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INCIDENCE OF EXTENDED SPECTRUM β-LACTAMASE AND AMPC β-LACTAMASE PRODUCING ESCHERICHIA COLI AND KLEBSIELLA SPP ISOLATED FROM NEONATAL SEPTICAEMIC CASES IN A TERTIARY CARE CENTRE, UTTAR PRADESH.

Microbiology

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ABSTRACT

Background: The array of β-lactamases is like ESBLs and AmpC produced by Gram negative bacilli causes many somber infections like neonatal sepsis and their increasing incidence major health concern.

Aim: To determine the incidence of ESBLs and AmpC β-lactamase producing *Escherichia coli* and *Klebsiella spp* isolated from neonatal sepsis cases.

Methodology: Blood samples from 382 clinically suspected neonatal septicaemic cases were processed by standard microbiological methods. Antimicrobial susceptibility testing was carried out by Kirby Bauer disk diffusion method as per CLSI guidelines. Screening and confirmation of ESBLs production was also done according to CLSI guidelines (2015). Disc antagonism test (DAT) and E test (Ezy MIC™ strip**)** tests were used for detection of AmpC.

Results: Of the 382 blood samples, 124 (32.46%) samples showed bacterial growth. Culture positivity in males was 34.36% and 29.67% in females. Culture positivity among the EONS and LONS cases was found to be 47.38% and 52.42%. There was preponderance of Gram negative isolates (58.87%) over Gram positive isolates (37.9%) and *Candida albicans* (3.23%). *E. coli* (41.09%) was mostly isolated from EONS cases while *Klebsiella spp* (31.51%) was isolated almost equally from both types of neonatal sepsis. The incidence of *E. coli* and *Klebsiella* spp was found to be 43.33% and 47.83% respectively. Pure AmpC as well as co-production of ESBL and AmpC was seen in 6.25% *E. coli* and 7.69% among *Klebsiella spp.*

Conclusion: It is very important to differentiate between ESBLs and AmpC producers so that the treatment guidelines can be wisely formulated. E strip test as well as disk antagonism test was found to be useful in detecting AmpC production.

KEYWORDS

AmpC, ESBL, E test, Neonatal sepsis.

INTRODUCTION

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]. Clinical manifestations vary from subclinical infection to severe presentations. The diverse etiological agents can be due to an in-utero infection, attainment from the maternal genital flora or postnatal acquisition from invasive procedures, environmental factors in hospital or community etc. The immature immune system among premature infants is responsible for higher susceptibility towards infection. The innate immune system is impaired in neonates due to impaired skin and gut flora and lower activity of dendritic cells, cytokines, neutrophils, and the complement system, especially in preterm infants [2].

Neonatal sepsis is broadly divided into two categories based on the onset of the infection. Early onset neonatal sepsis/septicaemia (EONS) defined as infection occurring in the first week of life while Late onset neonatal sepsis (LONS) is defined as sepsis occurring after 7 days. In developing countries *Escherichia coli, Klebsiella spp, Acinetobacter* are more responsible in causing EONS than Group B *Streptococcus* and Coagulase negative *Staphylococci* (CoNS). *Klebsiella spp* and *Pseudomonas spp, Salmonella spp* and *Serratia* precede Coagulase Negative *Staphylococcus* (CoNS) and *Staphylococcus aureus* in causation of LONS [3]. The greatest challenge nowadays is the emerging disaster of neonatal sepsis caused by drug resistant strains especially in *Escherichia coli* and *Klebsiella pneumoniae* as they are more prevalent than any other enterobacterial spp and outbreaks of infection caused by Extended spectrum β-lactamases (ESBL) producing *Klebsiella* spp have been widely reported[4]. The escalation in incidence of ESBLs and AmpC producers is a critical global health concern which has amputated treatment strategies and hence this study was aimed to detect the incidence of ESBLs and Amp C producing *Escherichia coli* and *Klebsiella spp* in neonates with sepsis which will help in formulation of the treatment plan.

MATERIAL AND METHODS

Sample Size & Study Design: A total of 382 blood samples were collected and processed in this cross sectional study carried out in the Department of Microbiology, Santosh Medical College Hospital, Ghaziabad in alliance 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*. AST was performed by Kirby Bauer disc diffusion method as per Clinical Laboratory and Standard Institute (CLSI) guidelines [5] 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 [5].

