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Research Article

Bacteriological Profile and Detection of β-Lactamase Producing Bacteria Isolated from Blood Samples of Neonates

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Abstract

The clinical impact of β-lactamase has become a public health problem around the world in terms of increased morbidity and mortality, especially in the child population. This study was aimed at determining the bacteriological profile and detection of β-lactamase producing bacteria isolated from the blood samples of neonates. For this study, a total of 1335 blood samples of neonates admitted in NICU, SCBU, and sepsis-suspected neonates visiting Paropakar Maternity and Women's Hospital, Thapathali, Kathmandu, Nepal were collected and processed. Blood culture was performed and the identification of bacteria was done by following standard microbiological methods. Antibiotic susceptibility testing was done by using the Kirby Bauer Disk Diffusion method and confirmation of ESBL, MBL, and KPC-producing bacteria was done by Combined Disk Test. The prevalence rate of neonatal sepsis was found to be 17%. *K. pneumoniae* 116 (50.2%) was the predominant Gram-negative bacteria followed by *K. oxytoca* 31 (13.4%) whereas *S. aureus* 39 (16.9%) was the predominant Gram-positive bacteria causing neonatal sepsis. Among 182 Gram-negative bacterial isolates, 69 (37.9%), 22 (12.1%), and 14(31.1%) were found to be ESBL, MBL, and KPC producers respectively. *K. oxytoca* (54.8%), *Enterobacter* spp. (25%) and *Citrobacter* spp. (14.3%) were the predominant ESBL, MBL, and KPC producers respectively. The co-production of ESBL, MBL, and KPC was also found among the 5 Gram-negative bacteria. Colistin, Meropenem, and Imipenem seem to be the choice of the drug against Gram-negative bacteria, whereas Vancomycin and Cefoxitin seem to be the choice of the drug against Gram-positive bacteria. Therefore, to lessen the burden of antibiotic resistance, it is essential to conduct regular antimicrobial susceptibility surveillance, periodic reviews of hospital settings, and early detection of beta-lactamase-producing bacteria.

Keywords: Neonatal sepsis; ESBL; MBL; Antibiotic Resistance; KPC

Abbreviations: MDR- Multi Drug Resistance; AST- Antibiotic Susceptibility Test; ATCC- American Type Culture Collection; BA- Blood Agar; BHIBrain Heart Infusion; CLSI- Clinical Laboratory Standard Institute; CoNS- Coagulase Negative Staphylococci; CRE- Carbapenem Resistant Enterobacteriaceae; CRP- C Reactive Protein; CSF- Cerebrospinal Fluid; EDTA- Ethylenediamine tetraacetic acid; EOS- Early Onset Sepsis; ESBLs- Extended Spectrum Beta Lactamases; GBS- Group B *Streptococcus;* GNB- Gram Negative Bacteria; LOS- Late Onset Sepsis; MA- Mac-Conkey Agar; MALDI-TOF- Matrix Assisted Laser Desorption -Time of Flight; MBLs- Metallo Beta Lactamases; MHA- Mueller Hinton Agar; NICU- Neonatal Intensive Care Unit; PBA- Phenyl Boronic Acid; PROM- Prolonged Rupture of Membrane; SCBU- Special Care Baby Unit; WHO- World Health Organization.

Introduction

Although there are different ongoing advances in medical technology, the combat against drug-resistant bacteria is always challenging and drug resistance is commonly regarded to be the next global pandemic (Aslam *et al.*, 2018). Carbapenems, polymyxin (colistin), and tigecycline are currently used as backup drugs for multidrug-resistant infections (Almohammady *et al.*, 2020). However, the continual use of Carbapenems and Polymyxins to treat bacterial infections, resistant to Penicillins, Cephalosporins, and Carbapenems are increasing the rate of resistance to these last-resort antibiotics (Meletis, 2016; Osei and Reta 2020). The emergence of multidrug-resistant bacteria is currently posing challenges in the treatment of neonatal sepsis (Yadav *et al.*, 2018).

Neonatal mortality rates have remained unacceptably high, with an estimated 2.9 million newborn deaths occurring within the first 28 days of life each year. Nearly a quarter of them are directly caused by infectious causes, with neonatal sepsis accounting for 15% (Lawn *et al.*, 2014). Neonatal sepsis is defined as a clinical syndrome in an infant younger than 28 days of age caused by a pathogen in the bloodstream (Simonsan *et al.*, 2014). There are two types of neonatal septicemia. Early onset neonatal septicemia refers to an infection that occurs within 72 hours of birth. Similarly, late-onset neonatal septic septicemia refers to an infection that occurs within 72 hours of birth. Similarly, late-onset neonatal septicemia refers to an infection that occurs after 72 hours of age (Thapa and Sapkota 2019).

In developing countries, neonatal sepsis is one of the most common reasons for admission to neonatal units (Struzek *et al.*, 2018). In both developed and developing countries, it is a major cause of morbidity and mortality. Over one-third of the global burden of child, mortality is attributed to neonatal deaths. In neonates, sepsis is a major cause of morbidity and mortality (Kumar and Bhat 2016). Prematurity, low birth weight, and a prolonged hospital stay are risk factors for neonatal sepsis. Early colonization and subsequent infection by resistant bacteria that cause high morbidity and mortality are caused by a lack of adequate space, shortage of staff, high occupation rates, extensive use of antibiotics, and increased susceptibility of the population (Kumar and Bhat 2016). Over 25% of neonatal deaths worldwide or one million deaths annually occur in the neonatal period (0-28 days) and 99% of these deaths take place in developing nations (Waters *et al.*, 2011). The World Health Organization estimates that one million deaths per year are due to neonatal sepsis and that 42% of these deaths occur in the 1st week of life (Lawn *et al.*, 2014).

