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# MOLECULAR CHARACTERIZATION OF EXTENDED-SPECTRUM B- LACTAMASES PRODUCING CTX-M-15 FROM GRAM NEGATIVE BACTERIA ISOLATED FROM PEDIATRIC AND NEONATAL INTENSIVE CARE UNIT

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**Abstract-** The rising rate of antimicrobial drug resistance in *Enterobacteriaceae* reduces the number of reliably effective drugs that can be used to treat infections. Gram negative bacteria producing β-lactamases that are resistant to many other antibiotics and very few antimicrobial agents remain effective as treatment option. Since the initial description of Extended Spectrum β-lactamases (ESBLs) production by *Klebseilla pneumonia* & *Escherichia coli* in the 1980s, strains of *Enterobacteriaceae* resistant to third-generation cephalosporins are increasingly being recognized globally. ESBL-producing *Enterobacteriaceae* strains have been frequently implicated in outbreaks in all intensive care units especially pediatric Intensive Care Unit (PICUs) and Neonatal Intensive Care Unit (NICU). Therefore, there have been many recent calls to intensify current infection control efforts aimed at reducing the emergence and dissemination of infections caused by antibiotic-resistant bacteria. The widespread use of Cefriaxone and/or cefotaximine has been proposed as a reason for the emergence of CTX-M enzymes. The increased frequency of isolation & reporting of CTX-M ESBLs is alarming and is likely to represent only the tip of iceberg for the underdeveloped continents where molecular technology for the analysis of ESBL enzymes is scares. To further delineate the mode of successful dissemination of ESBLs CTX-M-15 and to gain insights into the mechanism underlying this phenomenon we designed this study to assesses clonality & diversity of Enterobacteriaceae strains isolated from PICUs and NICUs of our hospital. Total of 100 blood samples were received from PICU and NICU during the study period of two months. 10 ESBLs were isolated from total 55 Gram negative bacteria, of that five were possessing CTX-M-15 by PCR methods.

**Keywords-** Extended-Spectrum β- Lactamases, CTX-M-15, Pediatric intensive care unit, Neonatal intensive care unit

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## Introduction

Worldwide, Intensive care units (ICUs) are faced with increasing rapid emergence and spread of antibiotic-resistant Gram-negative bacteria and Gram-positive bacteria. Gram negative bacteria producing β-lactamases that are resistant to many other antibiotics and very few antimicrobial agents remain effective as treatment option. Since the initial description of Extended Spectrum β-lactamases (ESBLs) production by *Klebseilla pneumonia* & *Escherichia coli* in the 1980s [1], strains of Enterobacteriaceae resistant to third-generation cephalosporins are increasingly being recognized [2]. ESBL-producing Enterobacteriaceae strains have been frequently implicated in outbreaks in Pediatrics Intensive Care Unit (PICUs) and Neonatal Intensive Care Unit (NICU) [3]. Therefore, there have been many recent calls to intensify current infection control efforts aimed at reducing the emergence and dissemination of infections caused by antibiotic-resistant bacteria [4-7]. The widespread use of cefriaxone and/or cefotaximine has been proposed as a reason for the emergence of CTX-M enzymes. The increased frequency of isolation & reporting of CTX-M ESBLs is alarming and is likely to represent only the tip of iceberg for the underdeveloped continents where molecular technology for the analysis of ESBL enzymes is scares. The loss of oxyaminocephalosporins for the treatment of infections represents a serious problem that seems to reach unprecedented level globally [4]. Currently, the most widely distributed CTX-M enzyme is CTX-M-15 which is detected in *E. coli* and other Enterobacteriaceae members from India in 2001 [5] and now also been reported in several countries, including the United Kingdom [6], Italy, Turkey, Japan, Norway, France, Canada [6].

Two recent studies using multilocous sequencing typing (MLST) Identified a single clone of CTX-M-15 producing *E. coli* named ST 131 [4-15]. This clone is associated with community as well as hospital acquired infections. In our hospital, we found a marked increased in the numbers of ESBL-producing Enterobacteriaceae in the PICUs & NICUs.

