Molecular Characterisation and Profile of Extended Spectrum Beta Lactamase Producing Enterobacteriaceae Isolates Causing Neonatal Sepsis at a Tertiary Care Center

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ABSTRACT

Introduction: Emergence of extended spectrum beta-lactamase producing strains of gram-negative bacteria is increasing and it affects outcome of neonatal sepsis. Present study was done to assess the prevalence, antibiogram and molecular characterisation of ESBL producing *enterobacteriaceae* organisms in neonatal sepsis.

Methods: A cross-sectional study was conducted at a tertiary care centre in Agra from January 2016 to June 2017. Total 700 patients satisfying inclusion and exclusion criteria were enrolled. Workup for sepsis screen, blood culture, antibiotic susceptibility, disk approximation test for detection of ESBL producing organism and polymerase chain reaction were performed.

Results: Out of 700 patients, blood culture was positive in 238 (34%) cases. Among all the blood-culture isolates, Gram - positive, Gram - negative and candida species were (54%), (39%) and (7%) respectively. Among gram - negative isolates, *Klebsiella* (16%), *E. coli* (10%), *Pseudomonas* (6%), and *Burkholderia* (5%) were isolated. Prevalence of ESBL producing *Enterobacteriaceae* isolates was 42.42%. Among ESBL producers 13 were E. Coli and 15 were *Klebsiella*. ESBL producing bacilli were more common in males ($p = 0.015$) and out born patients ($p = 0.042$). CNS symptoms were the commonest manifestations (35.71%). All ESBL producing *Klebsiella* and 92.3% of E. coli were resistant to co-amoxyclav. All the ESBL producing *Klebsiella* and *E. coli* were sensitive to imipenem and colistin. CTXm gene was the commonest gene present in ESBL producing E. coli (61.54%) and *Klebsiella* (26.67%).

Conclusions: ESBL producing *Klebsiella* and *E. coli* were resistant to most of the commonly used antibiotics but they were sensitive to carbapenems and colistin. CTXm gene was the commonest gene, associated with ESBL producing *enterobacteriaceae*.

Keywords: Blood culture; CTXm gene; *Enterobacteriaceae*; ESBL; Neonatal sepsis

INTRODUCTION

Neonatal sepsis is a clinical syndrome characterised by signs and symptoms of infection with or without accompanying bacteraemia in the first month of life. It encompasses septicaemia, meningitis, pneumonia, arthritis, osteomyelitis and urinary tract infection. Septicaemia is one of the most important causes of neonatal mortality and morbidity. Incidence of septicaemia varies between countries, but developing countries had reported higher incidence in comparison of developed nations.[1] In India also many studies had reported septicaemia as a leading cause of neonatal mortality and morbidity.[2] Both Gram-positive as well as Gram-negative bacteria and sometimes fungal infections are responsible for neonatal sepsis.[3] Because of the changing pattern of antibiotic use, the spectrum of organisms that cause neonatal sepsis change over times and vary regionally.[4]

Gram negative bacteria form important group of neonatal sepsis worldwide. Among Gram negative bacilli, the two most important bacterial pathogens causing neonatal sepsis in developed countries are Klebisella pneumoniae and *E. Coli*.[5,6] Sepsis with extended-spectrum beta-lactamase (ESBL) producing bacteria is a major problem worldwide.[7] ESBLs are plasmid-mediated enzymes that hydrolyse broad-spectrum beta-lactams.[8] More than 100 ESBL variants from different types are known, commonest being SHV, TEM, OXA and CTX-M. While TEM- and SHV-type ESBLs have developed from their ancestors SHV-1 and TEM-1/-2 by point mutations, the origin of the other enzymes is less clear.[9]

Molecular characterisation of ESBL from neonatal sepsis cases at Safdarjung Hospital, Delhi had shown CTX-M-15 as the most prevalent type, with other types being TEM-1, SHV-1, SHV-28, SHV-11 and SHV-12.[10] The present study was planned to know the prevalence and molecular characterisation of extended spectrum beta lactamase producing Enterobacteriaceae in newborns admitted in NICU with neonatal sepsis in our institute.

