were identified as *K. pneumoniae*. Screening for ESBL gave 23 isolates positive, for AmpC it was 20 isolates and for MBL, imipenem gave 2, meropenem gave 14 and ceftazidime gave 23 isolates positive.

Results of confirmatory tests of three  $\beta$ -lactamases are given in Table 1. Among 24 isolates, all except one isolate produced either type of  $\beta$ -lactamase. AmpC production was more (12.5%) compared to ESBL and MBL production (8% both), and the occurrence of co-production of AmpC + MBL was found in 16 isolates (67%).

Results of antimicrobial susceptibility to cephalosporins and other antimicrobials are given in Tables 2 and 3. All the isolates except one, that is, total 23 (96%) isolates were found to be multiple drug resistant, as they were resistant to more than two drug classes as per CDC guidelines.<sup>[15]</sup>

## DISCUSSION

Septicemia is the major problem in the neonatal population around the world. Timely detection and initiation of proper antimicrobial therapy can be helpful in saving the life. In our study, the incidence of neonatal sepsis confirmed by

**Table 1: Results of  $\beta$ -lactamase production**

$\beta$ -lactamase	Number of isolates (%)
ESBL	02 (8)
AmpC	03 (12.5)
MBL	02 (8)
AmpC+MBL	16 (67)
Negative	01 (4)
Total	24 (100)

Test applied  $\chi^2=32.191$ ,  $P<0.0001$ . ESBL: Extended spectrum  $\beta$ -lactamase, MBL: Metallo  $\beta$ -lactamase

**Table 2: Antimicrobial resistance in *Klebsiella pneumoniae* isolates (n=24) and in  $\beta$ -lactamase producing isolates to penicillins and cephalosporins**

Antibiotic	Resistant (n=24) (%)	ESBL positive (n=2)	AmpC positive (n=3)	MBL positive (n=2)	AmpC+MBL (n=16)
Ampicillin	24 (100)	02	03	02	16
Piperacillin	22 (92)	02	02	02	15
Ticarcillin	23 (96)	02	02	02	16
Cefuroxime	23 (96)	02	02	02	16
Cefotaxime	22 (92)	01	02	02	16
Ceftriaxone	22 (92)	01	02	02	16
Ceftazidime	23 (96)	02	02	02	16
Cefoperazone	23 (96)	02	02	02	16
Cefixime	23 (96)	02	02	02	16
Cefpodoxime	23 (96)	02	02	02	16
Cefepime	22 (92)	01	02	02	16
Cefoxitin	20 (83)	02	02	01	16

ESBL: Extended spectrum  $\beta$ -lactamase, MBL: Metallo  $\beta$ -lactamase

**Table 3: Antimicrobial resistance in *Klebsiella pneumoniae* isolates (n=24) and in  $\beta$ -lactamase producing isolates to other antimicrobials**

Antibiotic	Resistant (n=24) (%)	ESBL positive (n=2)	AmpC positive (n=3)	MBL positive (n=2)	AmpC+MBL (n=16) (%)
Aztreonam	22 (92)	02	02	02	15 (94)
Imipenem	02 (8)	00	00	00	02 (12.5)
Meropenem	14 (58)	02	02	01	11 (69)
Amikacin	16 (67)	00	01	02	12 (75)
Tetracycline	16 (67)	00	03	02	11 (69)
Ciprofloxacin	17 (71)	01	02	02	12 (75)
Co-trimoxazole	06 (25)	01	01	00	04 (25)
Chloramphenicol	01 (4)	00	00	00	01 (6)
Tigecycline	05 (21)	00	01	02	02 (12.5)

ESBL: Extended spectrum  $\beta$ -lactamase, MBL: Metallo  $\beta$ -lactamase

blood culture was 36% (114/318) and we could obtain 21% ( $n = 24$ ) isolates of *K. pneumoniae*. There has been a wide variation in the growth positivity and the prevalence of *K. pneumoniae* isolates. Culture positivity from 37% to 53% and the rate of prevalence of *K. pneumoniae* from 26% to 48% has been reported by various authors.<sup>[9,16,17]</sup>

As the report of blood culture and antibiotic susceptibility are available after 72 h or more, any delay in the initiation of correct empirical therapy or improper choice of antimicrobials cannot be justified. A common practice is to use ampicillin and an aminoglycoside or a third-generation cephalosporin in neonatal sepsis. Widespread use of third-generation cephalosporins and aztreonam is believed to be the major cause of the mutations in TEM and SHV enzymes that has led to the emergence of the ESBLs.<sup>[8]</sup> ESBL screen test gave 23 (96%) isolates positive. Study done by Modi *et al.*, reported 89% positive in ESBL screening.<sup>[8]</sup> ESBL production was confirmed in only 2 isolates [Table 1] that is, its prevalence in Neonatal Intensive Care Unit was 8.3%, which was remarkably less compared to other studies (31–47.8%).<sup>[5,9,17]</sup>

