Isolation Of Beta Lactamases Engendering Lactose Fermenters From Different Categories Of Neonatal Sepsis Cases

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Abstract: Background: The escalating incidence of ESBLs and Amp C producers in various grievous clinical conditions has convoluted treatment strategies. The objective of this study is to determine the incidence of ESBLs and AmpC β-lactamase producing *Escherichia coli* and *Klebsiella spp* from different types of neonatal sepsis cases. Methodology: This cross sectional study included 382 blood samples which were processed by standard microbiological methods. A.S.T was carried out by Kirby Bauer disk diffusion method as per CLSI guidelines. The presumptive producers of ESBLs were confirmed as per CLSI guidelines (2015). Inducible AmpC were detected by Disc antagonism test (DAT) and E test. Results**:** The blood culture positivity was 32.46% (n=124/382) which was higher in males (34.36%) as compared to females (29.67%). Culture positivity among the EONS and LONS cases were found to be 47.38% and 52.42% respectively. There was dominance of Gram negative isolates (58.87%) over Gram positive isolates (37.9%) and Yeast like fungi (3.23%). *E. coli* (41.09%) was significantly isolated from EONS cases while *Klebsiella spp* (31.51%) was isolated almost dispassionately from both types. Among the 45.28% ESBL producing isolates, 58.33% (14) isolates of *E.coli* (09) and *Klebsiella species* (05) were recovered from EONS cases while 10 (41.67%) strains of *E.coli* (04) and *Klebsiella* species (06) were recuperated from LONS cases. The isolation of inducible AmpC was found to be 13.79%. Pure AmpC (both from EONS cases) as well as co-production of ESBL and AmpC (01 from LONS and 01from EONS cases) was seen in 01 each of *E.coli* and *Klebsiella* spp. Conclusion: The differentiation of ESBL from AmpC β-lactamases is necessary for formulation of treatment guidelines as the incidence is escalating.[Mishra P Natl J Integr Res Med, 2019; 10(2):29-34]

Key Words: AmpC, ESBL, *E.coli*, *Klebsiella*, Neonatal sepsis.

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Introduction: Neonatal sepsis is a clinical condition which is characterized by systemic signs and symptoms due to invasion of blood by microbes in the neonatal life. Neonatal sepsis is the 3rd most common cause of death with an estimated 0.4 million of deaths in 2015, the majority of which are in developing countries [1].

Innate factors like feebly developed immune system, immature skin barrier, mucosal defense mechanisms and blood brain barrier predisposes the neonate to the increased susceptibility to infection. Neonatal sepsis is broadly categorized into two categories on the basis of timing of onset of the infection. First, Early onset neonatal sepsis (EONS) which is defined as infection occurring in either the first 48-72 hours of life or the first week of life. Late onset neonatal sepsis (LONS) is defined as sepsis occurring after 7 days. The modes of transmission, etiology, and treatment for EONS and LONS are different [2,3].

In developing countries *Escherichia coli, Klebsiella spp, Acinetobacter* accounted in causing EONS as compared to Group B *Streptococcus* and Coagulase negative *Staphylococci* (CoNS). *Klebsiella spp, Pseudomonas spp, Salmonella spp* and *Serratia* precede Coagulase Negative *Staphylococcus (*CoNS) and *Staphylococcus aureus* in causing LONS [5]. ESBLs are more prevalent in *Klebsiella* spp and *E.coli* than any other enterobacterial species and outbreaks of infection caused by ESBL producing *Klebsiella* spp have been widely reported [6]. There are several published reports on the detection of ESBLs among various clinical samples from different region of India but most of them have not differentiated the β-lactamases types especially in isolates from neonatal septicaemic cases. The precise detection of ESBL types helps in institution of antibiotic policies as well as in tracking trends and evolution of β-lactamases. Keeping in view the above facts, this study is undertaken to detect the incidence of ESBL producing *E. coli* and *Klebsiella spp* from different types of neonatal sepsis cases.

