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Pattern of clinical drug resistance and occurrence of Gram negative bacterial neonatal sepsis at a tertiary care hospital

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Abstract: Sepsis is a leading cause of neonatal deaths across the world. Gram-negative rods such as *Klebsiella* and *E. coli* are major cause of sepsis in neonates. With a mortality rate of 1-4 deaths per thousand live births, sepsis is the second most important cause of neonatal deaths in the developing countries. The present study was designed to determine the occurrence of Gram-negative bacteria in neonatal sepsis and to find antibiotic susceptibility of isolated microbes. Blood samples of 100 neonates (1-89 days old) were sub cultured on MacConkey's and Blood agar for isolation of Gram-negative bacteria. A total of four bacterial species were isolated including *Klebsiella* (35.71%), *E. coli* (28.57%), *Acinetobacter* (21.42%) and *Proteus* (14.28%). Gram-negative bacteria were isolated more commonly from EOS (early onset sepsis) as compared to LOS (late onset sepsis). *Klebsiella* isolates from neonates showed sensitivity to imipenem (70%) followed by ceftazidime (40%) and cefotaxime (40%) and high resistance was shown by sulfamethoxazole (80%) and amikacin (70%). *E. coli* associated with neonatal sepsis were sensitive to imipenem (63%) while highly resistant to cefotaxime (75%) and ciprofloxacin (62%). For *Acinetobacter* high sensitivity was found for ceftazidime (50%) and resistance was shown to ciprofloxacin and sulfamethoxazole (100%). *Proteus* showed high sensitivity to amikacin (75%) and high resistance to imipenem and ciprofloxacin (75%). In conclusion, Gram-negative associated neonatal sepsis was found in the studied subjects and drug resistance was observed to clinically used antibiotics.

Keywords: Drug resistance, neonatal sepsis, frequency, bacterial infection.

INTRODUCTION

Sepsis is characterized by signs and symptoms that is associated with bacterial, viral and fungal infections and colonization of these microbes in the host. Neonatal sepsis is a leading cause of death all over the world, mainly in developing countries (Thapa and Sapkota, 2019) and it is categorized as early onset sepsis (EOS) *i.e.* beginning of symptoms within 72 hours of birth and late onset sepsis (LOS) in which symptoms appeared after 72 hours of birth (Chaurasia *et al.*, 2019). In 50% of sepsis cases, lethality is due to hospital acquired sepsis. It is more severe form of sepsis and it showed more resistance to antibiotics as compared to type of sepsis present upon admission (Meyer *et al.*, 2018). Risk factors responsible of causing neonatal sepsis include premature birth, low birth weight and perinatal asphyxia. More factors like umbilical cord care practice, prenatal care, delivery type and settings are also considered the cause of neonatal sepsis (Thapa and Sapkota, 2019). Pathogens involved in neonatal sepsis differ all over the world. Gram negative organisms are more commonly reported in developing countries (Mohsen *et al.*, 2017). Gram negative organisms involved in sepsis are *Escherichia coli*, *Pseudomonas spp*, *Neisseria meningitides*, *Klebsiella spp*, *Enterobacter spp*, *Salmonella spp* and *Haemophilus influenzae* (Yadav *et al.*, 2018). EOS is considered to be vertically transferred from mother and *E. coli* is the leading cause among Gram-negative organisms, while LOS is considered to be transmitted horizontally or vertically. *E. coli* and other Gram negative organisms along with coagulase-negative Staphylococci *(CoNS),* Staphylococcus aureus and Candida albicans are responsible for sepsis (Akbarian-Rad et al., 2020).

Lymphocytes and neutrophils are the most important elements of immune system which fight with the pathogens at initial stage. In case of sepsis a strong adhesion is formed between the endothelium and neutrophil causing failure in migration of neutrophil to site of infection. Release of cytokines and bacterial products in sepsis are involved in delay of neutrophil apoptosis resulting in increase of sepsis severity (Wilar, 2019).

Diagnostic tests for neonatal sepsis involve inflammatory biomarkers (procalcitonin test, C- reactive protein) and complete blood count. The most commonly used method for diagnosis in neonatal intensive care unit is by blood culture also called as rule out sepsis. This method is used as a reference standard for diagnosis of neonatal sepsis (Durrani *et al.*, 2019).

