Research Article

Nepal Journal of Health Sciences

Submitted:15/09/2021
Accepted:30/10/2021
Published:31/12/2021

Detection of Extended Spectrum β-Lactamases (ESBL) Producing Bacteria in Sepsis Suspected Neonates

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ABSTRACT

Introduction: Neonatal sepsis is a clinical syndrome which is caused when blood stream of infant is invaded by the bacteria in first month after birth.

Objective: The objective of the study was to identify bacteria involved in the infection and to determine "*extended spectrum beta-lactamase*" (ESBL) producing bacteria from blood samples of sepsis suspected neonates in Neonatal Intensive Care Unit and Special Care Baby Unit.

Methods: This cross-sectional study was conducted from January to July 2019 at Microbiology laboratory of Paropakar Maternity and Women's Hospital. A total of 380 venous blood specimens were included in the study. The blood culture was performed and organisms were identified with standard microbiological methods. The Antibiotic susceptibility test was performed using modified Kirby Bauer disk diffusion method. Screening of the organisms was done using *cefotaxime* and *ceftazidime* antibiotic disc and confirmation of ESBL was done by combined disk test. The data were considered statistically significant if p- value was < 0.05.

Results: Out of a total of 380 blood specimens, the prevalence of neonatal sepsis was found to be 21.05% among which 57.5% were EOS type and 42.5% were LOS type. In EOS, E. coli (72.73%) was the predominant isolate while CoNS (100%) was the predominant isolate in LOS. Of the total 80 isolates, 65% isolates were found multidrug-resistant (MDR) whereas 58.75% of isolates were found to be ESBL producers.

Conclusions: This study concludes that routine bacterial surveillance and study of their resistance patterns is an essential component of neonatal care unit

Keywords: *Extended spectrum β-Lactamases;* neonates; neonate intensive care unit; special care baby unit; sepsis.

INTRODUCTION

Sepsis is a deregulated response to infection that may accompany to organ failure, responsible for sixth leading cause of neonatal death within 28 days and the eighth leading cause of infant's death within first year of life.[1] Neonatal sepsis is a bloodstream infection occurring in infants within 28 days of birth and is one of the major causes of neonatal morbidity and mortality each year.[2] Gram negative bacteria are the major cause of neonatal sepsis in developing countries whereas Group B *Streptococcus* is the major cause of neonatal sepsis in developed countries.[3] On the basis of infant's age during onset of symptoms, neonatal sepsis is divided into two types: Early-onset sepsis that occur within first 72

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Citation
Nepal S, Koirala S, Thakur S, Bhattarai S, Dhungana S, Giri S, Khanal S. Detection of Extended Spectrum β-Lactamases (ESBL) Producing Bacteria in Sepsis Suspected Neonates. Nepal J Health Sci. 2021 Jul-Dec; 1(2):7-14.

hours of birth and late-onset sepsis that occur after 72 hours of birth.[4]

In Asia, the study has reported the incidence of neonatal sepsis of 7-38 in every 1000 live birth.[3] However, in Nepal, limited information is available about extended spectrum beta lactamase (ESBL) producing bacterial isolates in neonates. Thus, the present study was carried out to identify ESBL-producing bacteria isolated from neonatal sepsis in a tertiary care hospital in Nepal.

METHODS

The cross-sectional study was conducted from January to July 2019 at Microbiology laboratory of Paropakar Maternity and Women's Hospital. All neonates who were suspected with neonatal sepsis, admitted to Neonatal Intensive Care Unit (NICU) and Special Care Baby Unit (SCBU) were included in the study. Patients were selected on the basis of clinical decision of sign and symptoms of sepsis at the time of admission or during their hospital stay. Those patients whose parents refused to be part of the study and those who were already on antibiotics therapy after sepsis were excluded from the study.

Total number of 380 estimated blood samples was included as a sample for the study which was calculated using a single proportion formula[5] keeping 95% confidence level and 5% confidence interval. The blood samples were collected by a well-trained nurse and Pediatrician by using aseptic technique. The total of 0.5-1ml venous blood was drawn from the antecubital vein. The blood was then inoculated into Brain Heart Infusion Broth and the blood culture bottle was transported to the Microbiology Laboratory of Paropakar Maternity and Women's Hospital for the culture and antibiotics susceptibility tests. All blood culture bottles were incubated aerobically at 37°C and were observed daily for up to 5 days for perceptible microbial growth by observing any one of the following: coagulation of broth, turbidity, hemolysis and gas production. Since growth on only one bottle is considered as contaminant, blood was inoculated on two aerobic culture bottles from every single patient and growth on both bottles were considered as pathogen. That blood culture bottle with signs of microbial growth was subcultured onto Blood Agar (BA), Chocolate Agar (CA) and MacConkey Agar (MA).