Material And Method: Sample Size & Study Design: This cross sectional study included 382 blood samples which were collected and processed in the Departments of Microbiology, Santosh Medical College Hospital, Ghaziabad in

collaboration with Rohilkhand Medical College and Hospital, Bareilly from May 2015 to May 2017 after ethical clearance by institutional ethics committee of both the institutes.

Blood samples (1-2 ml) from the neonates were collected and inoculated on blood culture bottle containing Brain Heart Infusion (BHI) broth (Himedia, Mumbai) and were incubated aerobically at 37˚ C for 7 days. The samples were subcultured onto 5% sheep blood agar and Mac Conkey agar. The culture isolates were identified by colony characteristics, Gram staining, motility and standard biochemical tests for confirmation of *Escherichia coli* and *Klebsiella spp*. They identified isolate was further confirmed by Vitek system. AST was performed by Kirby Bauer disc diffusion method as per Clinical Laboratory and Standard Institute (CLSI) guidelines [6] using Mueller Hinton Agar plates (MHA) and commercially available antibiotic discs (Himedia).

Screening of ESBL was done according to the CLSI guidelines by "Disc Diffusion Method". Isolates showing zone of inhibition of ≤ 22 mm against Ceftazidime (30 µg), ≤ 25 mm against Ceftriaxone (30 µg), and ≤ 27 mm against Cefotaxime (30 µg) were recognized as potential ESBL producers [6].

Confirmation of ESBLs and AmpC:

(1) "**Combined Disc Diffusion Method**". This test was done by using a disk of Ceftazidime (30µg) alone and a disk of Ceftazidime + Clavulanic acid (30 µg/10 µg) is used. A disk of Cefotaxime (30µg) alone and a disk of Cefotaxime+ Clavulanic acid (30 µg/10 µg) were also used. Both pair of disks were placed at least 25 mm apart, center to center, on a lawn culture of the test isolate on Mueller Hinton Agar (MHA) plate and incubated overnight at 37°C. A difference in zone diameters with and without clavulanic acid of ≥ 5 mm confirmed ESBL production [6].

(2) By "**Disc Antagonism Test (DAT)" for Inducible ß-lactamases (AmpC)**: Cefoxitin (inducer) disc was placed at a distance of 2.5 cm from cephalosporin disc [7]. Production of inducible ß-lactamase was indicated by flattening of the zone of inhibition of the cephalosporin disc towards inducer disk by >1 mm.

(3) **E test (Triple ESBL detection Ezy MIC™ Strip (MIX+/MIX) EM079)** (Ceftazidime, Cefotaxime & Cefepime Mix: 0.125-16) (Ceftazidime, Cefotaxime & Cefepime Mix + Clavulanic acid: 0.032- 4). (**ESBL & AmpC detection Ezy MIC™ Strip (MIX+/MIX) EM081) MIX +:** Ceftazidime, Cefotaxime, Cefepime, Cloxacillin + Clavulanic acid (0.032 - 4) MIX: Ceftazidime, Cefotaxime, Cefepime & Cloxacillin (0.125 -16) (Himedia, Mumbai). The test was performed and AmpC was interpreted as described in kit literature.

The data was entered and analyzed using SPSS, version 22. Statistical analysis was performed using descriptive statistics such as mean, frequency, percentage etc. The value P ≤ 0.05 was considered to be statistically significant.

Results : Of the 382 samples, 124 (32.46%) samples were culture positive. The culture positivity was more in males (34.36%) as compared to females (29.67%) as shown in Figure 1. From the 124 isolates n=124/382 (32.46%), a preponderance of Gram Negative isolates (58.87%) was observed over Gram positive isolates (37.9%) and *Candida albicans* (3.23%). The distribution of the isolates is depicted in Figure 2