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 [5].

(2) By "**Disc Antagonism Test (DAT)" for Inducible ß-lactamases**

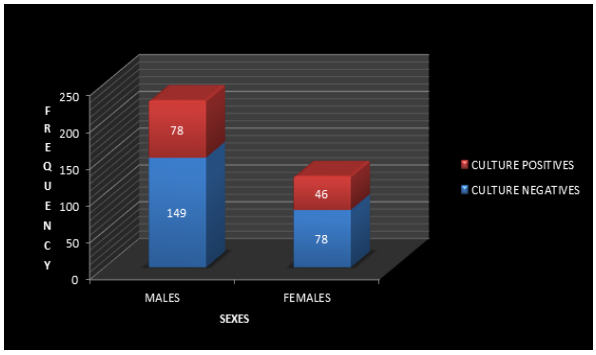
(AmpC): Cefoxitin (inducer) disc was placed at a distance of 2.5 cm from cephalosporin disc [6]. Production of inducible ß-lactamase was indicated by flattening of the zone of inhibition of the cephalosporin disc towards inducer disk by >1 mm.

(4) **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) Standard bacterial suspension was made and inoculated on MHA plate. Triple ESBL detection strip was placed on Muller Hinton agar plate. Plates were incubated overnight at 37°C aerobically. The presence of ESBL was confirmed by the appearance of a phantom zone or when the minimum inhibitory concentration (MIC) of antibiotic mixture side was reduced by ≥ 8 times in the presence of β-lactamases inhibitor. 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 blood samples processed from neonatal cases, 124 (32.46%) samples showed bacterial growth. Culture positivity in males was 34.36% while in females it was 29.67% (Figure 1). The association between the different sexes and blood culture in the current study was not significant (p>0.05).

Figure 1: Positive Blood Cultures Among Sexes



Mean age of the neonates included in the study was found to be 1.95 days and the age ranged from 1 day to 28 days. Of the 382 suspected cases of neonatal sepsis, culture positivity among EONS and LONS cases was found to be 47.38% and 52.42% respectively. The association between types of neonatal sepsis and blood culture findings was found to be insignificant (p>0.05) as shown in Table 1.

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

Sl. 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%)	
$\chi 2 = 2.383$ (using chi square corrected test, Yates test)					

Note: # EONS = Early onset neonatal sepsis ## LONS= late onset neonatal sepsis

Of the 124 culture positive isolates, a preponderance of Gram Negative isolates (58.87%) was observed over Gram positive isolates (37.9%) and *Candida albicans* (3.23%). 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. Isolation of *E. coli* was more common from EONS cases (55 %) as compared to LONS cases (24.24%) while *Klebsiella* spp had comparatively similar distribution among LONS and EONS cases. The difference in distribution of the Gram negative isolates with relation to types of neonatal sepsis was found to be statistically significant (p<0.05).

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
1.	*Staphylococcus aureus*	11 (57.89%)	13 (46.43%)	24 (51.06%)	0.001
2.	*Coagulase Negative Staphylococci*	01 (5.26%)	13 (46.43%)	14 (29.79%)	
3.	*Group B Streptococci*	05 (26.32%)	00 (0.00%)	05 (10.64%)	
4.	*Enterococci*	02 (10.53%)	02 (7.14%)	04 (8.51%)	
	Total	19 (40.42%)	28 (59.57%)	47 (100%)	

Note: # EONS = Early onset neonatal sepsis ## LONS= late onset neonatal sepsis

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
1.	*Escherichia coli*	22 (55.00%)	08 (24.24%)	30 (41.10%)	0.02
2.	*Klebsiella spp* *(K. oxytoca,* *K.pneumoniae)*	10 (25.00%) 04 06	13 (39.39%) 03 10	23 (31.51%) 07 16	
3.	*P.aeruginosa*	01 (2.50%)	07 (21.21%)	08 (10.96%)	
4.	*Proteus spp* *(P.mirabilis,* *P.vulgaris)*	03 (7.50%) 02 01	03 (9.09%) 02 01	06 (8.22%) 04 02	
5.	*Citrobacter spp*	03 (7.50%)	01 (3.09%)	04 (5.48%)	
6.	*Acinetobacter*	01 (2.50%)	01 (3.09%)	02 (2.74%)	
	Total	40 (54.79%)	33 (45.21%)	73 (100%)	