METHODS

A cross-sectional, prospective study was carried out among patients admitted with sepsis in NICU in Sarojini Naidu Medical College, Agra, India. All neonates with clinical features suggestive of sepsis like alteration in the established feeding behaviour, tachypnea, chest retraction, grunting, excessive or high pitched cry, seizure, blank look or bulging anterior fontanel, lethargy, hypothermia, fever, diarrhoea, vomiting and abdominal distension, sclerema, episodes of apneic spells or gasping were included in study during January 2016 to June 2017. Neonates who were already on antibiotics or those who developed signs of sepsis within 72 hours of discontinuation of antibiotics, neonates with major congenital anomalies, severe birth asphyxia were excluded. After taking written and informed consent from parents, a semi-structured questionnaire was filled that consisted of basic socio demographic profile. Sepsis screening was carried out in all the newborns. Blood samples were also collected for blood culture and antibiotic sensitivity. The isolates of culture were further analysed for identification of gram negative Enterobacteriaceae and ESBL production. Molecular characterisation of ESBL producers was analysed by PCR.

Neonates with two or more of the following were considered positive sepsis screen - Absolute neutrophil count < 1800 cell/mm^3, total leucocyte count < 5000/mm^3, ratio of immature to total neutrophil count > 0.2, micro ESR > 15 mm and CRP > 10 mg/l. Blood cultures that flagged positive were subjected to Gram staining and sub cultured on appropriate medium. Mac-Conkey agar and 5% sheep blood agar was used for sub culturing Gram-negative and Gram-positive organisms, respectively. Blood culture bottles incubated in the system for up to seven days.

Antibiotic susceptibility testing was done by Kirby Bauer Disk Diffusion method on Mueller Hinton Agar as per Clinical and Laboratory Standard Institute guidelines (CLSI). *Escherichia coli* ATCC 25922 was used as control. Samples showing an inhibition zone size of ≤ 22 mm with ceftazidime and ≤ 27 mm with cefotaxime were considered as potential ESBL producers and were further investigated for confirmation.[11] Isolates that showed resistance to third generation cephalosporins were screened to detect ESBL production. A modified double disk synergy (Disk Approximation Test) was carried out on resistant

isolates. The ESBL producing isolates were further processed for genetic characterisation. Genetic material isolation was done using miniprep (reference) method.[11]

Three sets of primers were used to amplify internal region of the TEM, SHV and CTX-M genes. PCR amplification was performed in 25 µl reaction mixture. A negative (no template) control and a positive control containing a DNA mixture of three control strains (SHV-1, TEM-2 and CTX-M beta-lactamase producers) were included. DNA was amplified with the BIO-RAD C1000™ thermal cycler using 3 cycles. The PCR products were analysed on 2% Agarose gel with 1U Tris-borate-EDTA buffer. The results were confirmed for CTX_m, SHV and TEM gene by appearance of band near 544, 928 and 837 base pair marker ladder respectively. Data were presented as frequency and percentage, Chi square test was used to find out association between study groups and p value < 0.05 was considered as statistically significant. Present study was approved by Ethical research committee of the institute.

RESULTS

Out of 700 cases, sepsis screen was positive in 52.5% (n = 368) cases and blood culture was positive in 34% (n = 238) cases (Figure 1). 60.1% (421) patients were males, 63.9% (447) were preterm, 65% (465) were LBW (< 2500 grams), 47.9% (335) patients were of lower class, 37.3% (261) were of lower middle class and 73.4% (514) were out born. There was statistically significant association of blood culture positivity with sepsis screen, onset of sepsis and place of delivery (p < 0.05) (Table 1). The clinico - pathological features of the cases have been depicted in table 2.