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The intense increase of antimicrobial resistance in pathogens is a worldwide concern of this era which needs urgent strategies at national and international level. The cases of infections due to multidrug resistant bacteria (MDR) are increasing day by day both in developing and highly developed countries (Folgori and Bielicki, 2019). The first line antibiotic for neonatal sepsis has been penicillin along with aminoglycoside but there is unrestrained resistance in public for these antibiotics. For second line drugs; carbapenems and third- generation cephalosporins are used but due to spread of ESBL enzyme, E. coli, Acinetobacter and Klebsiella became resistant to these drugs (Sands et al., 2021). Currently there is no vaccine available for the control of neonatal sepsis in developing countries or low-to-middle-income countries (LMICs). However, choosing broad spectrum antibiotics as 1st line treatment against these resistant organisms is better option as compared to second line empiric antibiotics along with preventative strategies. The present study aimed to determine the occurrence of neonatal sepsis and antimicrobial susceptibility pattern of Gram negative associated neonatal sepsis.

MATERIALS AND METHODS

The study was carried out in accordance with Institutional Bioethics Committee (IBC) of the University of Agriculture Faisalabad and Allied Hospital Faisalabad, Pakistan (No., 13409-12, dated as 02-04-2019). The neonatal blood samples were collected after taking an informed consent from the parents of the neonates with full compliance.

Sample collection from neonates

A total of 100 blood samples were collected aseptically from neonatal sepsis cases from pediatric ward of tertiary care Allied Hospital Faisalabad. The inclusion criteria were the neonates both males and females with age ranging from 0-89 days. Blood samples were collected before the beginning of antimicrobial therapy (Li *et al.*, 2019) with the help of trained paramedical staff by using aseptic techniques (Dalal *et al.*, 2017). One ml of blood samples from neonates were taken in 9ml of Brain Heart Infusion Broth (Oxoid Ltd) having ratio 1:10. Blood samples were transferred to the Institute of Microbiology for further processing.

Phenotypic examination of neonatal blood samples

The broth containing blood samples were incubated at 37℃ for 7 days and observed daily to check the turbidity. Each sample was sub cultured on 3rd, 5th and 7th day on MacConkey's agar and Eosin Methylene Blue agar (Oxoid Ltd.) for isolation of Gram-negative bacteria. Gram negative bacteria were further purified on above mentioned agars (fig. 1). Bacterial colonies were characterized on the basis of morphology, Gram's staining and biochemical tests which include catalase test, coagulase test, citrate utilization test, triple sugar iron agar test, indole test, methyl red test, oxidase test and urease test. Blood cultures that do not show any growth after sub culturing were considered negative. However, samples that show growth on 3rd and 5th day were considered positive for sepsis.

Antibiotic susceptibility testing

Antibiotic susceptibility testing for Gram negative bacteria was performed by Kirby Bauer disc diffusion method. Antibiotic discs (Oxoid, UK) including imipenem 10µg, amikacin 30µg, ciprofloxacin 5µg, ceftazidime 30µg, sulfamethoxazole 30µg and cefotaxime 30µg were applied. Zones of inhibition of different antibiotic discs were measured and interpreted as resistant, susceptible an intermediate according to CLSI 2017 criteria.

STATISTICAL ANALYSIS

The data of neonatal sepsis and association of risk factor was analyzed by Graphpad Prism version 5 using chi square test and P value < 0.05 was considered to be statistically significant.

RESULTS

Frequency of Gram-negative bacteria in blood samples of neonates

Out of 100 blood samples 28 were confirmed for microbial growth and 72 samples showed no growth on culture media. Out of 28 positive blood samples, 10

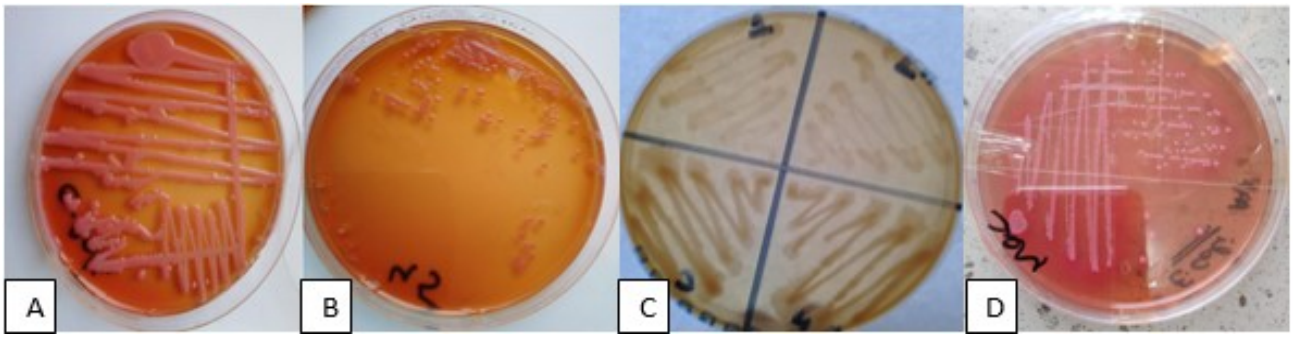

Fig. 1: Representative agar colony morphology of Gram-negative isolates from neonatal sepsis on specific media (A) *Klebsiella* (B) *Acinetobacter* (C) *Proteus* (D) *E. coli.*

isolates were identified as *Klebsiella* (10%), along with *E. coli* (8%), *Acinetobacter* (6%) and *Proteus* (4%) as shown in fig.1.