The most common bacterial organisms responsible for neonatal septicemias in developed countries include coagulase negative *Staphylococcus* and group B *Streptococcus* while in developing countries like Nepal, Pakistan, India, Nigeria, Bangladesh etc are *Escherichia coli*, *Klebsiella* species, *Enterobacter* species, etc. (Huynh *et al.*, 2015). As part of the target set by the Sustainable Development Goal for health, Nepal is aiming to lower newborn death rate to 12 per 1000 live births or fewer by 2030, according to the Ministry of Health and Population Nepal and National Planning Commission. Between 2001to 2016, the neonatal mortality rate in Nepal declined from 39 to 21 deaths per thousand (KC *et al.*, 2020). However, neonatal infection is one of the leading causes of hospital admissions and neonatal deaths in Nepal (Chapagain *et al.*, 2015; Yadav *et al.*, 2018). The prevalence of neonatal infections in Nepal is 2-4%, with 37.1% of infections occurring in neonatal intensive care units of tertiary referral hospitals (Chapagain *et al.*, 2015).

Multiple drug-resistant (MDR) organisms that cause newborn sepsis are becoming more prevalent in developing nations and *K. pneumoniae* is frequently documented in this setting. *K. pneumoniae* is resistant to a variety of antibiotics by a variety of methods, including the production of extended-spectrum beta-lactamases (ESBLs), AmpC beta-lactamases, 16S rRNA methylases, aminoglycoside modifying enzymes and carbapenemases (Roy *et al.*, 2013).

The emergence of ESBLs is crucial in the management of infections associated with sepsis. Additionally, ESBL-producing isolates show resistance to third-generation cephalosporins and other beta-lactam antibiotics. They also tend to exhibit resistance to other classes of drugs, including Aminoglycosides, Cotrimoxazole, Tetracycline, and Fluoroquinolones. As a result, they provide a difficult problem with few treatment alternatives, especially in nations with low resources (Chandel *et al.*, 2011).

In many clinical settings during the past ten years, the usage of Carbapenems like Ertapenem, Meropenem, and Imipenem has grown due to the proliferation of highly resistant ESBL-expressing organisms. It is possible that as a result, carbapenem-resistant Enterobacteriaceae (CRE) has emerged and is beginning to spread globally (Drew *et al.*, 2013). *K. pneumoniae* and *Enterobacter* spp. are the two bacteria most frequently reported as exhibiting carbapenem resistance (Sievert *et al.*, 2013).

Metallo-beta lactamases (MBLs) have the propensity to hydrolyze almost all beta-lactam drugs, including Carbapenems. MBLs have recently emerged as one of the most threatened resistance mechanisms. Its spread on highly mobile gene elements in nosocomial pathogens limits the therapeutic options (Thapa *et al.*, 2017). It has thus become essential to be alert about the trend in susceptibility patterns of organisms to save the therapies.

The clinical impact of Carbapenem resistance has become a public health problem around the world in terms of increased mortality, longer hospital stays, and higher costs. The child population in this issue is of great concern as it is

a naturally vulnerable population in which the risk may vary, depending on immunological maturity, the presence of comorbidities, the presence of invasive medical devices, and even the prior use of antibiotics. Due to limited antibiotic stockpiles, increasing antimicrobial resistance poses a major challenge in the control of neonatal sepsis. Knowledge of prevalent bacterial isolates and their antibiotic susceptibility pattern is crucial when choosing the appropriate empirical therapy in order to decrease morbidity and mortality. So, it is of utmost importance to determine the prevalence of culture-positive neonatal sepsis, its clinic bacteriological profile and antibiotic susceptibility pattern of the isolates along with the detection of ESBL, MBL and KPC producing bacteria.

Methodology

Study Site and Period

The study was carried out at the Microbiology Department, Clinical Laboratory section of Paropakar Maternity and Women's Hospital, Thapathali, Kathmandu, Nepal from 1st March, 2021 to 30th August, 2021.

Study Design

This research was a hospital based cross-sectional study.

Study population

Sepsis-suspected neonates admitted in Neonatal Intensive Care Unit (NICU), Special Care Baby Unit (SCBU), and sepsis-suspected neonates visiting Paropakar Maternity and Women's Hospital within the study period were considered as study population.

Ethical approval

Ethical approval was taken from IRC of Paropakar Maternity and Women's Hospital, Thapathali, Kathmandu, Nepal.

Sample Types and Sample Size

Blood samples of neonates are the sample used in this study. The sample size was obtained by using the formula.

$$n = (Z_\alpha)^2 * p * \frac{1-p}{d^2}$$

where,

n= minimum sample size

d= desired level of significance (0.05)

Z= confidence interval (1.96)

P= prevalence rate i.e 16%

Sample size $n = (1.96)^2 * 0.16 * \frac{1-0.16}{0.05^2} = 206.5 \approx 207$

A total of 1335 blood samples were collected during the study for target bacteria isolation.

Inclusion and Exclusion Criteria

Inclusion Criteria: Only blood samples of neonates collected aseptically, in a clean, sterile, leakproof container along with neonate's demographic information and with no visible signs of contamination were included in the study.

Exclusion Criteria: Improperly labeled, low number of samples and leaked samples were excluded from the study. Samples not meeting inclusion criteria were rejected and requested for repetition if possible.

Study Variables

The study variable includes the type of pathogens, early-onset sepsis, late-onset sepsis, antibiotic susceptibility pattern, multi-drug resistance, and carbapenem resistance.

Laboratory analysis

For this study blood samples of neonate obtained in the laboratory were used and preceded as follows:

a. Sample collection

The blood samples of neonates were collected aseptically by experienced medical officers, nurses, or laboratory technicians by following strict aseptic conditions. Blood samples were labeled appropriately with the patient's identification number. The samples were processed immediately as soon as possible.

b. Sample processing

Standard laboratory protocols were followed for the processing of blood samples as recommended by Cheesbrough (2012).