Among blood culture positive cases (n = 238), Gram-positive bacteria were isolated in 54%, Gram-negative bacteria in 39% cases and candida in 6.7% cases. Among Gram - positive bacteria Staphylococcus (31.9%), Streptococcus (15.1%), Enterococcus (7.6%) were isolated. Among Gram-negative bacteria *Klebisella pneumoniae* (16.8%),

Table 1. Demographic profile of study population

		Blood culture			
		Total n = 700 (%)	**Positive n = 238 (%)**	**Negative n = 462 (%)**	**p value**
Gender	Male	421 (60.1)	148 (62.2)	273 (59.1)	0.428
	Female	279 (39.8)	90 (37.8)	189 (40.9)	
Gestational age	Preterm	447 (63.9)	162 (68.1)	285 (61.7)	0.096
	Term	253 (36.1)	76 (31.9)	177 (38.3)	
Place of delivery	Inborn	186 (26.6)	77 (32.4)	109 (23.6)	0.047*
	Outborn	514 (73.4)	161 (67.7)	353 (76.4)	
Socioeconomic status	Upper class	0	0	0	
	Upper middle class	0	0	0	
	Middle class	104 (14.8)	33 (13.9)	71 (15.4)	
	Lower middle class	261 (37.3)	96 (40.3)	165 (35.7)	
	Lower class	335 (47.9)	109 (45.8)	226 (48.9)	
Birth weight	<1500 grams	163 (23.3)	60 (25.2)	103 (22.3)	0.375
	1500 – 1999 grams	145 (20.7)	54 (22.7)	91 (19.7)	
	2000 – 2499 grams	147 (21.0)	46 (19.3)	101 (24.9)	
	Normal (2500-3999 grams)	245 (35.0)	78 (32.8)	167 (36.2)	
Onset of sepsis	Early onset sepsis	413 (59.0)	153 (64.3)	260 (56.3)	0.041*
	Late onset sepsis	287 (41.0)	85 (35.7)	202 (43.2)	
Sepsis Screen	Positive	368 (52.5)	210 (88.2)	158 (34.2)	<0.001*
	Negative	332 (47.5)	28 (11.8)	304 (65.8)	

** p value < 0.05 considered as statistically significant*

Table 2. Clinicopathological profile of study population

		Frequency (n = 700)	Culture positive cases (n = 238)	ESBL Positive Cases (n = 28)
Symptoms and Signs of sepsis	General symptoms (Refusal to feed, lethargy)	268 (38.28%)	96 (40.34%)	8 (28.57%)
	CNS (seizures, bulging fontanels)	137 (19.57%)	53 (22.57%)	10 (35.71%)
	Respiratory symptoms	132 (18.85%)	35 (14.70%)	3 (10.71%)
	Hypothermia	93 (13.28%)	31 (13.03%)	2 (7.14%)
	Haematological (Bleeding, Jaundice)	36 (5.14%)	10 (04.20%)	2 (7.14%)
	Abdominal distension	18 (2.57%)	9 (03.78%)	3 (10.71%)
	Sclerema	16 (2.28%)	5 (2.10%)	0
Onset of sepsis	Early onset sepsis (< 72 hours of life)	413 (59%)	153 (64.29%)	17 (60.71%)
	Late onset sepsis (> 72 hours of life)	287 (41%)	85 (35.71%)	11 (39.29%)
Blood culture	Positive	238 (34%)		
	Negative	462 (66%)		
Total Leucocyte count (TLC)	< 5000 or > 20000 per mm^3*	154 (22%)	55 (23.11%)	9 (32.14%)
	5000 – 20000 per mm^3	546 (78%)	183 (76.89%)	19 (67.85%)
Absolute neutrophil counts (ANC)	< 1800 per mm^3*	139 (19.85%)	48 (20.17%)	7 (25%)
	> 1800 per mm^3	561 (80.15%)	190 (79.83%)	21 (75%)
Micro ESR (Erythrocyte sedimentation rate)	> 15 mm *	245 (35.00%)	92 (38.70%)	11 (39.28%)
	< 15 mm	455 (65.00%)	146 (61.30%)	17 (60.71%)
I/T ratio	> 20%*	63 (09.00%)	22 (9.24%)	4 (14.28%)
	< 20%	637 (91.00%)	216 (89.02%)	24 (85.71%)
C- Reactive Protein (CRP)	> 10 mg/l*	260 (37.15%)	98 (41.18%)	11 (39.28%)
	< 10 mg/l	440 (62.85%)	140 (58.82%)	17 (60.71%)