Frequency of Gram-negative bacteria with respect to the age of neonates

The frequency of *Klebsiella* (10%) and *E. coli* (8%) were found high in neonates of age 1 to 4 days. *Acinetobacter* showed high positivity (6%) in neonates of age 5-8 days followed by *Proteus* (4%) from age 5 to 12 days. The significant frequency of Gram-negative bacteria was found in blood samples of neonates as shown by chi square test (P =.005) (table 1).

Frequency of EOS and LOS in neonates with respect to age and gender

The frequency of EOS was found to be (60%) as compared to LOS (40%). Neonates of 5-8 days old showed high prevalence and significant results (P=.020) in EOS (37%) and (19%) in LOS (table 2). The significant presence (P=.027) of EOS and LOS were high in female (55%) as compared to male neonates (45%) as shown in table 3.

Antibiotic susceptibility pattern of Gram-negative bacteria isolated from neonatal sepsis

The antibiotic susceptibility pattern of *Klebsiella* isolates showed highest susceptibility (70%) to imipenem followed by (40%) susceptibility to ceftazidime and cefotaxime as shown in table 4. The *Klebsiella* isolates (n=2) showed intermediate susceptibility (30%) to ciprofloxacin and cefotaxime (40%). Antibiotic susceptibility pattern of *E. coli* isolates from neonates showed high susceptibility (63%) to imipenem and sulfamethoxazole followed by (50%) susceptibility to amikacin as shown in table 4. *E. coli* isolates showed intermediate susceptibility to ceftazidime and cefotaxime (25%). Neonatal sepsis associated *Acinetobacter* was found to be sensitive to ceftazidime (50%), imipenem and cefotaxime (34%) (table 4). Intermediate susceptibility pattern (17%) was shown for amikacin by Acinetobacter isolates. The *Proteus* isolates from neonates were sensitive (75%) to amikacin, ceftazidime and sulfamethoxazole. However, these were intermediately susceptible (25%) to imipenem and ciprofloxacin.

DISCUSSION

Sepsis is major cause of morbidity and mortality in neonates globally ranging from 30-50% in developing countries (Klick and Guins, 2021). Gram negative bacteria play an important role in causing nosocomial and non- nosocomial infections (Rasool *et al.*, 2019). Death rate in neonates comprise of 41% (3.6 million) in children below 5 years. About 1 million of these deaths are linked with infectious diseases like pneumonia, neonatal sepsis and meningitis (Getabelew *et al.*, 2018). The present study revealed the occurrence and antimicrobial susceptibility pattern of Gram-negative bacteria in neonates in a tertiary care hospital in Pakistan.

Gram negative bacteria were isolated from 28% of neonatal blood samples. A study conducted in Kolkata, India reported (42.94%) cases of Gram negative bacilli much higher than that reported in present study (Dutta *et al.*, 2020). The rate of isolated Gram negative bacteria from neonatal sepsis had been reported high (69.6%) in Nepal (Thapa and Sapkota, 2019).

Neonatal sepsis is categorized as EOS and LOS and we found that Gram negative bacteria were more common in EOS (60%) as compared to LOS (40%). A study conducted in Nigeria reported incidence of Gram negative bacteria in EOS as (77.2%) (Sa'adu *et al.*, 2019). Study conducted in Nepal reported high cases of EOS (78.3%) (Pokhrel *et al.*, 2018). However, study in Switzerland reported prevalence of Gram-negative bacteria in EOS as (20%) lower than that recorded in present study. This might be related with variation in area, hygienic conditions and use of antibiotics (Giannoni *et al.*, 2018).

In sepsis multi drug resistant organisms are crucially involved in causing high mortality as compared to non-multi drug resistant bacteria (Yusef *et al.*, 2018). A study in India showed that use of broad-spectrum antibiotics leads to high resistance in *K. pneumoniae*. Aminoglycosides, carbapenem, 3rd generation cephalosporins, fluoroquinolones along with other drugs showed susceptibility against *K.* pneumoniae (Patidar and Patidar, 2020). We found high sensitivity (70%) against imipenem and resistance (70%) against amikacin, which was reported in previous study (Marando *et al.*, 2018). Study from Ghana reported that *Enterobacteriacea* showed 100% resistance to cefotaxime, 50% to ceftriaxone and 50% to gentamicin. However *Proteus mirabilis* did not show resistance to cefotaxime in a previous study (Aku *et al.*, 2018). In our study, the *E. coli, Klebsiella,* and *Proteus* isolates from neonatal sepsis showed 75%, 60% and 50% resistance to cefotaxime. Another study from India revealed that *Enterobacteriacea* showed high resistance (90%) to ciprofloxacin as compared to meropenem (49%). Resistance to sulfamethoxazole by *E. coli* and *Klebsiella* was 69% and 89% respectively (Mitra *et al.*, 2019) . We found that neonatal sepsis associated *Klebsiella* showed resistance to sulfamethoxazole (80%) and to ciprofloxacin (70%). However, the resistance for sulfamethoxazole and ciprofloxacin in *E. coli* was 37 and 62%, respectively.