About 1 ml of blood from sepsis-suspected neonates was collected by following strict aseptic conditions. After collection, the blood was transferred into a culture bottle containing BHI (Brain heart infusion) broth. The culture bottles were incubated at 37°C aerobically. After overnight incubation, the subculture was done on to fresh 5% sheep Blood agar (BA) and MacConkey agar (MA). MA plate was incubated aerobically whereas BA plate was incubated in a CO_2 jar at 37°C for 24 hours. After overnight incubation, colonies of the isolates were identified on the basis of colony characteristics on Blood Agar, MacConkey Agar, Gram's reaction and biochemical tests. If growth was not seen on plates after overnight incubation, subcultures were repeated from the broth on day 3, day 4, and finally on day 7 (Cheesbrough, 2012).

In the case of the blood sample collected in BACTEC bottle, the blood sample was kept in BACTEC for 72 hours. The samples detected positive in BACTEC was cultured in BA and MA. MA plate was incubated aerobically whereas BA plate was incubated in CO_2 jar at 37°C for 24 hours. After overnight incubation, colonies of the isolates were identified on the basis of colony characteristics on Blood Agar, MacConkey Agar, Gram's reaction and biochemical tests (Messbarger and Neeman, 2018).

Identification of Bacteria

The identification of various bacterial isolates was done by using standard microbiological techniques as described in Bergey's manual of systemic bacteriology which comprises

of studying the colonial morphology, staining reactions and various biochemical properties (Bergey's manual, 2nd edition, volume 2). Isolated colonies from the pure culture were identified by performing the standard conventional biochemical tests; catalase test, oxidase test, coagulase test, O/F test, sulphur indole motility test, MR-VP test, TSIA test, urease test and Bile Esculin agar test.

Antibiotic Susceptibility Testing

Isolates were subjected to in-vitro antibiotic susceptibility tests by Kirby Bauer disk diffusion method as recommended by Clinical Laboratory Standard Institute (CLSI, 2018). During each test, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as quality control to ensure accuracy of the antimicrobial susceptibility assays. In this method, the broth culture of test organism (comparable to McFarland tube number 0.5; inoculums density (1.5×10^8 organisms/ml) were uniformly carpeted on the surface of Mueller Hinton agar. Then, antibiotics discs were placed over the lawn culture of the test organism by sterile forceps. Antibiotics were used according to the organism isolated. The antibiotics for disk diffusion test were in the following concentration; Ampicillin (AMP) (30µg), Ciprofloxacin (CIP) (5µg), Cotrimoxazole (COT) (30µg), Cefotaxime (CTX) (30µg), Ceftazidime (CAZ) (30µg), Gentamicin (GEN) (10µg), Amikacin (AK) (30µg), Piperacillin/tazobactam (PIT) (10µg), Meropenem (MRP) (10µg), Cefoxitin (CX) (30µg), Cefepime (CPM) (30), Vancomycin (VA) (5µg) and Colistin (CL) (10µg). The inoculated and seeded MHA plates then were incubated at 37° C for 24 hours (or overnight). Diameters of the zone of inhibition around the disks were measured in millimeter (mm) and the organism was reported as "Resistant", "Intermediate" or "Susceptible". Isolates resistant to three or more than three antibiotics were classified as multidrug resistance (CLSI 2018; Magiorakos *et al.*, 2012).

ESBL: Screening and Confirmatory Test

Screening test for the production of ESBL was performed by using Ceftazidime (CAZ) (30µg) and Cefotaxime (CTX) (30µg) discs. The zone of inhibition ≤22 mm for Ceftazidime and ≤27 mm for Cefotaxime of the isolate was considered as a potential ESBL producer as recommended by CLSI. The confirmations of ESBL were done by combination disk method in which CAZ and CTX alone and in combination with Clavulanic Acid (CA) (10µg) was used. An increased zone of inhibition ≥5 mm for either antimicrobial agent in combination with CA versus its zone when tested alone was considered to be confirmed ESBL producers (CLSI, 2018).

MBL: Screening and Confirmatory Test

MBL screening was determined by using Imipenem (IPM) (10µg) and Meropenem (MRP) (10µg). In susceptibility testing by disk diffusion, zone of diameter ≤23 mm on for both Imipenem (10µg) and Meropenem (10µg) were reported as probable MBL producers (CLSI, 2018).

MBL production was determined by combined disk method. In this method, test strains with 0.5 McFarland standard suspensions were lawn cultured on MHA plates. Two Imipenem discs (10µg) were placed on MHA plate, one combined with 10µl of 0.5M EDTA. After overnight incubation, the inhibition zone of Imipenem and Imipenem + EDTA discs were compared. An increase in zone of inhibition by ≥7 mm in combined disc compared to Imipenem alone was considered as MBL positive (Sachdeva *et al.*, 2017).

KPC: Screening and Confirmatory Test

Carbapenem resistance among isolates was determined by using Imipenem (10µg) and Meropenem (10µg). In susceptibility testing by disk diffusion, zone of diameter ≤23 mm on for both Imipenem (10µg) and Meropenem (10µg) were reported carbapenem resistant. Carbapenem resistant Gram-negative isolates were tested for possible KPC production (CLSI 2018).

All carbapenem resistant isolates were tested for KPC production by combined disk method. A lawn culture of test strain, equivalent to 0.5 McFarland in nutrient broth, was done in the MHA plate. From the stock solution of 20mg/ml PBA, 20µl (400µg of PBA) was dispensed onto one of the Meropenem disc and allowed to dry. Both Meropenem discs with and without PBA was placed onto MHA plate. An increase of ≥7 mm inhibitory zone of diameter around the Meropenem disc combined with PBA compared to Meropenem alone was reported as KPC producer strain (Sood, 2014).

Quality Control

During this study, a strict aseptic condition was maintained in the collection and processing of the specimens in order to ensure the good microbiological results. Each agar, antibiotic disk and biochemical reagents were checked for their respective lot number, manufacture date, expiry date and for proper storage conditions. Each batch of culture and biochemical media was incubated at 37˚C for 24 hours to ensure the proper quality. For the standardization of Kirby Bauer Disk Diffusion Test and for performance testing of antibiotics and MHA, control strain of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were tested primarily. Quality of sensitivity test was maintained by maintaining the thickness of MHA at 4mm and pH 7.2-7.4.