* *Taken as Positive*

E. Coli (10.9%), *Pseudomonas* (6.3%) and *Burkholderia* (4.6%) were isolated.

In our study, isolated organisms of E*nterobacteriaceae* family (66) were *Klebsiella pneumoniae* (40) and E. coli (26)**.** Out of 66 culture positive cases of *Enterobacteriaceae,* 28 (42.42%) isolates were ESBL producers. Fifty percent (13/26) of E. coli and 37.5% (15/40) of *Klebsiella pneumoniae* were ESBL producers. ESBL producers were more common in males (p = 0.015) and out born patients (p = 0.042) (Table 3). Common clinical and laboratory parameters of babies with ESBL positive sepsis (n = 28) has been shown in table 2.

Among ESBL producing *E. Coli*, eight isolates had CTX_m gene, two isolates had TEM gene and three isolates had other genes. Among ESBL producing *Klebsiella*, four isolates had CTX_m gene, one isolate had TEM gene, one isolate had SHV gene and nine had other genes (Figure 2). As shown in figure 3, all CTXm ESBL producing *E. coli* and *Klebsiella pneumoniae* were resistant to amoxyclav and sensitive to imipenem and colistin. Similar pattern was also seen with TEM positive E coli and *Klebsiella pneumoniae* isolates and SHV positive *Klebsiella pneumoniae* isolates, however all the isolates were sensitive to meropenem.

DISCUSSION

Antibiotic resistance is one of the major concerns in the management of neonatal sepsis and an important confounding factor is the development of ESBL producing organisms.[4] The most evolving mechanism of ESBL production among *Enterobacteriaceae* is the selective pressure

Table 3. Demographic profile of newborns with Enterobacteriaceae isolates

		Total Enterobacteriaceae n=66 (%)	ESBL		
			Positive n=28 (%)	Negative n=38 (%)	P value
Gender	Male	31 (47.0)	18 (64.3)	13 (34.2)	0.015*
	Female	35 (53.0)	10 (35.7)	25 (65.8)	
Gestational age	Preterm	43 (65.2)	19 (67.9)	24 (63.2)	0.692
	Term	23 (34.8)	9 (32.1)	14 (36.8)	
Place of birth	Inborn	18 (27.3)	4 (4.3)	14 (36.8)	0.042*
	Outborn	48 (72.7)	24 (85.7)	24 (63.2)	
Birth weight	< 1500 grams	16 (24.2)	7 (25)	9 (23.7)	0.149
	1500 – 1999 grams	14 (21.2)	9 (32.1)	5 (13.2)	
	2000 – 2499 grams	13 (19.7)	6 (21.4)	7 (18.4)	
	Normal (2500 - 3999 grams)	23 (34.9)	6 (21.4)	17 (44.7)	
Socioeconomic Status (Modified BG Prasad, 2016)	Upper class	0	0	0	0.0084*
	Upper middle class	0	0	0	
	Middle class	8 (12.12)	3 (10.7)	5 (13.16)	
	Lower middle class	20 (30.30)	9 (32.1)	11 (28.95)	
	Lower class	38 (57.58)	16 (57.2)	22 (57.89)	
Onset of sepsis	Early onset sepsis	35 (53.0)	17 (60.7)	18 (63.2)	0.283
	Late onset sepsis	31 (47.0)	11 (39.3)	20 (63.8)	

** p value < 0.05 considered as statistically significant*

imposed by inappropriate use of third generation cephalosporins, most often encountered in ICU settings.[12]

In present study neonatal sepsis was found to be more common among males, preterm, outborn and LBW babies and these findings were similar to several other studies.[13,14] Highest number of