Neonatal Blood culture is considered "Gold standard" in diagnosis of sepsis but the inadequate amount of blood limits the accuracy of test. Other tests like procalcitonin, complete blood count and C-reactive protein are useful in diagnosis but are not helpful to choose the antibiotic, to start the initial treatment of suspected neonates with sepsis (Popescu *et al.*, 2020).

Table 1: Frequency of Gram-negative bacteria with respect to the age of neonates

		Organisms					Total
		Klebsiella	*E. coli*	*Acinetobacter*	*Proteus*	No Growth	
Age of Neonates	1-4 D	5	4	1	0	20	30
	5-8 D	3	2	3	2	33	43
	9-12 D	1	1	2	2	10	16
	13-16 D	1	1	0	0	9	11
Total		10	8	6	4	72	100

Chi-Square Test			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	18.379[a]	18	.005
Likelihood Ratio	17.869	6	.007
Linear-by-Linear Association	1.695	1	.193
N of Valid Cases	144		
a. 2 cells (16.7%) have expected count less than 5. The minimum expected count is 2.40.			

Table 2: Frequency of EOS and LOS in neonates with respect to age

		Stage		Total
		EOS	LOS	
Age of Neonates	1-4 D	23	0	23
	5-8 D	37	19	56
	9-12 D	0	17	17
	13-16 D	0	4	4
Total		60	40	100

Chi-Square Test			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	16.728[a]	7	.020
Likelihood Ratio	17.217	12	.142
Linear-by-Linear Association	.562	1	.453
N of Valid Cases	144		
a. 9 cells (45.0%) have expected count less than 5. The minimum expected count is 1.15.			

Table 3: Frequency of EOS and LOS in neonates with respect to gender

		Stage		Total
		EOS	LOS	
Male/Female	Male	24	21	45
	Female	36	19	55
Total		60	40	100

Chi-Square Test			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	23.143[a]	3	.027
Likelihood Ratio	29.245	3	.004
Linear-by-Linear Association	.403	1	.526
N of Valid Cases	144		
a. 10 cells (50.0%) have expected count less than 5. The minimum expected count is .94.			

EOS= Early Onset Sepsis; LOS= Late Onset Sepsis

Table 4: Antibiotic susceptibility of Gram-negative bacteria isolated from neonatal sepsis

Bacteria	IPM% S(n)	AMK% S(n)	CIP% S(n)	CAZ% S(n)	CTX% S(n)	SXT% S(n)
Klebsiella (n=10)	7(10)=70%	3(10)=30%	3(10)=30%	4(10)=40%	4(10)=40%	2(10)=20%
E. coli (n=8)	5(8)=63%	4(8)=50%	3(8)=38%	2(8)=25%	2(8)=25%	5(8)=63%
Acinetobacter (n=6)	2(6)=34%	1(6)=17%	0(6)=0%	3(6)=50%	2(6)=34%	0(6)=0%
Proteus (n=4)	1(4)=25%	3(4)=75%	1(4)=25%	3(4)=75%	2(4)=50%	3(4)=75%

IPM=Imipenem; AMK= Amikacin; CIP= Ciprofloxacin; CAZ= Ceftazidime; CTX= Cefotaxime; SXT= Sulfamethoxazole

Excessive use of antibiotics in neonatal intensive care units for treating neonatal sepsis leads to multi drug resistance in pathogens. A previous study showed that cefuroxime can be used as a prophylactic or therapeutic treatment for Gram negative and Gram positive bacteria in neonatal sepsis as *E. coli* showed (14%) resistant to intravenous cefuroxime and *Klebsiella* showed no resistance (Li *et al*., 2019).

Gram negative bacteria showed resistance (80%) to commonly used antibiotics in Pakistan, India, Nigeria, China and Congo. The data showed approximately 3 million cases of neonatal sepsis each year (Popescu *et al.*, 2020).

CONCLUSION

In conclusion, for managing the burden of neonatal sepsis we must need to take preventive management which includes antenatal care, intrapartum care and neonatal care. Use of broad-spectrum antibiotics must be limited in neonatal care/ management so that future treatment option will be left for treatment of neonatal sepsis.

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