There has been a paucity of data about the prevalence of AmpC producing strains in India, and very little information is available in neonatal group. In the present study 20 (83%) isolates were AmpC screen positive. Out of these, only 3 (12.5%) isolates were positive by confirmatory test [Table 1]. The rate of AmpC production was more compared to other reports from India suggesting broader spectrum of resistance in our isolates.<sup>[1,8,16]</sup>

The production of MBLs in strains largely limits therapeutic options. In screening of MBL, we found 2 (8.3%) isolates resistant to imipenem, 14 (58%) isolates resistant to meropenem, and 23 (96%) isolates resistant to ceftazidime. Confirmatory test gave positive results for only 2 (8.3%) isolates [Table 1]. In a study carried out by

Vinod Kumar *et al.*, 20% resistance to imipenem and 17% rate of MBL production was reported.<sup>[9]</sup> Similarly, Kamble *et al.*, reported 20% MBL production.<sup>[17]</sup>

In our study, co-production of two  $\beta$ -lactamases in single isolates was observed. This was an alarming finding with very large number, that is, 16 isolates (67%) producing both AmpC + MBL [Table 1]. There was a significant difference between  $\beta$ -lactamase productions with a maximum number of isolates co-producing two enzymes ( $\chi^2 = 32.191$ ,  $P < 0.0001$ ). One prominent finding was that, among these 16 isolates; 13 isolates were sensitive to imipenem, 4 isolates were sensitive to meropenem, and 2 isolates were intermediate sensitive to meropenem. These results indicate that if susceptibility reports are being referred for treatment without laboratory testing of  $\beta$ -lactamases, it can result in serious therapeutic failures. Isolates producing more than one  $\beta$ -lactamase have been reported in few studies, however rarely any study had documented multiple productions to a larger extent in the neonatal population. Study done by Singh *et al.*, reported co-production of ESBL + AmpC 14.8%, MBL + AmpC 18.5%, and ESBL + MBL 11.1% in neonatal sepsis.<sup>[16]</sup>

In most centers  $\beta$ -lactamase production is not routinely tested which ultimately results in the dissemination of  $\beta$ -lactamase producing strains in hospitals, and it remains undetected for longer periods. Inappropriate usage of antimicrobials leads to escalating percentage of  $\beta$ -lactamase production. Therefore, preventive antibiotics should be used as little as possible and specific therapeutic antibiotics should be used for short period as suggested by Singh *et al.*,<sup>[16]</sup> In conditions, wherein the use of antibiotics is necessary, rotation of antibiotic regimens is suggested.

Antimicrobial susceptibility profile of total 24 isolates and selectively of the isolates producing three  $\beta$ -lactamases and AmpC + MBL showed a high degree of resistance to antimicrobials. The resistance for penicillins, aztreonam, and cephalosporins were highest (92–100%) [Table 2]. In neonatal sepsis, the third-generation cephalosporins have been used extensively as a first-line antibiotic; as a result of which they are rendered uselessly. Our isolates showed least resistance for imipenem, co-trimoxazole, followed by meropenem (8–12.5%, 25% and 58–69% respectively) [Table 3]. The rate of resistance to the various drugs was in co-ordinance with other studies.<sup>[5,8,16,17]</sup> Amikacin and ciprofloxacin are good alternatives, and they will also provide some economic relief to the poor patients.

In our study, the lesser number of ESBL positivity may be due to high-level expression of AmpC or MBL,



which can mask the phenotype of ESBL. This masking effect can be detected by methods using boronic acid, however it has been stated that confirmation of ESBL production by clavulanic acid inhibition can be difficult not only because the activity of the  $\beta$ -lactamase varies with different substrates, but also because organisms may contain additional resistance mechanisms that can mask the presence of ESBL activity. These could include AmpC-type enzymes, porin changes, and TEM and SHV  $\beta$ -lactamases that are no longer inhibited by clavulanic acid due to mutations in the coding sequences.<sup>[18]</sup>

Though molecular techniques are the gold standard, it is difficult for most of the diagnostic laboratories to do on a routine basis. On the other hand, although phenotypic methods give little imprecise results, they are widely being used because of their simplicity and cost-effectiveness. ESBL detection by only CLSI method and lack of molecular data is the limitation of the study.