The MA and BA agar plates were incubated in aerobic condition and CA in the micro-aerophilic atmosphere using a candle jar at 37°C for 24-48 hours. The observation was repeated until 7 days before discarding the blood culture bottle where growth was not observed and declaring them to be microorganism free after getting the result of routine subculture on BA, MA, and CA as no growth. All positive blood cultures were further sub-cultured on Nutrient agar (NA) and were incubated at 37°C for 24 hours so as to obtain pure bacterial colonies. Isolated bacteria were identified by their morphological characteristic on the media, Gram staining reaction and confirmed by different biochemical reactions. As per the recommendation of Clinical and Laboratory Standards Institute[6], the antibiotic susceptibility testing was performed using Modified Kirby-Bauer Disk Diffusion Method. A suspension was made by inoculating 3-4 pure bacterial colonies into the nutrient broth. Before distributing the suspension on the surface of Muller Hinton agar, the turbidity was matched with 0.5 McFarland standards.

After the turbidity was matched, the suspension was inoculated on MHA and the plates were left at room temperature for 5-10 minutes to dry and 5 antibiotic discs were incorporated on each 90 mm MHA plate. The antibiotics discs used during the test were Ciprofloxacin (CIP) (5µg), Cotrimoxazole (COT) (30 µg), Piperacillin/tazobactam (PIT) (10µg), Amikacin (AK) (30µg), Gentamicin (GEN) (10µg), Cefotaxime (CTX) (30µg), Ceftazidime (CAZ) (30µg), Erythromycin (E), Meropenem (MRP) (10µg), Vancomycin (VA) (5µg), (15µg), Oxacillin (OX) (10µg). The plate was incubated at 37°C for 24 hours. Diameters of the zone of inhibition of the incorporated discs were measured according to the CLSI (2018) and reported to be resistant, intermediate and sensitive.

Bacteria which were resistant to three or more than three antimicrobial classes were considered as Multidrug resistant bacteria.[7]If bacteria were resistant to any of Ceftazidime and Cefotaxime disc with zone of inhibition ≤ 22 mm for ceftazidime and ≤ 27 mm for cefotaxime, then they were suspected as ESBL producer and were confirmed by combined disc test using Cefotaxime- clavulanate and Ceftazidime-clavulanate as per recommendation of CLSI guideline 2017.[7]A positive ESBL test was confirmed when the zone of inhibition was greater than or equal to 5 mm around combined disk (Cefotaxime-clavulanate or Ceftazidime- clavulanate) than single disk (Cefotaxime or Ceftazidime).

The quality control procedures of the standard medical microbiology laboratory were strictly applied during the collection, transport and processing of the specimens for standard results. The adequate control of all equipment and material was done during the research. Each agar, biochemical reagents and antibiotic disks were checked for their respective manufacture date, expiry date, lot number and for appropriate storage condition. Five percentages of media were incubated at 37°C for 24 hours from each and every batch of the prepared media to ensure the purity of the culture media. The purity plate was used to ensure that the colony used for biochemical tests was pure. The inoculum density of bacterial suspension during AST test was standardized by comparing it to 0.5 McFarland standards. The quality of the AST test was ensured by maintaining the thickness of MHA at 4mm and pH at 7.2-7.4. Double data entry method was used to ensure the accuracy of data.

All the data were entered and analyzed using Statistical Package of Social Science (SPSS) software version 16 and the inferential statistics such as Chi-square test was used to find the association between the variables at 95% confidence level (p-value < 0.05)

The ethical approval was obtained from Institutional Review Committee of Paropakar Maternity and Women's Hospital's development Board (Ref number 59-11-1874). The permission to conduct the research was obtained from director of the hospital. Consent was taken from the parents to include neonates on the study. Demographic, clinical and other relevant data were obtained by pediatrician/s for the study. The study was conducted as per the ethical regulations and principles of the Institutional Review Committee of Paropakar Maternity and Women's Hospital Women's Hospital.

