

diagnostic methods available for neonatal septicemia include CRP, CBC, platelet count, and ESR, but the blood culture is the gold standard. There are epidemiological differences in the incidence, risk factors, microbial pattern, and antimicrobial susceptibility pattern of neonatal sepsis among different regions and countries in the world².

Aim

The objective of the present task is to study the microbial profile, antibiotic susceptibility pattern, risk factors, the incidence of neonatal septicemia. Also, limit the injudicious use of antimicrobial agents by reviewing the antibiotic prophylaxis policy.

Materials and Methods

Source of data

The study includes 2550 neonates with clinical manifestation of septicemia from neonatal intensive care unit at the Tertiary care teaching center, western side of India: December 2017 to May 2019.

Type of study: A prospective observational study.

Inclusion criteria

- The clinicians diagnosed neonates with clinical signs of sepsis and evidence of positive blood culture results.

Exclusion criteria

- Neonates were clinically diagnosed as sepsis but not confirmed by blood culture and were born outside of our center.

The proforma include age of onset, sex, type of isolates & associated risk factors.

The study has three parts

1. Sample collection
2. Sample processing and identification
3. Antibiogram

1] Sample collection and receiving³: The clinicians suspected signs of neonatal septicemia, then 0.5 to 5 ml of blood collected with aseptic precaution before starting antimicrobial therapy by the resident doctors and the nursing staff. The collected blood inoculates into pediatric BacT/ALERT blood culture bottles figure:1. The samples with duly filled LRF are received and accepted in the laboratory, then sample ID is generated using LIS.

2] Processing of samples done in the following ways³:

The labeled bottle loads into the BacT/ALERT 3D continuous automated monitoring system with expandable detection units with self-contained incubation (37°C) chambers that analyze the color changes every ten minutes. The machine emits light and beep sound as soon as the threshold value reaches. That indicates positive growth. The bottles that did not show emission after seven days were considered no growth. The principle of BacT/ALERT is colorimetric detection of CO₂ produced by growing microbes. Each bottle contained specialized Liquid Emulsion Sensors at the bottom of the culture bottle, visibly changing color in response to shifts in pH by rising CO₂ levels.



Direct microscopy:

Smears were prepared from the positively signaled bottle using a needle syringe, on a clean glass slide; Gram staining was done and examined under the light microscope.

Processing of sample for culture³:

The blood from the all-positive signaled bottle was inoculated on MacConkey, blood, and chocolate agar plates. The plates were incubated aerobically at 37°C in an incubator, and results were read after

24 hours. All positive cultures were, identified by their characteristic colony morphology, compared gram staining from the direct and culture smears, and by the battery of biochemical reactions.

The following conventional biochemical tests were done:

Gram-positive bacteria: catalase, coagulase (tube & slide), mannitol fermentation, bile esculin slant & pyrrolidinyl peptidase test.

Gram-negative bacteria: Oxidase, Triple Sugar Iron, Citrate utilization, Phenylalanine Deaminase, Urease, Indole, Methyl Red, Voges-Proskauer, Sugar fermentation, Nitrate Reduction, Acetamide, Amino Acid Decarboxylase, and Oxidation/Fermentation tests.

The Yeast isolates: Germ tube test, sugar assimilation test. The yeast isolates were also inoculated into Sabouraud dextrose agar, corn meal agar, and CHROM agar for spp. identification.

3] Antibiogram of isolates³: Kirby Bauer disk diffusion test for antimicrobial susceptibility testing (AST) was performed after bacterial growth on the mentioned agar plates.

Suspension: At least 3-5 well-isolated colonies were selected using a sterile cotton swab and were transferred into the normal saline tube and mixed well. The turbidity of the suspension was adjusted to 0.5 % McFarland turbidity standard for bacteria and 5% McFarland turbidity standard for candida spp. A lawn culture was done on MHA for bacteria and MHA with glucose (2%) and methylene blue (50 µg/ml) for Candida species, then antibiotic disc was applied according to organisms & inverted plates were incubated aerobically at 37°C, and results were read after 18-24 hours.

Reading of plates and interpretation of results:

After incubation of plates, the results were read by the naked eye by using an antibiotic zone scale, and the zone of inhibition was measured in mm. The results were reported as follows: Susceptible(S), Susceptible dose-dependent(SDD), Intermediate(I), Resistant (R), and special tests were done for detecting MRSA, MBL, ESBL, and AmpC based on CLSI 2017 guidelines.