In the current scenario of burden of resistance, development of tigecycline drug is a significant advancement. In the total 24 study isolates, nonsusceptibility to tigecycline was low (5 isolates, 21%) [Table 3]. Two ESBL producing isolates were susceptible (0% resistance) to tigecycline whereas, one AmpC producing, two MBL producing, and two AmpC + MBL co-producing isolates were nonsusceptible (33%, 100%, 12.5% resistance respectively). This study showed good activity of tigecycline against *K. pneumoniae* producing ESBL, AmpC, as well as both AmpC + MBL co-producing isolates. It lost its activity against MBL producing isolates, although the numbers of MBL producers were less to arrive at the definite conclusion. The clinical efficacy of tigecycline in neonatal sepsis has not yet been established. *In vitro* evaluation of its efficacy in ESBL and MBL producing isolates in neonatal septicemia have been reported by Roy *et al.*, in two different studies.<sup>[7,19]</sup>

Tigecycline has not been approved by the FDA for the treatment of bloodstream infections.<sup>[10,20]</sup> It has unusual pharmacokinetic properties, resulting in low blood concentrations but somewhat higher tissue levels.<sup>[21]</sup> Hence, its extensive tissue distribution allows for eradication of the source of secondary bacteremia that is likely to result in the clinical resolution.<sup>[20]</sup> However, more clinical experience with this drug is required to understand its role in the treatment of infections caused by carbapenemase producing *K. pneumoniae*<sup>[10]</sup> and clinicians should be more cautious when implementing tigecycline for the treatment of suspected or proven bacteremia, and its use should be guided preferably by the minimum

inhibitory concentrations (MIC).<sup>[20]</sup> Hence the MIC study has been advocated unanimously for evaluating its efficacy. However, some studies have underlined the convenient and cost-effective disk diffusion method and proved its efficacy over the laborious MIC methods.<sup>[10,22,23]</sup>

Studies regarding the comparison of MIC and disk diffusion methods for tigecycline done by various authors have reported good correlation (92–100%).<sup>[10,22,23]</sup> Moreover, Kumar *et al.*, has stated that, the microbiology laboratories might use the relatively easier method of disc diffusion, when compared to the comparatively tedious method of MIC determination.<sup>[10]</sup> Similar to our study, use of disk diffusion method for tigecycline susceptibility has been reported by Filgona *et al.*<sup>[24]</sup>

In the present era, the emergence of MDR organisms and their spread in the community is of great concern. Infections by MDR organisms lead to prolonged hospitalization, increased mortality, morbidity and cost of treatment.<sup>[14]</sup> As per the definition of CDC guidelines formulated for “the management of MDR organisms in healthcare settings,” isolates that were resistant to two or more of the most commonly used antimicrobial classes for the treatment were placed in MDR category.<sup>[15]</sup> Isolates exhibiting co-resistance to at least any two of the following drugs were considered as MDR and these drugs were: Third generation cephalosporin (cefixime/ceftriaxone/ceftazidime), an aminoglycoside (amikacin), a fluoroquinolone (ciprofloxacin), and a folate pathway inhibitor (co-trimoxazole). In our study, all isolates except one, that is, total 23 (96%) isolates were found to be MDR. Various authors have reported high percentage of MDR in their study.<sup>[1,5,8]</sup> Our findings are in concordance with them.

## CONCLUSIONS

In our study, a high prevalence of AmpC + MBL co-producing isolates and MDR isolates shows that there is a grave trouble in the treatment of infections caused by MDR *K. pneumoniae*. Hence, antimicrobial stewardship should be implemented as it helps in the prevention of the emergence and the cross-transmission of MDR organisms. Carbapenems should be kept as a last resort drugs. Though this study has shown the trend of tigecycline susceptibility in *K. pneumoniae* causing neonatal septicemia, the clinical efficacy of tigecycline in neonatal sepsis has not yet been established and clinicians should be more cautious when implementing tigecycline for the treatment of neonatal septicemia.

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**How to cite this article:** Gajul SV, Mohite ST, Mangalgi SS, Wavare SM, Kakade SV. *Klebsiella pneumoniae* in septicemic neonates with special reference to extended spectrum  $\beta$ -lactamase, AmpC, metallo  $\beta$ -lactamase production and multiple drug resistance in tertiary care hospital. *J Lab Physicians* 2015;7:32-7.

**Source of Support:** Nil. **Conflict of Interest:** None declared.