Data Analysis

All the data of results were entered into excel and analyzed using statistical package for social science (SPSS) software (Version 21.0). Frequency and percentage were calculated. Comparison of nominal data was done using Chi-square test. P-value less than 0.05 was considered as statistically significant.

Results

Growth Pattern of Bacteria

Out of 1335 blood samples of neonates, 231 (17%) of blood samples showed growth and 1104 (83%) showed no growth of bacteria in blood culture. The prevalence of neonatal sepsis was 17%.

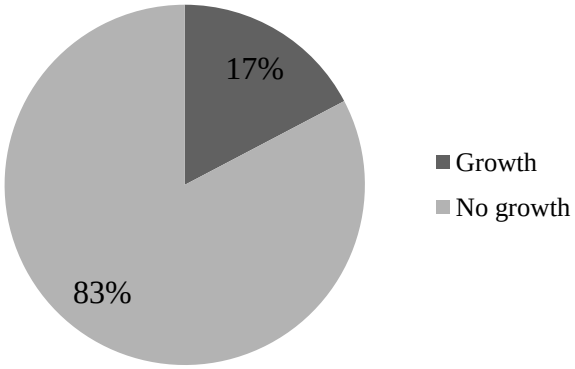


Fig. 1: Growth pattern of bacteria

Distribution of Early-Onset Sepsis and Late-Onset Sepsis

Out of 231 blood cultures positive, 138 (59.74%) were EOS and 93 (40.26%) were LOS.

Distribution of Bacteria According to Growth Profile

Among 231 positive blood culture isolates, *K. pneumoniae* was the predominant Gram-negative isolates 116 (50.2%) whereas *S. aureus* was the predominant Gram-positive isolate 39 (16.9%) (Table 1).

Table 1: Distribution of bacteria according to growth profile

Organism	Total No (%)
K. pneumoniae	116 (50.2%)
K. oxytoca	31 (13.4%)
Acinetobacter spp.	16 (6.9%)
Enterobacter spp.	12 (5.3%)
Citrobacter spp.	7 (3%)
Enterococcus fecalis	10 (4.3%)
S. aureus	39 (16.9%)
Total	231 (100%)

Antibiotic Susceptibility Pattern of Isolated Bacteria

Antibiotic susceptibility pattern of K. pneumoniae:

AST was performed on 116 *K. pneumoniae* isolates. *K. pneumoniae* were 100% resistant to Ampicillin followed by Gentamicin 100 (86.2%) and Cefepime 72 (62.1%) while 100% sensitive to Colistin followed by Meropenem and Imipenem with equal sensitivity of 91 (78.4%) (Table 2).

Table 2: Antibiotic susceptibility pattern of *K. pneumoniae*

Antibiotics	Resistance N (%)	Sensitive N (%)
Ampicillin	116 (100%)	0 (0%)
Gentamicin	100 (86.2%)	16 (13.8%)
Amikacin	39 (33.6%)	77 (66.4%)
Ciprofloxacin	35 (30.2%)	81 (69.8%)
Cefepime	72 (62.1%)	44 (37.9%)
Cefotaxime	53 (45.7%)	63 (54.3%)
Ceftazidime	51 (44.0%)	65 (56.0%)
Pipercillin/tazobactam	47 (40.5%)	69 (59.5%)
Meropenem	25 (21.6%)	91 (78.4%)
Imipenem	25 (21.6%)	91 (78.4%)
Colistin	0 (0%)	116 (100%)

Antibiotics susceptibility pattern of K. oxytoca:

Table 3 shows that *K. oxytoca* were found to be 100% resistant to Ampicillin followed by Pipercillin/tazobactam, Cefepime, Cefotaxime, Ceftazidime and Gentamicin with equal resistance of 29 (93.5%).

All the 31 isolates of *K. oxytoca* were sensitive to Colistin 31 (100%) followed by Meropenem, Imipenem, Amikacin and Ciprofloxacin with equal sensitivity of 20 (64.5%).

Table 3: Antibiotic susceptibility pattern of *K. oxytoca*

Antibiotics	Resistance N (%)	Sensitive N (%)
Ampicillin	31(100%)	0 (0%)
Gentamicin	29 (93.5%)	2 (6.5)
Amikacin	11(35.5%)	20 (64.5%)
Ciprofloxacin	11(35.5%)	20 (64.5%)
Cefepime	29 (93.5%)	2 (6.5%)
Cefotaxime	29 (93.5%)	2 (6.5%)
Ceftazidime	29 (93.5%)	2 (6.5%)
Pipercillin/tazobactam	29 (93.5%)	2 (6.5%)
Meropenem	11 (35.5%)	20 (64.5%)
Imipenem	11 (35.5%)	20 (64.5%)
Colistin	0 (0%)	31 (100%)

Antibiotic susceptibility pattern of other Gram-negative bacteria:

Most of the *Acinetobacter* spp. were resistant towards Ampicillin 13 (81.2 %) and least resistant towards Meropenem 4 (25.0%) and Imipenem 4 (25.0%). Similarly, *Enterobacter* spp. isolates were resistant towards Cefepime 9 (75.0%) and Ampicillin 9 (75.0%). Most of the *Citrobacter* spp. were resistant to Ampicillin 6 (85.7%) and

Gentamicin 6 (85.7%). Colistin was completely effective against all the isolates (Table 4).

Antibiotic susceptibility pattern of Gram-positive bacteria:

Most of the S. aureus were resistant to Gentamicin 19 (48.7%) and Ampicillin 19 (48.7%) whereas sensitive to Vancomycin 38 (97.4%) and Cefoxitin 35 (89.7%). Similarly, most of the *Enterococcus fecalis* were resistant to Gentamicin 8 (80%) and Ampicillin 8 (80%) whereas 100% sensitive to Vancomycin and Cefoxitin (Table 5).