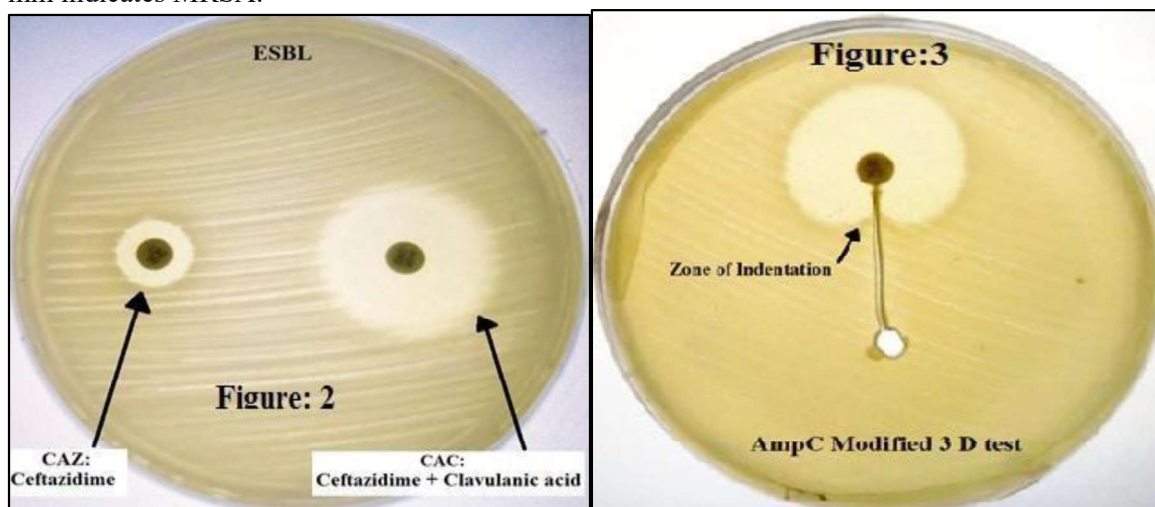
Quality control:

The reliability of the study findings was assured by implementing quality control (QC) measures throughout the whole laboratory works. All materials and equipment were validated and procedures aseptically managed. Culture media were checked for sterility and performance during the lot changes. CLSI recommended bacterial strains were used for quality control: Staphylococcus aureus ATCC 25923, Staphylococcus aureus ATCC 43300, Klebsiella pneumoniae ATCC 700603, Escherichia coli ATCC 25922, Enterococcus faecalis ATCC 29212, Pseudomonas aeruginosa ATCC 27853.

Detection of Various Resistance Pattern:

1. Detection of Methicillin resistance

A lawn culture of *S. aureus* was done on MHA then a 30µg cefoxitin disc was placed on it for checking methicillin resistance. For the quality purpose, *S. aureus* ATCC 25923 as a negative and ATCC 43300 as a positive control were used. If the zone of inhibition of 30µg cefoxitin disc was ≤ 21 mm indicates MRSA.



2. Detection of ESBL

Screening test: After a lawn culture of test isolates, discs of Ceftazidime (30 μ g) and Cefotaxime (30 μ g) were placed on the MHA plate. After incubation the zone of Ceftazidime \leq 22mm and Cefotaxime \leq 27mm were taken as an indicator of ESBL production which was confirmed by phenotypic combination disc test.

Phenotypic combination disc test: After a lawn culture of test isolates, discs of Ceftazidime (30 μ g), and Ceftazidime plus clavulanic acid (30 μ g/10 μ g) were placed on the MHA plate more than 25 mm apart. After incubation, if the zone diameter around the disc with clavulanic acid is increased by \geq 5 mm over the Ceftazidime (30 μ g) disc alone, it confirmed ESBL production (figure:2).

3. Detection of Amplified cephalosporinase (ampc)

Screening Test: Isolates having \leq 18mm zone to ceftazidime (30 μ g) disc indicate AmpC β -Lactamases producer.

Confirmatory test for AmpC β -lactamase: Modified three-dimensional test (MTDT)

After performing the lawn culture of ATCC 25922 E. Coli on MHA plates, the ceftazidime (30 μ g) disc was placed on the center. By using a sterile blade, a linear slit (3 cm long) was made up to a point 3 mm away from the edge of the ceftazidime disc. With a sterile Pasteur pipette, a well of 8 mm diameter was made at the outer end of the slit, at a distance of 0.5 cm from the edge of the plate. The wells were loaded with organisms' inoculum until the well was full. After incubation, presence of indentation of growth around slit indicates AmpC production (figure:3).

4. Detection of Carbapenemases

Modified Hodge Technique:

After doing a lawn culture of E. Coli ATCC 25922 on MHA plates, we placed an ertapenem (10 μ g) disc in the center of MHA and had streaked the test isolates from the edge of the disk to the edge of the plate. The Cloverleaf appearance noted at the streaking line towards the ertapenem disc indicates carbapenemases production.