Figure 1: Distribution Of Males And Females In Positive Cultures

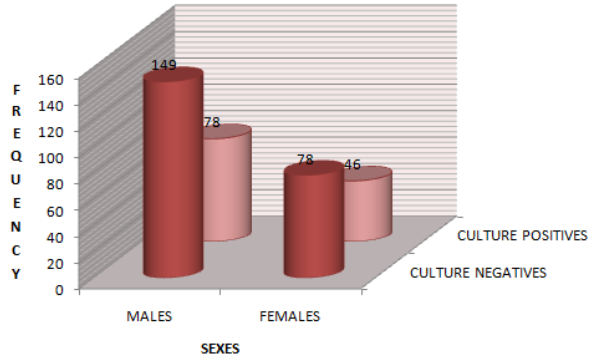
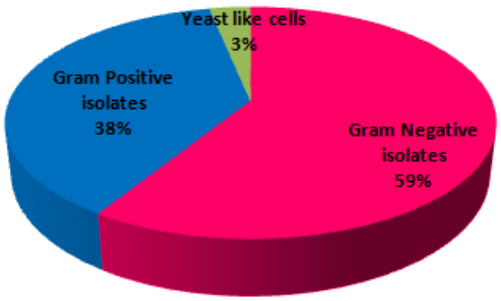


Figure 2: Percentage of different isolates among the neonatal sepsis cases



The mean age of the neonates was 1.95 days. The culture positivity among the EONS and LONS cases were 47.38% (n=59/124) and 52.42% (n=65/124) respectively (Table 1). The most common Gram Negative isolates was *E. coli (*41.09%) followed by *Klebsiella spp* (31.51%),

while *S. aureus* (51.06%) and Coagulase negative *Staphylococci* (CONS) (29.79%) were the common Gram Positive isolates as depicted in Table 2 & 3.

Table 1: Distribution of Culture Positive Cases among EONS and LONS Cases.

Sr No	Type of Neonatal Sepsis	Culture		Total	p value
		Negative	**Positive**		0.122
1	**EONS**	146 (71.22%)	59 (28.78%)	205 (100.00%)	
2	**LONS**	112 (63.28%)	65 (36.72%)	177 (100.00%)	
3	**Total**	258 (67.54%)	124 (32.46%)	382 (100.00%)	
Note: # EONS = Early onset neonatal sepsis ## LONS= late onset neonatal sepsis					

Table 2: Gram Positive Isolates among Early Onset and Late Onset Neonatal Sepsis cases.

Sl.No	Etiological Agents	Isolated From EONS Cases (N=19)	Isolated From LONS Cases (N=28)	Total	p value
	Staphylococcus aureus	11 (57.89%)	13 (46.43%)	24 (51.06%)	0.001
	Coagulase Negative Staphylococci	01 (5.26%)	13 (46.43%)	14 (29.79%)	
	Group B Streptococci	05 (26.32%)	00 (0.00%)	05 (10.64%)	
	Enterococci	02 (10.53%)	02 (7.14%)	04 (8.51%)	
	Total	19 (40.42%)	28 (59.57%)	47 (100%)	

Table 3: Gram Negative Isolates among Early Onset and Late Onset Neonatal Sepsis cases.

Sl.No	Etiological Agents	Isolated From EONS Cases	Isolated From LONS Cases	Total	p value
	Escherichia coli	22 (55.00%)	08 (24.24%)	30 (41.10%)	0.02
	Klebsiella spp	10 (25.00%)	13 (39.39%)	23 (31.51%)	
	(K. oxytoca, K.pneumoniae)	04 06	03 10	07 16	
	P.aeruginosa	01 (2.50%)	07 (21.21%)	08 (10.96%)	
	Proteus spp	03 (7.50%)	03 (9.09%)	06 (8.22%)	
	(P.mirabilis,	02	02	04	
	P.vulgaris)	01	01	02	
	Citrobacter spp	03 (7.50%)	01 (3.09%)	04 (5.48%)	
	Acinetobacter	01 (2.50%)	01 (3.09%)	02 (2.74%)	
	Total	40 (54.79%)	33 (45.21%)	73 (100%)	

A total of 53.33% isolates of *E.coli* and 56.52% of *Klebsiella* isolates were found to be presumptive producer of ESBLs. The isolation of ESBL producing *E.coli* and *Klebsiella* species from EONS cases were found to be 64.28% while 35.71% respectively while 40% and 60 % respectively from LONS cases [Table 4].