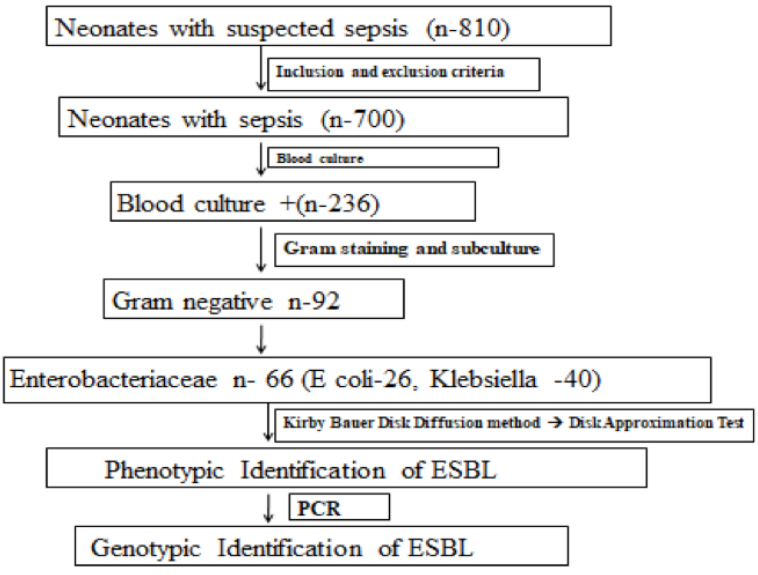

Figure 1. Schematic diagram of study design

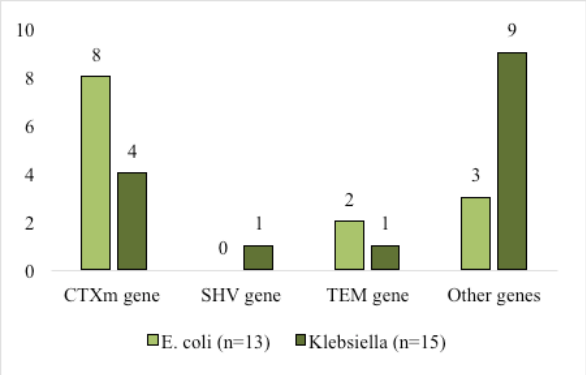

Figure 2. Molecular categorisation of ESBL positive isolates

patients were of lower socio-economic class, as in the study of Ahmed et al.[15] Most common presenting symptoms were refusal to feed and lethargy, followed by CNS and respiratory manifestation and these findings were similar to several other studies.[14,16]

The results of blood culture in our study were comparable to various other studies. In present study blood culture was positive more in outborn babies (p = 0.047), neonates with early onset sepsis (p = 0.041) and in neonates with positive sepsis screen (p < 0.001).[17,18] Among culture positive cases gram-positive bacteria were most common isolates. In this group Staphylococcus was most common organism followed by Streptococcus and Enterococcus. Similar results were seen in the study done by Kartikeyanet al.[19] An ESBL phenotype was recognized in 42.42% isolates in our study which was similar to the study done by Tofteland et al.[20] We found that among Gram negative isolates *Klebsiella* and *E. coli* were isolated most just like in other studies.[5,6] Prevalence of ESBL producing *E. coli* and *Klebsiella pneumoniae* isolates in our study was similar to various other studies.[10,21,22] The pattern of ESBL producing E Coli in our study was similar to study by Mardano et al. who noted that neonates having increased age and transferred from other centres had more prevalence of ESBL producing E Coli and significant morality rates.[23]

We found that CTXm gene was the commonest gene present in ESBL producing *enterobacteriace*