Distribution of ESBL Producing Gram Negative Bacteria

Out of 182 gram-negatives isolates, 97 (53.3%) were screened positive for ESBL production among which 69 (53.3%) of the isolates were confirmed to be ESBL producer (Table 6). The maximum production of ESBL was seen in case of *K. oxytoca* i.e. 17 (54.8%) while the least production was seen in case of *Acinetobacter* spp. i.e. 5 (31.1%).

Table 4: Antibiotic susceptibility pattern of other Gram-negative bacteria

Antibiotics	Bacteria					
	***Acinetobacter* spp.** N=16 (%)		***Enterobacter* spp.** N=12 (%)		***Citrobacter* spp.** N=7 (%)	
	R	**S**	**R**	**S**	**R**	**S**
Ampicillin	81.2	18.8	75	25	85.7	14.3
Gentamicin	75.0	25	58.3	41.7	85.7	14.3
Amikacan	43.8	56.2	33.3	66.7	28.6	71.4
Ciprofloxacin	31.2	68.8	33.3	66.7	14.3	85.7
Cefepime	56.2	43.8	75	25	57.1	42.9
Cefotaxme	31.2	68.8	58.3	41.7	42.9	57.1
Ceftazidime	31.2	68.8	58.3	41.7	42.9	57.1
Pipercillin/tazobactam	37.5	62.5	66.7	33.3	28.6	71.4
Meropenem	25	75	33.3	66.7	14.3	85.7
Imipenem	25	75	33.3	66.7	14.3	85.7
Colistin	0	100	0	100	0	100

Table 5: Antibiotic susceptibility pattern of Gram positive bacteria

Antibiotics	***S. aureus*** N=39 (%)		***Enterococcus fecalis*** N=10 (%)	
	R	**S**	**R**	**S**
Ampicillin	48.7	51.3	80	20
Gentamicin	48.7	51.3	80	20
Amikacan	10.3	89.7	20	80
Ciprofloxacin	2.6	97.4	10	90
Cefepime	25.6	74.4	20	80
Cefotaxime	12.8	87.2	20	80
Ceftazidime	12.8	87.2	20	80
Meropenem	2.6	97.4	10	90
Imipenem	2.6	97.4	10	90
Pipercillin/tazobactam	10.3	89.7	20	80
Cefoxitin	10.3	89.7	0	100
Vancomycin	2.6	97.4	0	100

Table 6: Distribution of ESBL producing Gram negative bacteria

Organism	**Total number of isolates**	**Screening positive No (%)**	**Phenotypic confirmation No (%)**
K. pneumoniae	116	53 (45.7%)	39 (33.6%)
K.oxytoca	31	29 (93.5%)	17 (54.8%)
Acinetobacter spp.	16	5 (31.3%)	5 (31.3%)
Enterobacter spp.	12	7 (58.3%)	5 (41.3%)
Citrobacter spp	7	3 (42.8%)	3 (42.8%)
Total	182	97 (53.3%)	69 (37.9%)

Table 7: Distribution of MBL producing Gram-negative bacteria

Organism	Total number of isolates	Screening positive No (%)	Phenotypic confirmation No (%)
K. pneumoniae	116	25 (21.5%)	10 (8.6%)
K. oxytoca	31	11 (35.5%)	7 (22.5%)
Acinetobacter spp.	16	4 (25 %)	1 (6.25%)
Enterobacter spp.	12	4 (33.3%)	3 (25%)
Citrobacter spp.	7	1 (14.3%)	1 (14.3%)
Total	182	45 (24.7%)	22 (12.1%)

Table 8: Distribution of KPC-producing Gram-negative bacteria

Organism	Total number of isolates	Screening positive No (%)	Phenotypic confirmation No (%)
K. pneumoniae	116	25 (21.5%)	8 (6.8%)
K. oxytoca	31	11 (35.5%)	3 (9.6%)
Acinetobacter spp.	16	4 (25 %)	1 (6.3%)
Enterobacter spp.	12	4 (33.3%)	1 (8.3%)
Citrobacter spp	7	1 (14.3%)	1 (14.3%)
Total	182	45 (24.7%)	14 (31.1%)

Table 9: Beta lactamase producers in MDR and Non-MDR Gram negative bacteria

Beta lactamase	Non-MDR	MDR	p-value
ESBL non-producer	68 (60.18%)	45 (39.82%)	
ESBL producer	0 (0%)	69 (100%)	0.000
MBL non-producer	68 (42.5%)	92 (57.5%)	
MBL producer	0 (0%)	22 (100%)	0.000
KPC non-producer	68 (40.48%)	100 (59.52%)	
KPC producer	0 (0%)	14 (100%)	0.003

Distribution of MBL Producing Gram Negative Bacteria

Out of 182 Gram negative bacterial isolates, 45 (24.7%) were probable MBL producer, while 22 (12.1%) were phenotypically confirmed MBL producer.

The most MBL-producing bacterial isolates were *Enterobacter* spp 3 (25%), while *Acinetobacter* spp. 1 (6.25%) produced the least MBL (Table 7).

Distribution of KPC-Producing Gram-Negative Bacteria

Data shown in Table 8 shows that out of 182 Gram-negative bacterial isolates, 45 (24.7%) were probable KPC producers and 14 (31.1%) were phenotypically confirmed KPC producers.

The highest KPC-producing bacterial isolates were *Citrobacter* spp. 1 (14.3%), while the least KPC-producing bacterial isolates were *Acinetobacter* spp. 1 (6.3%).