Table 4: **Screening and confirmatory tests for ESBL detection in different isolates**.

Sl.no	Name of the tests	Type of sepsis	*E.coli (n=30)*	*Klebsiella(n=23)*	Total (n=53)
1.	**Screening test**	EONS	12 (66.67%)	06 (33.33%)	18 (100%)
		LONS	04 (36.36%)	07 (63.64%)	11 (100%)
		Total	**16 (53.33%)**	**13 (56.52%)**	**29 (54.72%)**
Confirmatory test					
	CDDT	EONS	09 (64.28%)	05 (35.71%)	14 (100%)
		LONS	04 (40.00%)	06 (60.00%)	10 (100%)
		Total	**13 (43.33%)**	**11 (47.83%)**	**24 (45.28%)**

CDDT = Combined Disc Diffusion Test ***EONS = Early Onset Neonatal Sepsis***
LONS = Late Onset Neonatal Sepsis

The distribution of AmpC production among the isolates of *E.coli* and *Klebsiella* spp is tabulated in Table 5.

Table 5: **Distribution of Beta-Lactamases among *E.coli* and *Klebsiella* Isolates.**

S.no	Isolates	Type of sepsis	Screening test positive	ESBL*	AmpC**	ESBL + AmpC
1	***Escherichia coli***	**EONS**	12	09	01	00
		LONS	04	04	00	01
			16	**13**	**01**	**01**
2	***Klebsiella spp***	**EONS**	06	05	01	01
		LONS	07	06	00	00
			13	**11**	**01**	**01**
	Total		**29**	**24**	**02**	**02**
				P value=1 (Z test)	P value=1 (Z test)	P value=1 (Z test)

**ESBL= Extended Spectrum beta lactamases **AmpC= Inducible beta lactamases (Class C), EONS= Early onset neonatal sepsis, LONS= Late onset neonatal sepsis*

Discussion: Neonatal sepsis is one of the foremost causes of mortality of neonates which has to be addressed sincerely. It is life threatening emergency so the differentiation of the type of β-lactamases is necessary to formulate the antibiotic policy.

In the present study the blood culture positivity rate among neonates was found to be 32.46% which is in line with the study by Khanna A et al which reported a rate of 31.5% [8]. The studies from various centers showed an extensive variation in blood culture positivity, a higher positivity of 55.8% was reported from Vadodara, Gujarat in 2018 [9] while a low positivity rate has been reported from Kathmandu, Nepal [10]. This study inferred a higher positivity of 34.36% among males in contrast to 29.67% in females which can be due to the fact that higher proportions of males were embraced in the study sample. The differences seen in distribution with respect to sex has no statistical significance (p>0.005). This is comparable with the study of Monica Lazarus et al which reported a higher male to female ratio [11].

The current study revealed preponderance of Gram negative isolates (58.87%) over the Gram positive isolates (37.9%) and *Candida* (3.22%). This is in line with the findings of the study in 2018 which reported 56% of cases by Gram negative isolates, 46% cases by Gram Positive isolates, [10] while a deviating percentage of 40% and 60% reported by Thakur et al in 2016 [12]. Among the Gram positive isolates the most frequent isolate was *S. aureus* (51.06%) which is almost comparable among EONS (57.89%) and LONS (46.53%) cases, followed by CoNS (29.79%) isolated mostly from LONS (46.43%) cases then EONS 5.26% cases which is statistically significant (p<0.05). These results are analogous to the studies conducted by Reddy KA et al from Telangana [13] and Kumar R et al from Bihar [14]. Health care workers carry CoNS and *S. aureus* on the skin and nasopharynx which are transmitted to neonates during the invasive procedures and lack of proper disinfection practice leading to higher isolation from LONS cases. Among the Gram negative isolates *E. coli* 41.09% and *Klebsiella* spp 31.51% were most common which is similar to the findings reported by a previous study [15], this might be due to the higher number of cases of EONS as compared to LONS in current study.