RESULTS

Figure 1 demonstrates that of 380 blood samples, 80 blood culture specimens were positive. Among blood culture positive, 43 (53.75%) were from males and 37 (46.25%) were from females. The prevalence rate of neonatal sepsis was found to be 21.05%.

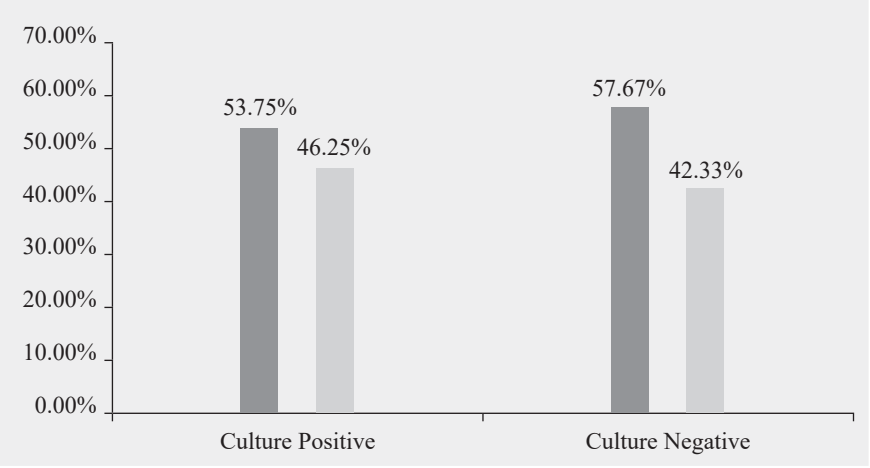

Figure 1: Bacterial growth pattern among the patients.

Neonatal sepsis was divided into two different categories on the basis of days of birth as early onset sepsis (EOS) and late onset sepsis (LOS) of neonates. Out of 80 blood culture positive samples, 46 (57.5%) were EOS type and 34 (42.5%) were LOS type. In EOS, *E. coli* (72.73%) was predominant isolate followed by *K. oxytoca* (71.43%) and *Acinetobacter spp.* (68.75%). CoNS (100%) were also predominant isolate in LOS followed by *S. aureus* (85.71%). There was significant association between isolated organisms and types of neonatal sepsis (Table1).

Table 1: Bacteriological profiles of early - onset sepsis (EOS) and late - onset sepsis (LOS).

Organisms	EOS N (%)	LOS N (%)	Total N	p-value
E. coli	8 (72.73)	3 (27.27)	11	
K. pneumoniae	18 (60)	12 (40)	30	
K. oxytoca	5 (71.43)	2 (28.57)	7	
Acinetobacter spp.	11 (68.75)	5 (31.25)	16	0.001
Enterobacter spp.	2 (33.33)	4 (66.67)	6	
Citrobacter spp.	1 (50)	1 (50)	2	
S. aureus	1 (14.25)	6 (85.71)	7	
CoNS	0	1 (100)	1	
Total	46 (57.5)	34 (42.5)	80 (100)	

Table 2: Antibiotic resistant pattern of isolated organisms.

Organisms Antibiotics	*K. Pneumoniae* n=30	*E. coli* n=11	*Acinetobacter spp.* n=16	*K. oxytoca* n=7	*Enterobacter spp.* n=6	*Citrobacter spp.* n=2	*S. aureus* n=7	CoNS n=1
Ampicillin (AMP)	NT	11 (100%)	NT	NT	NT	NT	NT	NT
Ciprofloxacin (CIP)	10 (33.33%)	7 (63.64%)	5 (31.25%)	4 (57.4%)	3 (50%)	1 (50%)	2 (28.57%)	0 (0.00%)
Cotrimoxazole (COT)	20 (66.67%)	7 (63.64%)	9 (56.25%)	6 (85.7%)	5 (83.33%)	1 (50%)	2 (28.57%)	0 (0.00%)
Piperacillin /tazobactum (PIT)	6 (20%)	0 (0.00%)	0 (0.00%)	2 (28.57%)	0 (0.00%)	0 (0.00%)	NT	NT
Amikacin (AK)	13 (43.33%)	7 (63.64%)	5 (31.25%)	6 (85.7%)	5 (83.33%)	1 (50%)	1 (14.29%)	0 (0.00%)
Gentamicin (GEN)	17 (56.67%)	6 (54.55%)	8 (50%)	5 (71.43%)	5 (83.33%)	1 (50%)	2 (28.57%)	0 (0.00%)
Cefotaxime (CTX)	28 (93.33%)	9 (81.82%)	15 (93.75%)	7 (100%)	6 (100%)	2 (100%)	4 (57.14%)	0 (0.00%)
Ceftazidime (CAZ)	27 (90%)	10 (90.90%)	14 (87.5%)	7 (100%)	6 (100%)	2 (100%)	NT	NT
Erytromycin (E)	23 (76.67%)	8 (72.73%)	11 (68.75%)	6 (85.7%)	4 (66.67%)	2 (100%)	2 (28.57%)	0 (0.00%)
Meropenem (MRP)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	NT	NT
Oxacillin (OX)	NT	NT	NT	NT	NT	NT	2 (28.57%)	0 (0.00%)
Vancomycin (VA)	NT	NT	NT	NT	NT	NT	0 (0.00%)	0 (0.00%)