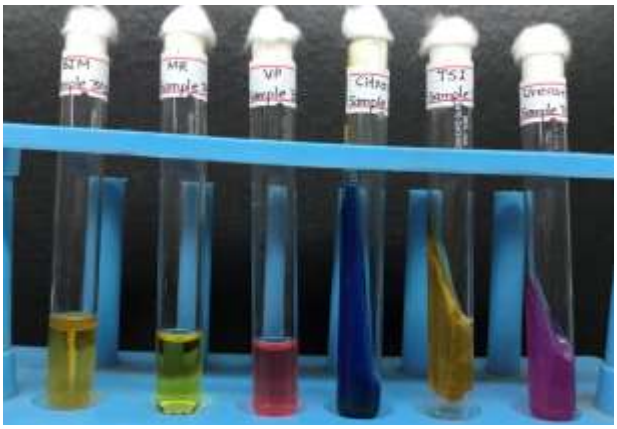
Beta-lactamase producers in MDR and Non-MDR Gram-negative bacteria

Out of 114 MDR Gram-negative bacterial isolates, 69, 22, and 14 were ESBL, MBL, and KPC-producing Gram-negative bacterial isolates respectively. The association between ESBL and MDR, MBL and MDR, and KPC and MDR was significant statistically (Table 9).

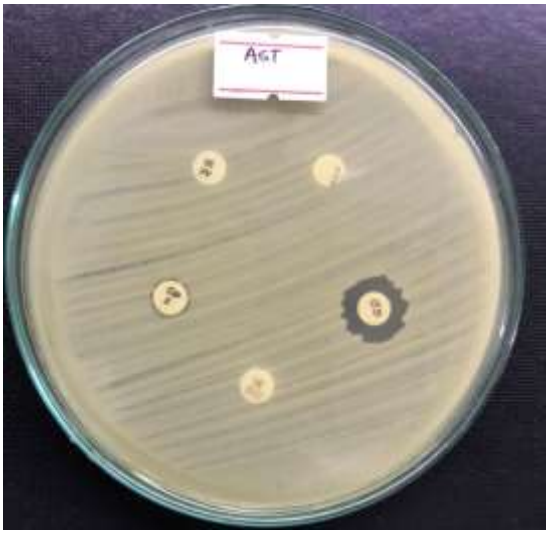
Co-production of ESBL, MBL and KPC-producing Gram-negative bacteria

Out of 182 Gram-negative bacterial isolates, 16 Gram-negative bacteria were both ESBL and MBL-producing bacteria, 12 were ESBL and KPC-producing bacteria, 6 were MBL and KPC-producing bacteria and 5 were ESBL, MBL, and KPC-producing bacteria.

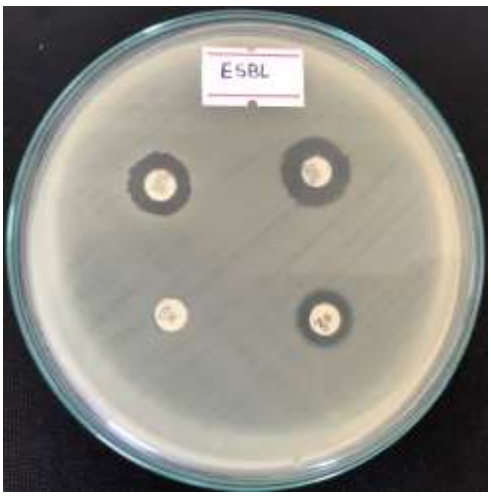
Photograph 1: Colonies of *Klebsiella pneumoniae* isolated from the blood sample of Neonate on Macconkey Agar



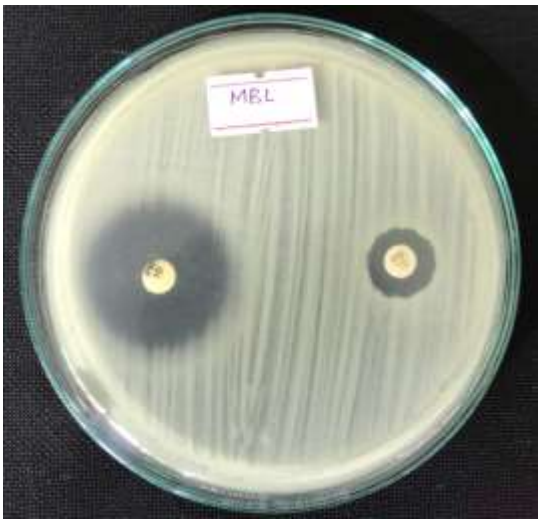
Photograph 2: Biochemical Test of *Klebsiella pneumoniae* [From left: SIM -ve, MR –ve, VP +ve, Citrate +ve, TSIA (A/A, Gas +ve, H_2S –ve) and Urease +ve]



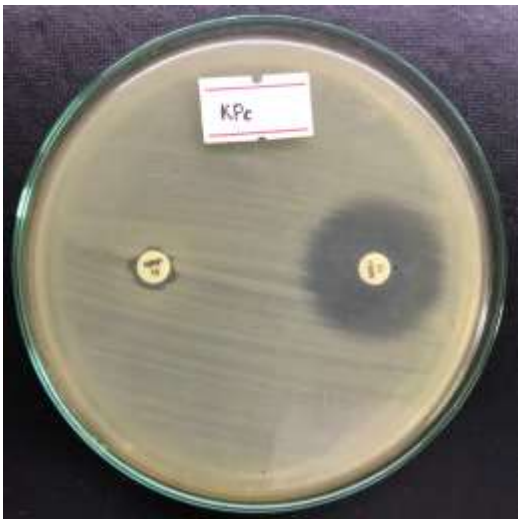
Photograph 3: Antibiotic susceptibility test of *Klebsiella oxytoca*, isolate number 113 [Ampicillin (Amp)–R, Amikacin (AK)–R, Ciprofloxacin (CIP)–R, Cefotaxime (CTX)–R And Colistin (CL)–S]



Photograph 4: ESBL producing *Klebsiella pneumoniae*, zone of inhibition ≥5 mm for Ceftazidime with Clavulanic acid (CAC), Cefotaxime with Clavulanic acid (CEC) than alone by Ceftazidime (CAZ) and Cefotaxime (CTX) by isolate number KP 227



Photograph 5: MBL producing *Enterobacter* spp., zone of inhibition of Imipenem + EDTA (IMP+EDTA) ≥7 than Imipenem (IMP) alone by isolate number 39



Photograph 6: KPC producing *Citrobacter* spp., zone of inhibition of Meropenem + Phenyl Boronic Acid (MRP+PBA) ≥7 than Meropenem (MRP) alone by isolate number 191

Discussion

Neonatal bacterial sepsis is a leading cause of death in developing countries like Nepal. Neonates are more susceptible to severe infections and the progression of the disease is more rapid due to developmental immunodeficiency and also, a significant proportion of infections may arise early after vertical transmission from the mother resulting in a high case fatality rate (Budhathoki *et al.* 2020).