According to World Health organization (WHO) estimates, there are about 5 million neonatal deaths a year, 98% occurring in developing countries. Infection, prematurity, and birth asphyxia are the main causes. The most common cause of death in the neonatal period are infections, including septicaemia, meningitis, respiratory infections, diarrhea and neonatal tetanus. Overuse of antimicrobial agents has been identified as an important factor in the emergence of antibiotic resistant bacterial infections in the ICUs. Several investigators have demonstrated a close association between previous use of antibiotics and the emergence of subsequent antibiotic resistance in both gram-negative and gram-positive bacteria. Nosocomial blood stream infections are among the most serious infections acquired by ICU patients. ESBLs are now problem in hospitalized patients worldwide. The ESBL phenomenon began in Europe, most likely because expanded-spectrum β-lactam antibiotics were first used there. The prevalence of ESBLs among clinical isolates varies from country to country and from institution to institution. According to CDC-National Nosocomial Infections surveillance), in the United States occurrence of ESBL production in Enterobacteriaceae ranges from 0 to 25%, depending on the institution, with the national average being around 3%. Major risk factors for colonization or infection with ESBL producing organisms include length of hospital stay, severity of illness, long stay in the ICU, incubation and mechanical ventilation, urinary or arterial catheterization, surgery, extended abuse of broad spectrum antibiotics [5-15]. More recently systematic attempts have been made to look at clinical outcomes of bacteremia caused by ESBL-producing strains *E. coli* and *Klebseilla pneumonia* strains. Epidemiological data are limited regarding ESBL CTX-M-15 from India. To further delineate the mode of successful dissemination of ESBLs CTX-M-15 and to gain insights into the mechanism underlying this phenomenon we designed this study to assesses clonality & diversity of Enterobacteriaceae strains isolated from PICUs and NICUs of our hospital.

## Methodology

**Place of Conducting Research-** Department of microbiology, Pad. Dr. D.Y. Patil Medical College and Hospital, Pimpri, Pune-18, MS, India.

**Ethics Statement-** Written informed consents were obtained from all patients and study protocol were approved by the institutional ethics committee of Dr. D.Y. Patil Medical College, Pune.

**Duration-** July 2012-August 2012 (two months). This work was part of short term studentship (STS)-2012 project by ICMR India.

**Sample Size-** 100 blood samples received in the Microbiology Department from PICU and NICU Department of Pad. Dr. D.Y. Patil Medical College and Hospital, Pimpri, Pune-18.

**Sample Processing-** Blood sample were incubated in Bact T alert 3D and will be process on Blood Agar and MacConkey agar and incubated at 37°C. Identification and confirmation will be done by standard conventional methods.

Isolation, identification and confirmation of gram negative bacteria were done by standard conventional methods [11-13]. Antibiotic susceptibility testing were done by Kirby Bauer s disc diffusion technique and interpretations of susceptibility will be done according to the CLSI (Clinical Laboratory Standard Institute) 2008 guidelines. The following antibiotic disc manufactured by Hi-Media will be used for testing the isolates [1-5].

**Double Disk Approximation Test (DDAT) [for ESBL Phenotypic Detection]**

**PCR Detection of O25b-ST131 CTX-M-15 Clone Isolates**

The newly described O25b O type *E. coli* were detected by using the following primers

gndbis.f (5'ATACCGACGACGCCGATCTG-3') and

rfbO25b.r (5'TGCTATTCATTATGCGCAGC-3').

Annealing temperature of 60ºC was used to generate a PCR product of 300bp with the conditions as described [7]. The reaction mixture (total volume 10ul) contained 1ul of 10X PCR buffer, 0.2 µl of 10 mM dNTP mixture, 0.2 ul of 10 p mol/ul primers, 100 ng of genomic DNA and 0.5 unit of Taq DNA polymerase. PCR amplification was performed in eppendorf thermo cycler with the following temperature profile: initial denaturation at 95°C for 5 mins, followed by 35 cycles each of denaturation at 94°C for 45 sec, annealing at 59°C for 45 sec and extension at 72°C for 45 sec, and a final extension at 72°C for 10 min. The amplified products obtained were checked by running on a 1.5% Agarose gel [1-8].

## Observations and Results

Total of 100 blood samples were received from PICU and NICU during the study period of two months. Of this 80(80%) samples showed growth in culture and sensitivity testing and male predominance was seen i.e. male 50(62.5%) and female 30(37.5%) [Fig-1].

