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...
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.[1] 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.[2] 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 1st week of life.[3]

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.[4] According to the data of National Neonatal Perinatal Database 2005, 40% of neonatal deaths were ascribed to sepsis in India.[5] Klebsiella pneumoniae was the most frequently isolated pathogen (32.5% and 30.1%) among both intramural and extramural admissions.[6]

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 β-lactamases (ESBLs), AmpC β-lactamases, 16S rRNA methylases, aminoglycoside modifying enzymes and carbapenemases.[7] Emergence of ESBLs is a vital factor in the treatment of infections associated with sepsis. ESBL producing isolates show resistance to β-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.[8]

AmpC β-lactamases are cephalosporinases that are poorly inhibited by clavulanic acid and can be differentiated from ESBLs by their ability to hydrolyze cephemycins. 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.[9]

Recently, metallo β-lactamases (MBLs) have emerged as one of the most feared resistance mechanisms due to their ability to hydrolyze virtually all β-lactam agents including carbapenems. Its spread on highly mobile gene elements in nosocomial pathogens limits the therapeutic options.[10] 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 glycyclcline 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.[10] 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 β-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 (bioMe’rieux, Marcy l’Etoile, France). These broth 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 β-lactamase**

The screening for ESBL production was done as per Clinical, and Laboratory Standards Institute (CLSI)
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 β-lactamas are given in Table 1. Among 24 isolates, all except one isolate produced either type of β-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.[13]

### 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 β-lactamase production

| β-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 $\chi^2=32.193, P<0.0001$. ESBL: Extended spectrum β-lactamase, MBL: Metallo β-lactamase

### Table 2: Antimicrobial resistance in *Klebsiella pneumoniae* isolates (n=24) and in β-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                  | 02                  | 02                  | 02              |
| Piperacillin| 22 (91)              | 02                  | 02                  | 02                  | 02              |
| Ticarcillin | 23 (96)              | 02                  | 02                  | 02                  | 02              |
| Cefuroxime | 23 (96)              | 02                  | 02                  | 02                  | 02              |
| Cefotaxime | 22 (92)              | 01                  | 02                  | 02                  | 02              |
| Ceftriaxone | 22 (92)              | 01                  | 02                  | 02                  | 02              |
| Cefazidime | 23 (96)              | 02                  | 02                  | 02                  | 02              |
| Cefoperazone | 23 (96)            | 02                  | 02                  | 02                  | 02              |
| Cefixime | 23 (96)              | 02                  | 02                  | 02                  | 02              |
| Cefpodoxime | 23 (96)              | 02                  | 02                  | 02                  | 02              |
| Cefepime | 22 (92)              | 01                  | 02                  | 02                  | 02              |
| Cefotixin | 20 (83)              | 02                  | 02                  | 02                  | 02              |

ESBL: Extended spectrum β-lactamase, MBL: Metallo β-lactamase

## 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%)...
Table 3: Antimicrobial resistance in *Klebsiella pneumoniae* isolates (n=24) and in β-lactamase producing isolates to other antimicrobials

| Antibiotic   | Resistant (n=24) (%) | ESBL positive (n=2) | AmpC positive (n=2) | MBL positive (n=2) | AmpC+MBL (n=16) (%) |
|--------------|----------------------|---------------------|--------------------|-------------------|----------------------|
| Aztreonam    | 22 (92)              | 02                  | 02                 | 02                | 15 (94)              |
| Imipenem     | 02 (8)               | 00                  | 00                 | 02                | 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                 | 01                | 06 (6)               |
| Tigecycline  | 05 (21)              | 00                  | 01                 | 02                | 02 (12.5)            |

ESBL: Extended spectrum β-lactamase, MBL: Metallo β-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.[9,16,17]

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.[8] ESBL screen test gave 23 (96%) isolates positive. Study done by Modi et al., reported 89% positive in ESBL screening.[8] 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%).[5,9,17]

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.[1,8,16]

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 cefazidime. 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.[9] Similarly, Kamble et al., reported 20% MBL production.[17]

In our study, co-production of two β-lactamas 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 β-lactamase productions with a maximum number of isolates co-producing two enzymes (χ² = 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 β-lactama, it can result in serious therapeutic failures. Isolates producing more than one β-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.[16]

In most centers β-lactamase production is not routinely tested which ultimately results in the dissemination of β-lactamase producing strains in hospitals, and it remains undetected for longer periods. Inappropriate usage of antimicrobials leads to escalating percentage of β-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.,[16] 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 β-lactamas 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-ordination with other studies.[5,8,16,17] 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 β-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 β-lactamases that are no longer inhibited by clavulanic acid due to mutations in the coding sequences.[10]

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 sepsis have been reported by Roy et al., in two different studies.[7,19] Tigecycline has not been approved by the FDA for the treatment of bloodstream infections.[10,20] It has unusual pharmacokinetic properties, resulting in low blood concentrations but somewhat higher tissue levels. [21] Hence, its extensive tissue distribution allows for eradication of the source of secondary bacteremia that is likely to result in the clinical resolution.[20] However, more clinical experience with this drug is required to understand its role in the treatment of infections caused by carbapenemase producing K. pneumoniae[10] 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).[20] 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.[10,22,23] Studies regarding the comparison of MIC and disk diffusion methods for tigecycline done by various authors have reported good correlation (92–100%).[10,22,23] 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.[10] Similar to our study, use of disk diffusion method for tigecycline susceptibility has been reported by Filgona et al.[24]

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.[14] 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.[15] Isolates exhibiting co-resistance to at least any two of the following drugs were considered as MDR and these drugs were: Third generation cephalosporin (cefotaxime/ceftiraxone/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.[1,5,8] 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 sepsis, 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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