In this study, among 1335 blood samples of neonates, 231 (17%) blood samples showed growth, and 1104 (83%) showed no growth. Hence, the prevalence of neonatal sepsis was 17%. A similar finding was reported by Yadav *et al.* (2018) and Nepal *et al.* (2021) where the prevalence rate of neonatal sepsis was 16.9% and 21.05% respectively. Few research depicted a higher incidence rate of neonatal sepsis in other developing countries such as Bangladesh (34.88%),

Uganda 37%), Ethiopia (44.7%), and Nigeria (45.9%) (Shehab El-Din *et al.* 2015) while lower incidence rate was depicted in Southern Africa (9.8%) (Mudzikati and Dramowski, 2015), Nepal (12.6%) (Ansari, 2015) and Pakistan (8.9%) (Raha *et al.*, 2014). Neonatal sepsis incidence rates may vary due to sampling size differences, antibiotic use prior to sample collection, anaerobe, viral or fungal pathogen infections, and effective nosocomial infection control measures (Thapa and Sapkota, 2019).

A total of 138 neonates (59.74%) had EOS whereas 93 (40.26%) had LOS. This study found that EOS incidence was higher than LOS incidence, which is consistent with prior studies (Naher and Khamael, 2013, Al-Shamahy *et al.*, 2013; Ansari *et al.*, 2015). Ansari *et al.* (2015) reported 82 (70.7%) of neonates accounted EOS and 34 (29.3%) of neonates accounted LOS.

Out of 231 positive blood culture isolates, *K. pneumoniae* was predominant Gram-negative isolates with number 116 (50.2%) followed by *K. oxytoca* 31 (13.4%), *Acinetobacter* spp. 16 (6.9%), *Enterobacter* spp. 12 (5.2%) and *Citrobacter* spp. 7 (3%). Likewise, *S. aureus* was predominant Gram-positive isolate with number 39 (16.9%) followed by *Enterococcus fecalis* 10 (4.3%). In the study conducted in India, the most common Gram-negative bacteria isolated were *Klebsiella* spp. (22.2%) whereas *S. aureus* (15.7%) was the most predominant Gram-positive bacteria recovered from neonatal septicemia patients (Khanna *et al.*, 2016). In the study conducted by Haque (2015), *K. pneumoniae* accounted highest prevalence rate 69.30% among Gram-negative isolates and *S. aureus* accounted for 1.75% among Gram-positive isolates. The etiology of newborn septicemia has varied throughout time and may differ regionally. This fluctuation can be caused by variations in the research environment, study participants, and hand hygiene practices.

The antibiotic susceptibility pattern of both Gram-negative and Gram-positive bacterial isolates was performed. Colistin was found to be the most effective antibiotic against all the Gram-negative isolates, as all the isolates were (100%) sensitive to this antibiotic. *K. pneumoniae* was 100% resistant to Ampicillin followed by Gentamicin 100 (86.2%), Cefepime 72 (62.1%), Cefotaxime 53 (45.7%), Ceftazidime 51 (44.0%), Piperacillin/tazobactam 47 (40.5%), Amikacin 39 (33.6%), Ciprofloxacin 35 (30.2%), Meropenem 25 (21.6%) and Imipenem 25 (21.6%). A similar finding was reported by Dutta *et al.* (2020) where most of the *K. pneumoniae* were found to be sensitive to Meropenem 77.27% and Imipenem 76.74%.

K. oxytoca was found to be equally sensitive to Meropenem, Imipenem, Amikacin, and Ciprofloxacin 20 (64.5%). *K. oxytoca* were 100% resistant to Ampicillin followed by Piperacillin/tazobactam, Cefepime, Cefotaxime, Ceftazidime and Gentamicin with equal sensitivity of 29 (93.5%). Similar findings were reported where *K. oxytoca* were demonstrated to be 100% sensitive to Meropenerm and resistant to Cefotaxime 7(100%) and Ceftazidime 7(100%) (Nepal *et al.*, 2021).

Colistin was completely effective against all the isolates of *Acinetobacter* spp. And was most resistant to Ampicillin 13 (81.2 %) whereas least resistant to Meropenem and Imipenem 4 (25.0%). In the study conducted in India, all the strains of *Acinetobacter* were 100% sensitive to Colistin while least sensitive to Ampicillin (Nazir, 2019).

Enterobacter spp. were sensitive to Colistin 12 (100%) followed by Meropenem 8 (66.7%), Imipenem 8 (66.7%), Ciprofloxacin 8 (66.7%), and Amikacin 8 (66.7%) while least sensitive to Cefepime 3 (25.0%) and Ampicillin 3 (25.0%). In the study conducted in South Arabia, *Enterobacter* spp. was sensitive to Meropenem 1(50%) and resistant to Ampicillin 2(100%) (Alharbi 2022). Nepal *et al.* (2021) reported *Enterobacter* spp. was highly resistant to Cefotaxime 6(100%) followed by Ceftazidime 6 (100%) and Amikacin 5(83.33%).

Most of the *Citrobacter* spp. demonstrated susceptibility towards Colistin 7 (100%) followed by Meropenem 6 (85.7%), Imipenem 6 (85.7%), and Ciprofloxacin 6 (85.7%) and it showed the least susceptibility to Gentamicin 1 (14.3%) and Amikacin 1 (14.3%). In the study reported by Yadav *et al.* (2018), *Citrobacter* spp. were 100% sensitive to Meropenem and Imipenem whereas 100% resistant to Ampicillin.