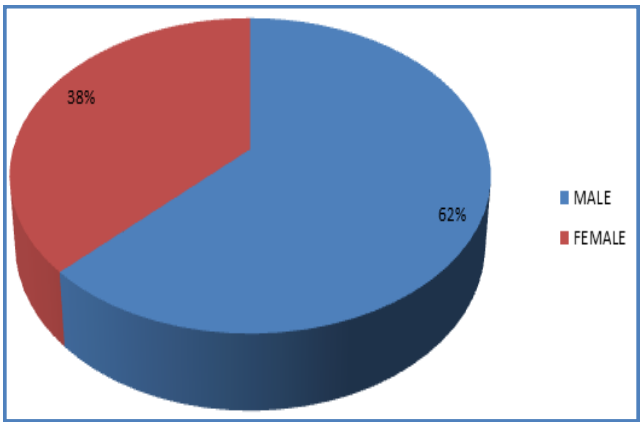


**Fig. 1-** Gender wise Distribution of Clinical Samples Received from NICU and PICU

Among these 80 isolates, distributions of Gram-negative bacteria were 55 (68.75%) while Gram-positive bacteria were 25(31.25%). Among Gram negative bacteria, *E. coli* and *Klebseilla pneumonia* were predominantly isolated followed by *Acinetobacter spp.* while in Gram-positive bacteria, Methicillin resistant *Staphylococcus aureus* (MRSA) were predominantly isolated followed by methicillin sensitive *Staphylococcus aureus* (MSSA) [Table-1].

*Table 1- Distribution of isolated organisms from PICU and NICU*

| Organisms | Distribution of Organisms | Number of Isolates |
|---|---|---|
| Gram-negative bacteria | *Klebseilla pneumonia* | 17 |
| | *E. coli* | 13 |
| | *Acinetobacter spp.* | 10 |
| | *Citrobacter spp.* | 3 |
| | *Enterobacter spp.* | 1 |
| | *Salmonella typhi* | 1 |
| Gram-positive bacteria | *MRSA* | 14 |
| | *MSSA* | 8 |
| | *Streptococci spp.* | 3 |

All 10 ESBLs were subjected for CTX-M-15 PCR and all 4 strains of ESBL *E. coli* were subjected for Sequence type lineage ST-131 by PCR. Results showed that of the 10 ESBL GNBs, 5 were positive for CTX-M-15 and of the 4 strains of *E. coli* 2 were positive for ST-131. All 10 ESBLs were resistant for aminoglycosides. CTX-M-15, the enzyme most closely associated with ST131.

## Discussion

The emergence and spread of infections caused by resistant microorganisms has been rising worldwide. Since 1980s ESBLs in *Klebseilla pneumonia* and *E. coli* had been documented and recent reports indicates that the widespread use of carbapenem, the agents reliably active against these bacteria, resulted in the emergence of a new resistance of metallo-β-lactamases. Recently, the increase of ESBLs and MBLs production in Enterobacteriaceae has become a major concern globally. In our hospital, we found alarming increasing in the number of the ESBL and MBL producing Enterobacteriaceae in PICU and NICU.

In the present study 80% samples showed growth and followed for identifications and sensitivity. We found male preponderance i.e. 50(62.5%) and female were 30(37.5%). Among 80 bacterial isolates, 55(68.75%) were Gram negative bacteria while 25(31.25%) were gram positive cocci [Table-1]. Of the total 55 Gram negative bacteria, *Klebseilla pneumonia* were 17(30.90%), *E. coli* 13 (23.63%) *Acinetobacter spp.* 10(18.18%), *Citrobacter spp.* 3 (5.45%) *Enterobacter cloacae* 1(1.81%) and *Salmonella typhi* 1 (1.81%). All isolates were screened for detection of production of ESBL and results were recorded. It showed that of the 55 GNB isolates-17 (63.63%) were resistant to ceftazidime and 10 (18.185%) were confirmed phenotypically as ESBL producers and only one isolate of *Acinetobacter baumanii* were MBL producer [Table-2]. Of the 10 confirmed ESBLs. 4 were *E. coli*, 3 were *Klebseilla pnemoniae*, 2 were *Citrobacter frundii*, and 1 was *Acinetobacter baumanii*. All 10 ESBLs were subjected for CTX-M-15 PCR and all 4 strains of ESBL *E. coli* were subjected for Sequence type lineage ST-131 by PCR. Results showed that of the 10 ESBL GNBs, 5 were positive for CTX-M-15 and of the 4 strains of *E. coli* 2 were positive for ST-131. All 10 ESBLs were resistant for aminoglycosides. CTX-M-15 [Fig-2], the enzyme most closely associated with ST131,was first identified in India in 1999 (Karim A., et al 2001 and Poirel L., et al 2002). It is now the most widely distributed CTX-M worldwide. The present study detected five strains those were possessing CTX-15 enzyme i.e. pandemic strains from received clinical sample from NICU and PICU. This distressing the situation and affecting on social as well as economical burden. Our hospital is tertiary care hospital and we are also receiving admission of patents those were critically ill and received treatment from outside most of the time. We are now taking efforts to reduce the spreading and disseminating such strains inside the hospital and among hospitalized patients.