Table 3: Bacteriological profile of Multidrug Resistant (MDR) and Extended Spectrum β-lactamase (ESBL) producing organisms.

Organisms	Number	MDR N (%)	ESBL-producer N (%)	p-value
E. coli	11	7 (63.63)	5 (45.45)	
K. pneumoniae	30	23 (76.67)	23 (76.67)	
K. oxytoca	7	4 (57.14)	3 (42.86)	
Acinetobacter spp.	16	9 (56.25)	9 (56.25)	0.001
Enterobacter spp.	6	6 (100)	6 (100)	
Citrobacter spp.	2	1 (50)	1 (50)	
S. aureus	7	2 (28.57)	NT	
CoNS	1	0 (0)	NT	
Total	**80**	**52 (100.0)**	**47 (100)**	

K. pneumoniae was resistant to cefotaxime (93.33%) followed by ceftazidime (90%) erythromycin (76.67%) while sensitive to meropenem (100%). All isolated *E.coli* were resistant to Ampicillin (100%) and followed by ceftazidime (90%) and cefotaxime 81.82%). *Acinetobacter spp.* was sensitive to piperacillin/tazobactum and meropenem (100%) where as 100% resistance to cefotaxime and ceftazidime. Remaining other isolated Gram negative bacteria such as *K. oxytoca, Acinetobacter spp., Citobacter spp.* and *Enterobacter spp.* were 100% resistance to cefotaxime and ceftazidime while Gram positive bacteria such as *S. aureus* and CoNS were sensitive to Vancomycin (100%). CoNS were sensitive to all treated antibiotics (Table 2).

Out of total 80 isolated organisms, 52 organisms were found multidrug resistant (MDR). *Enterobacter spp.* (100%) were predominant MDR bacteria followed by *K. pneumoniae* (76.67%). The numbers of ESBL-producing bacteria were 47 and among them, *Enterobacter spp.* (100%) was predominant ESBL-producing bacteria which were followed by *K. pneumoniae* (76.67%). The association between MDR and ESBL-producing organisms were statistically significant (p=0.001) (Table3).

DISCUSSION

Although considerable practice in hygiene has been adopted and new antimicrobial agents have been introduced, the most common cause of death among newborns was reported to be neonatal infections like sepsis in both developed and developing countries as well which accounted for 6% to 25%.[8] Thus, it is very crucial to have information on the bacteriological profile and the appropriate antibiotics to treat sepsis in order to overcome mortality and morbidity due to neonatal sepsis.[8] In the current study, 80 blood culture specimen were found to be positive among 380 blood specimens taken as samples among which the highest proportion with 53.75% was from males while 46.25% was from females. This finding of our study is in harmony with the study conducted by Ansari et al[9] and Al-Shamahy et al.[10]