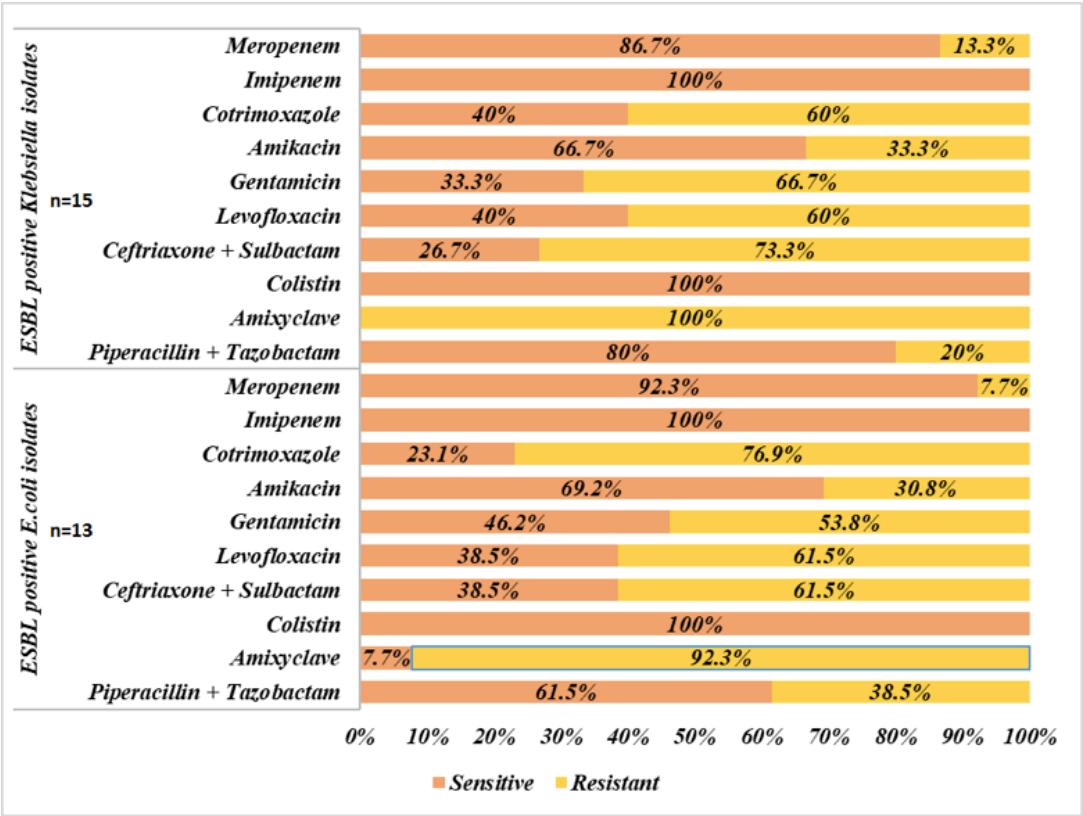

Figure 3. Distribution of cases based on Antibiogram of ESBL Positive isolates

isolates followed by TEM and SHV gene. CTXm gene was associated with resistance to most of the first line cephalosporins and amikacin and fluroquinolones and sensitive to imipenem, meropenem and pipercillin – tazobactam.[10,24] This fact is also supported in our study. Several studies have reported that carbapenems along with β lactam / β lactamase inhibitors combination could be used as empirical therapy for suspicion of ESBL producing bacteria.[25,26]

Although we tried to elaborate the organisms implicated in neonatal sepsis, our study is relatively small and single centric. We also could not identify gene responsible for ESBL production in three cases of ESBL producing *E. coli* and nine cases of ESBL producing *Klebsiella* because of resource constraints. Despite these limitations, we are hopeful that our research light help to shed more light on the topic of neonatal sepsis in resource limited set up like ours.

CONCLUSIONS

Among ESBL producers, *E. coli* and *Klebsiella* were the commonest organisms isolated and were more prevalent in outborns and male babies. These isolates were resistant to commonly used antibiotics like penicillin, cephalosporines, fluoroquinolons and aminoglycosides and were sensitive to carbapenems and colistin. CTXm gene was most common gene associated with ESBL producing *E. coli* and *Klebsiella*.

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