The present study established *E.coli* (37.29%) as the leading pathogen causing EONS while *Klebsiella* spp (15.0%) as the foremost causing LONS which is parallel to the finding of Porta et al. in 2017 [16] which avowed *E. coli* as main culprit causing EONS. This might owe to the verity that coliforms, especially *E. coli*, are frequent colonizers of the maternal vaginal canal which the new born acquire during delivery. It was found that the isolation of *E.coli* was significantly (<0.05%) more common in EONS cases 55.00% as compared to LONS cases 24.24% while *Klebsiella spp* (statistically insignificant, p>0.05%) were

isolated more from LONS cases (39.39%) than from EONS (25.00%) cases. A varied result of isolation of *Klebsiella spp* among LONS (30%) cases and EONS (16.6%) cases was reported by Hematyar M et al. [17].

The prevalence of ESBL producing *Escherichia coli* and *Klebsiella* species among the neonatal cases of sepsis were found to be 45.28%. Individually the prevalence for *E. coli* and *Klebsiella species* were found to be 43.33% and 47.83% respectively. Of the 29 (n=16 *Escherichia coli* & n= 13 *Klebsiella spp*) isolates that were positive in the screening test, 24 (82.76%) isolates were phenotypically confirmed as ESBL producers by the CLSI phenotypic confirmatory combined disc diffusion method. A varied isolation of ESBL producers among the positively screened isolates ranged from 67.57% to 91.1% in various other studies [18,19]. Fourteen (58.33%) isolates of *E.coli* (09) and *Klebsiella species* (05) were recovered from EONS cases while 10 (41.67%) strains of *E.coli* (04) and *Klebsiella* species (06) were recuperated from LONS cases. There was high isolation of ESBL strains from EONS cases which may due to maternal exposure to cephalosporins or their infection with ESBL strains during caesarian section or PROM. This is in line with the study conducted by Deepak Sharma et al in 2016 stating the high incidence of ESBL producing strains from EONS [20]. By using Disc antagonism test for inducible AmpC ß-lactamases and E- strip test, 6.89% (6.25% of *E. coli* and 7.69% of *Klebsiella species)* isolates were detected each for co-production of both ESBL and AmpC β-lactamase (01 from LONS and 01from EONS cases) and AmpC alone (both from EONS cases) among the 29 isolates. In total the prevalence of inducible AmpC in the present study was found to be 13.79% which is comparable to the study from Bijnaur which reported 12.5% of AmpC production and a lower percentage (8%) of isolation of ESBL among 24 isolates from neonatal sepsis cases [21]. Conversely Qadeer S et al from Lahore reported pure Amp C production in 30.7% and 20% of *E.coli* and *K.pneumoniae* respectively and co-production was found in 23% of *E.coli* and 20% of *K.pneumoniae* isolates respectively[22].

Conclusion: here was considerable incidence of beta lactamases (ESBLs and AmpC) producing *Escherichia coli* and *Klebsiella spp* causing neonatal sepsis in the study centre. The incidence is substantially marked in both types of neonatal sepsis. The presence of AmpC beta lactamases causes false positive screening test for ESBLs which causes hindrance in diagnosis and formulation of empirical therapy for treatment of such deadly conditions. Early diagnosis with the type of beta lactamase will help in reducing neonatal morbidity and mortality and prevent emergence of drug resistant strains.

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Conflict of interest: None

Funding: None

Cite this Article as: Mishra P, Bisht D, Sharma V P, Goel V. Isolation Of Beta Lactamases Engendering Lactose Fermenters From Different Categories Of Neonatal Sepsis Cases. Natl J Integr Res Med 2019; Vol.10(2): 29-34