Enterococcus fecalis demonstrated 100% susceptibility to Cefoxitin and Vancomycin followed by Meropenem 9 (90%), Imipenem 9 (90%), and Ciprofloxacin 9 (90%). Most of the *Enterococcus fecalis* were resistant to Gentamicin 8 (80%) and Ampicillin 8 (80%). In the study conducted by Raghubanshi *et al.* (2021), *Enterococcus* spp. were 100% resistant to Ampicillin, Amikacin, and Cefotaxime and 100% sensitive to Imipenem, Meropenem and Vancomycin.

Most of the *S. aureus* were sensitive towards Vancomycin 38 (97.4%) followed by Meropenem 38 (97.4%), Imipenem 38 (97.4%) and Ciprofloxacin 38 (97.4%) whereas resistant towards Gentamicin 19 (48.7%) and Ampicillin 19 (48.7%) followed by Cefepime 10 (25.6%), Cefotaxime 5 (12.8%) and Ceftazidime 5 (12.8%). A similar finding was reported by Ranghubanshi *et al.* (2021) where *S. aureus* was 100% susceptible to Vancomycin followed by Meropenem and Imipenem and resistant to 7 (90%) Ampicillin and 7 (90%) Cefotaxime.

Other studies carried out both inside and outside of Nepal revealed a similar finding, reporting that Gram-negative bacteria were susceptible to Polymixin (Colistin) and Carbapenems (Imipenem and Meropenem) and Gram-positive bacteria were susceptible to Vancomycin followed

by Carbapenems (Fahmey, 2013). However, these antibiotics should be considered alternatives until other effective drugs that could be administrated safely. In the present study, antibiotic resistance among the Gram-positive and Gram-negative bacteria was quite high to recommend drugs like Ampicillin, Cephalosporins and Aminoglycosides.

The prevalence of multidrug-resistant bacteria in neonatal sepsis has increased progressively over the past few years and infections with bacterial strains producing ESBL and carbapenemases are of particular concern for clinicians and are a major threat worldwide. So, a phenotypic test for the screening and confirmation of ESBL, MBL, and KPC-producing bacterial isolates was performed in this study. One hundred and eighty-two isolates of Gram-negative bacteria were analyzed, 97 (53.3%) of those were screened as ESBL-producing bacteria and 69 (37.9%) isolates were phenotypically confirmed ESBL-producing bacteria. *Klebsiella*. *oxytoca* 17 (54.8%) had the highest proportion of confirmed ESBL producers, whereas *Acinetobacter* spp. 5(31.3%) had the lowest proportion. According to the research published by Zakir *et al.* (2021), *K. pneumoniae* 23 (31.9%) was the most common ESBL-producing strain with a prevalence rate of 72 (34%). According to a study conducted in Taiwan, 393 cases of Gram-negative bacteremia in NICU patients, ESBL production was the most common form of resistance accounting for 67.1% (Tsai *et al.*, 2014).

Among 182 Gram-negative bacterial isolates, 45 (24.7%) were probable MBL producers and 22 (12.1%) were phenotypically confirmed MBL producers. Most MBL-producing bacterial isolates were *Enterobacter* spp 3 (25%), while *Acinetobacter* spp. 1 (6.25%) were the least MBL-producing bacteria. A similar study conducted by Kumar *et al.* (2019) reported 17% of MBL production. Likewise, Kamble *et al.* (2014) reported 20% MBL production.

Out of 182 Gram-negative bacterial isolates, 45 (24.7%) Gram-negative isolates were probable KPC-producing bacteria, while 14 (31.1%) Gram-negative isolates were phenotypically confirmed KPC-producing bacteria. Likewise, 13 (36.1%) KPC production was reported by Azimi *et al.* (2012)

Among 114 MDR Gram-negative bacterial isolates, 69, 22, and 14 were ESBL, MBL, and KPC-producing Gram-negative bacterial isolates respectively. The association between ESBL and MDR, MBL and MDR, and KPC and MDR was significant statistically.

In this study, out of 182 Gram-negative bacterial isolates, 16 were both ESBL and MBL producers, 12 were ESBL and KPC producers, 6 were MBL and KPC producers and 5 were ESBL, MBL and, KPC producers. In a similar study conducted by Oberoi *et al.* (2013), co-production of ESBL, MBL, and AmpC beta-lactamase was seen in 52 (19.04%) of bacterial isolates. Another study conducted by Singla *et al.* (2014) reported 29% co-production of ESBL and AmpC beta-lactamase in *Acinetobacter* spp. The co-existence of different classes of beta-lactamases in a single bacterial isolate may pose diagnostic and treatment challenges. The KPC and MBL-producing organisms can act as a hidden reservoir for ESBLs. Also, the high-level expression of the MBL and KPC in bacteria may mask the recognition of the ESBLs or other beta-lactamases and it may result in fatal and inappropriate antimicrobial therapy.

Conclusion

It is evident from this study that *Klebsiella* spp. and *S. aureus* are the predominant bacteria causing neonatal sepsis and most of them are resistant to multiple antibiotics. There is a rise in ESBL, MBL, and KPC-producing Gram-negative bacteria in neonates causing neonatal sepsis. Therefore, to lessen the burden of antibiotic resistance, it is essential to conduct regular antimicrobial susceptibility surveillance, periodic reviews of hospital settings, and early detection of beta-lactamase-producing bacteria. The occurrence of co-production of ESBL, MBL, and KPC-producing bacteria seems to be alarming in a hospital setting. Colistin appears to be the antibiotic of choice for treating Gram-negative bacteria that produce ESBL, MBL, and KPC. However, until additional effective medications may be administered safely, these should be regarded as alternatives. Regular surveillance of MDR and beta-lactamase producers, as well as the adoption of hospital infection control strategies, is crucial for preventing the spread of such isolates.

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Authors' Contribution

All authors contributed equally at all stages of research work and manuscript preparation. Final form of manuscript was approved by all authors.

Conflict of Interest

The authors declare that there is no conflict of interest.

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