ESBL and AmpC Production:

Isolated *E.coli* and *Klebsiella* spp showing inhibition zone size of ≤ 22 mm with Ceftazidime (30 µg), ≤ 25 mm with Ceftriaxone (30 µg), ≤ 27 mm with Cefotaxime (30 µg), and ≤ 27 mm with Aztreonam (30 µg) were recognized as presumptive producers of ESBLs. In the screening test, which involved detection of resistance to one or more of the four cephalosporin antibiotics stated earlier, 29 (54.72%) were found to be resistant. Of the 30 *E. coli* isolates and 23 *Klebsiella* spp isolates, 16 (53.33%) and 13 (56.52%) isolates were found positive in screening tests respectively. The incidence of ESBL producing *Escherichia coli* and *Klebsiella* spp among the neonatal cases of sepsis was found to be 45.28%. Individually the incidence for *E. coli* and *Klebsiella* spp was found to be 43.33% and 47.83% respectively. Positivity of confirmatory tests by combined disc diffusion test (CDDT) was 45.28%, [*E.coli*= 13 (43.33%), *Klebsiella spp* 11 (47.83%)]. By using "Disc antagonism test" for inducible AmpC ß-lactamases and Ezy MIC™ strip test (EM079 and EM081) 2(6.89%) isolates [1 (6.25%) of *Escherichia coli* and 1 (7.69%) of *Klebsiella spp*] were detected for co-production of both ESBL and AmpC beta-lactamase among the 29 isolates. Fraction of ESBL and AmpC β-lactamases among the tested isolates is displayed in Table 4. By using both these methods, 01 (6.25%) *E. coli* and 01 (7.69%) *Klebsiella spp* isolates were identified

as only AmpC beta-lactamase producers. The prevalence of inducible AmpC was found to be 13.79%. There was no statistically significant difference in proportions of *E. coli* & *Klebseilla* spp with relation to positivity for only ESBL (p=1), AmpC (p=1) or both (p=1) through Z test.

Table 4: **Distribution of Beta-lactamases Among *E.coli* and *Klebsiella* Isolates.**

Sl no	Isolates	Screening Test Positive	ESBL* only	AmpC** only	ESBL + AmpC
1.	***Escherichia coli***	16	13	01	01
2.	***Klebsiella spp***	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)

DISCUSSION

Neonatal sepsis is a foremost contributor to mortality of neonates which should be and has to be grappled wisely. The prime focus of the present study was to find out the incidence of ESBLs and AmpC β-lactamases in *Escherichia coli* and *Klebsiella* spp from suspected cases of neonatal septicemia as ESBLs producing *E.coli* and *Klebsiella* spp are the leading cause of neonatal infections around the world [7].

The current study inferred blood culture positivity rate among neonates to be 32.46% which is in line with the rate of 31.5% reported by Khanna A et al [8]. An extensive variation in blood culture positivity has been delineated over the past decade from various centers across the globe. From our country, a higher positivity of 55.8% was reported from Vadodara, Gujarat in 2018 [9]. The lower blood culture positivity in present study might be due to a slightly lesser number of risk factors associated while the higher rate of sepsis among various studies can be poor antenatal care, high use of invasive procedures, illiteracy among the patients, low socioeconomic conditions around the study centre. The higher positivity of 34.36% was found in males in contrast to 29.67% in females which can be due to of higher proportion of males included in the study sample. No statistical significance (p>0.005) was attributed to the differences seen in distribution with respect to sex. This is in accordance with the study of Monica Lazarus et al which reported a higher male to female ratio [10].