*Table 2- Antimicrobial Susceptibility of Clinical Isolates of Gram-negative bacteria*

| Name of Antibiotics | Susceptibility pattern of GNBs (n=55) | |
|---|---|---|
| | Susceptibility (%) | Resistance (%) |
| Amikacin | 17 | 38 |
| Amoxycillin | 18 | 37 |
| Cefotaxime | 38 | 17 |
| Ceftriaxone | 33 | 22 |
| Ciprofloxacin | 25 | 30 |
| Cotrimaxazol | 20 | 35 |
| Ceftazidime | 38 | 17 |
| *Ceftazidime+Calvulanic acid (n=258) | 10 | 7 |
| Gentamycin | 19 | 36 |
| Imipenem | 51 | 4 |
| *Imipenem + EDTA(n=100) | 1 | 3 |
| Tetracycline | 51 | 4 |

**Ceftazidime + Calvulanic acid disc susceptibility testing were done on only ceftazidime resistance strains. Imipenem + EDTA disc susceptibility testing were done on only Imipenem resistance strains.*

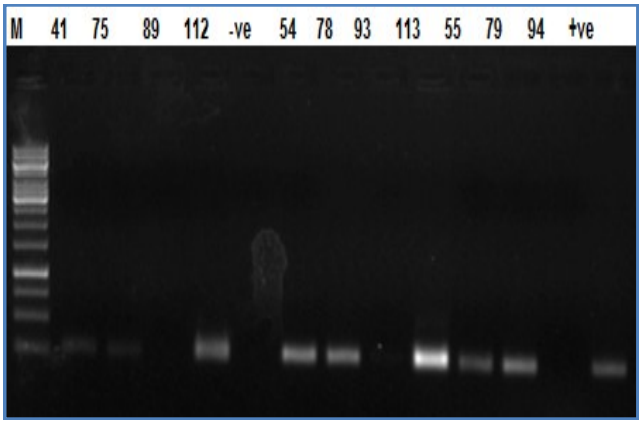


**Fig. 2-** PCR based detection ST131-CTX-M-15 clonal group of strains.

More recently, systematic attempts have been made to look at clinical outcomes of bacteria caused by ESBL-producing *E. coli* and *Klebseilla pneumonia* strains reviewed by Peterson, et al [19]. The studies described by Nichols Chanoin, et al [20] that bacteremia caused by ESBL-producing strains is associated with a higher mortality rate and early administration of carbapenem use may reduce the rate of mortality among patients with infections caused by ESBL-producing organisms [15-20]. Tsu-Lan Wu, et al (2003) reported growing trend of ESBL producing Enterobacteriaceae in PICU in Taiwan [20-22] while Yun- Kyung Kim, et al (2002) reported high prevalence of ESBL-producing *E. coli* and *Klebseilla pneumonia* in PICU in Korea [21]. E. Lebessi, et al (2002) have reported high prevalence of ESBL-producing *E. coli* and *Klebseilla pneumonia* in NICU from Athens, Greece [22]. From India, Savita Jadhav, et al have reported increasing incidence of multidrug resistance *Klebseilla pneumoiae* infections in hospital and community and reported MBL producers from NICU and PICU [22-25].

## Implementation

The increase in the number of ESBL- producing isolates by *Entero-*

*bacteriaceae* in PICUs outbreaks were reported by several authors worldwide. The dissemination of ESBL-producing *Enterobacteriaceae* is a consequence of the clonal expansion of a few epidemic strains and the spread of resistance plasmids among bacterial organisms which has been associated with community as well as hospital acquired. Since the resistance displayed by bacteria reflects the environment in which the organism thrives, immediate action, including reinforcement of infection control measures, should be taken to prevent further spread of the resistant bacteria. This study will highlights the need for monitoring the spread of this multidrug -resistant clonal complex throughout the nation and provides better understanding of the contribution of clonal dissemination among multidrug resistance Gram negative pathogens.

**Conclusion**

The emergence and spread of antimicrobial resistance is promoted by two factors: lapses in infection control and antibiotic selective pressure. ESBL producing gram negative bacteria should be best controlled by limiting the use of extended-spectrum cephalosporins in general and Ceftazidime in particular.

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