The prevalence rate of neonatal sepsis was found to be 21.05% which is more in comparison to the study conducted by Ansari et al.[9] The reason for more prevalence of neonatal sepsis in our study is due to differences in geographic location and differences in time during infection as stated by Shrestha et al.[11] Among total neonatal sepsis, the higher prevalence was found in Early onset of sepsis in comparison to the late onset of sepsis which is in contrast to the finding reported by Yadav et al.[12] However, this reporting was found to be accord with the reporting done Chaudhary et al.[13] The highest prevalence of *K. pneumonia* followed by *Acinetobacter spp., E. coli, K.oxytoca, S. aureus, Enterobacter spp., Citrobacter spp.*, and CoNS was shown by the

bacterial profile. These species of organisms have been reported as predominant causative agents by numerous studies.[11, 14] The highest proportion of gram-negative isolates was isolated from the study in comparison to the gram-negative isolates which is attributable to the exposure of neonates to a large number of gram-negative bacteria of the vagina.[11] The most common organism isolated in our study was *Klebsiella pneumoniae* however, the study performed by Olorukooba et al identified *E. coli* as the most predominant isolates with 31%.[15] Likewise, the study conducted by Onalo et al reported *S. aureus* as a predominant organism with 18%.[16]The reason for this different reporting is due to the change in etiology over the years.[15]

In this current study,Meorpenem and Piperacillin/tazobactam were observed to be the most effective antimicrobial agents against Gram-negative bacteria while Cefotaxime and Ceftazidime were found to be the least effective antimicrobial agents. Similar effectiveness of Meropenem and Piperacillin/tazobactam was reported to all Gram-negative organisms by a study performed by Shrestha et al.,[17] Desai and Malek.,[9]Ozkan et al.[18] On the other hand, in the case of Gram-positive bacteria, Vancomycin were found to be the most effective antibiotic in comparison to other antibiotics. A diverse antibiotic-resistant pattern has been reported in the different studies over different times. The rationale for this is due to the emergence of antibiotic-resistant strains which is due to the irrational use of antimicrobials.[9]

Similarly, of the total of 80 positive isolates, 65% isolates were found multidrug-resistant (MDR) among which Enterobacter spp. with 100% were predominant MDR. Those bacteria were considered MDR in this research study if they exhibit the ability to induce resistance to at least one antibiotic from distinct classes of common antimicrobial agents[20]. Moreover, 58.75% of isolates were found to be ESBL producers of a total of 80 positive isolates which is very high when compared to the finding reported by the study conducted by Dolma et al.[21] *Enterobacter spp.* (100%) was the most common ESBL-producer in comparison to other isolates which is contrary to the study performed by Zakariya where *K. pneumoniae* was reported to be the predominant ESBL producer with total 54.5%[22].

Limitation of the study

In this study, the molecular level study could not be performed due to the unavailability of a molecular lab on the hospital and the sample could not transport to other molecular laboratories for further study from the hospital due to the stringent rules and regulations of that hospital. Furthermore, the further characterization of CoNS could not be performed up to species level.

CONCLUSIONS

To conclude, the bacterial pathogens were isolated from 21.05% specimens which indicate the prevalence rate of neonatal sepsis to be 21.05% of which, the higher prevalence was found in the early onset of sepsis. The predominant organism that was responsible for causing neonatal sepsis was found to be *K. pneumoniae*. Meropenem and Piperacillin/tazobactam were observed to be the most effective antimicrobial agents against Gram-negative bacteria while Vancomycin was found to be the most effective antibiotic in the case of Gram-positive bacteria. The prevalence of MDR and ESBL among bacteria in sepsis suspected neonates in this study was very high with 65% and 54.5% respectively. The significant rise in MDR and ESBL in sepsis suspected neonates can be mitigated by making the rational choice of antibiotics and by identifying appropriate strategies to minimize the infection. Neonatal sepsis is difficult to identify and can be asymptomatic. So, laboratory testing with blood culture should be done immediately. If sepsis is suspected clinically, empirical treatment with appropriate antibiotics which is guided by the AST pattern of common bacteria in NICU must be started. Moreover, clinical and epidemiological investigations are necessary to assess changes in the organisms that cause neonatal sepsis. Further

studies using PCR should be performed including genotypic characterization of organisms involved in neonatal sepsis. Agents with high intrinsic activity against Enterobacter spp. should be selected for severely ill patients. Rapid diagnostic tests are highly needed in our country to detect ESBL-producing bacteria from neonate's blood in order to reduce the mortality with ESBL-producing bacterial neonatal sepsis.

ACKNOWLEDGEMENT

We would like to acknowledge the microbiology department of Paropakar Maternity and Women's Hospital for allowing us to perform research and to collect data from the laboratory.

Conflict of Interest: None

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