The present study showed preponderance of Gram negative isolates (58.87%) in contrast to Gram positive isolates (37.9%) and yeast like fungi (3.22%). This is in line with the findings of the study conducted in 2016 which reported 53.93 % of cases by Gram negative isolates, 24.72% cases by Gram Positive isolates, and 21.35% by yeast like fungi i.e. *Candida* spp [8] while a divergent percentage of 40% and 60% reported by Thakur et al in 2016 [11]. In the present study the most frequently isolated Gram positive bacteria 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. These finding are comparable to the studies by Reddy KA et al. from Telangana [12] and Kumar R et al. in Bihar [13]. Similar findings have been reported by Hasibuan B S et al from Indonesia which reported isolation rate of *S. aureus* as 5.1% and 4.0% among EONS and LONS. CoNS were isolated in 6.33% and 14.67% among EONS and LONS cases respectively [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. This difference in distribution among various Gram positive bacteria was found to be statistically significant (p<0.05). In the present study, *E. coli* spp 41.09% and *Klebsiella* spp 31.51% were most common Gram negative isolates which is similar to the findings reported by a past study [15], this might be due to the higher number of cases of EONS as compared to LONS in current study. In contrast to current findings Khanna et al. reported *Klebsiella* spp (20.2%) as predominant pathogen while *E.coli* was isolated from 14.6 % cases [10]. Similarly Reddy KA et al. isolated *Klebsiella* spp from 25% cases followed by *Escherichia coli* from 8.33% cases [14].

The present study established *E.coli* (37.29%) is the prime pathogen causing EONS while *Klebsiella* spp (15.0%) as the leading pathogen causing LONS. Similar finding has been reported by Porta et al. in 2017 [16] which affirmed *E. coli* as main cause of sepsis in the first 72 hours of neonatal life. This might owe to the fact that coliforms, including *E. coli*, are frequently colonizers of the maternal vaginal canal and the new born acquire them 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%) in the current study. A proportionate result of isolation of *Klebsiella* spp in (30%) among LONS cases (16.6%) among EONS cases was reported by the study of Hematyar M et al. [17]. A disparate finding has been reported by studies of Muhammad et al. & Hasabuan B. S et al. which stated a higher incidence of *Klebsiella spp* in EONS (68.49) & LONS (70.73%) cases and EONS (20.3%) & LONS (18.7%) respectively [18,14].

Of the 29 (n=16 *Escherichia coli* & n= 13 *Klebsiella spp*) isolates that were positive in the preliminary screening test in the current study, 24 (82.76%) isolates were phenotypically confirmed as ESBL producers by the CLSI phenotypic confirmatory combined disc diffusion method. Detection of ESBL producers among the positive screened isolates ranged from 67.57% to 91.1% in other studies [19,20]. Of the 16 *E. coli* and 13 *Klebsiella* isolates, ESBL production was confirmed in 13 (81.25%) and 11 (84.61%) isolates. Thus, from the total population of *E.coli* and *Klebsiella* spp included in the present study, ESBLs were detected in 24 (45.28%) isolates. Individually ESBL was detected in 13(24.53%) *E. coli* and 11(20.75%) *Klebsiella* isolates.

By using "Disc antagonism test (DAT)" for inducible AmpC ß-lactamases and Ezy MIC™ strip test in the present study, 2 (6.89%) isolates (1 *E. coli* and 1 *Klebsiella spp*) were found as co-producers of both ESBL and inducible AmpC β-lactamase. By using both these methods, 01 (6.25%) *E. coli* and 01 (7.69%) *Klebsiella spp* isolates were identified as only producers of inducible AmpC beta-lactamase which is statistically insignificant through Z test. This is higher when compared to another study which reported 2.33% isolates as producers of inducible AmpC as detected by DAT [21]. The incidence 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 [22]. While 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 [23].

CONCLUSION:

The current study establishes a discernible incidence of ESBL as well as AmpC among the *E.coli* and *Klebsiella* spp isolates from neonatal sepsis cases. It was observed that the E test and DAT were equally effective for AmpC detection. These tests are simple to perform and easy to interpret, and requires less expertise for detection of isolates harboring AmpC. The presence of AmpC give positive ESBLs screening but fail to confirm, giving ambiguous results and ultimately poses hindrance in formulation of treatment. Hence it should be compulsory to detect these resistant strains separately and differentiate between Amp C and ESBLs producers.

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