Detection of Class B Carbapenemase by phenotypic method combined disc synergy test:

A lawn culture of the test strain had done on the MHA plate, then Imipenem (10mcg) and Imipenem with EDTA were placed. After incubation, if the Zone diameter increased around the disc of the Imipenem with EDTA \geq 5 mm over the Imipenem disc alone, indicate Metallo beta-lactamase production.

5. Colistin sulfate agar testing³

For colistin agar testing, powder forms of 2 μ g/ml Colistin had incorporated into the MHA plate. 32-36 inoculums of test organisms applied simultaneously into colistin-containing MHA plate by inoculums loop wire. After 24 hours of incubation at 37 $^{\circ}$ C, if growth occurs indicates the organism is resistant to Colistin. Such strains under AMR Surveillance Program forward to NCDC New Delhi.

Antibiotics used: For Gram-positive organisms: Gentamicin (10 μ g), amikacin (30 μ g), tetracycline (30 μ g), ampicillin (10 μ g), ampicillin-sulbactam (20 μ g), co-trimoxazole (25 μ g), cefuroxime (30 μ g), (ciprofloxacin, levofloxacin ((5 μ g) each)), Penicillin G (10 units), erythromycin (15 μ g), clindamycin (2 μ g), ceftazidime (30 μ g), (vancomycin, linezolid (30 μ g) each)), high-level gentamicin (120 μ g), for Gram-negative organisms: Gentamicin (10 μ g), amikacin (30 μ g), tetracycline (30 μ g), ampicillin (10 μ g), ampicillin-sulbactam (20 μ g), co-trimoxazole (25 μ g), cefuroxime (30 μ g), (ciprofloxacin, levofloxacin ((5 μ g) each)), Ceftazidime (30 μ g), cefotaxime (30 μ g) & cefoperazone (75 μ g), cefepime (30 μ g), and imipenem (10 μ g) used. The anti-Pseudomonas antibiotics like piperacillin (100 μ g), ticarcillin (75 μ g), mezlocillin (25 μ g), netilmicin (30 μ g) & cefepime-tazobactam (40 μ g) were used. The Antifungal discs like (Amphotericin & Nystatin (100 units, each)), (Ketoconazole & Miconazole (30 μ g, each)), Fluconazole & Clotrimazole (10 μ g, each)) and Voriconazole (1 mcg) were used.

Results

Among 2550 patients, 675(26.47%) were culture positive. Our study found gram-negative isolates 435(64.4%) as compared to gram-positive isolates 125 (18.5%) and Candida spp. 115 (17.03%).

Among 675 cases, Klebsiella pneumoniae 170 (25%), E. coli 155 (22.9%), Candida spp. 115 (17.03%), S. aureus 110 (16.29%), Pseudomonas aeruginosa 85 (12.59%), Acinetobacter spp. 25 (3.7%) and Enterococcus spp. 15 (2.2%) were isolated. The incidence of EONS was 419 (62.07%), and LONS was 256 (37.9%). The following risk factors, Preterm 155(22.9%), Low birth weight

85(12.59%), Fever with Jaundice 42(6.2%), Fever of unknown origins 22(3.25%), Poor feed 19(2.8%), Respiratory distress 18(2.6%) and Premature Rupture of Membrane 6(0.8%), Perinatal asphyxia 5 (0.7%) noted.

Antimicrobial Susceptibility Pattern:

Table 1: Antibiotics susceptibility pattern of Gram-positive organisms

Sr. No	Antibiotic	S. aureus resistance		Enterococcus spp. resistance	
		Number (n=10)	%	Number (n=15)	%
1	Penicillin G	85	77.2	07	46.6
2	Ampicillin	85	77.2	07	46.6
3	Cefuroxime	69	62.72	-	-
4	Cefoxitin	25	22.7	-	-
5	Ampicillin-sulbactam	69	62.72	-	-
6	Gentamycin	52	47.2	-	-
7	Amikacin	37	33.6	-	-
8	Tetracycline	16	14.5	05	33.3
9	Co-trimoxazole	52	47.2	-	-
10	Ciprofolxacin	29	26.3	05	33.3
11	Levofloxacin	29	26.3	04	26.6
12	Clindamycin	29	26.3	-	-
13	Erythromycin	78	70.9	06	40.0
14	Vancomycin	00	0.00	01	6.6
15	Lenozolid	00	0.00	00	00
16	High level Gentamycin	-	-	01	6.6

Table 2: Antibiotics susceptibility pattern of Gram-negative organisms

Sr. No	Antibiotic	Klebsiella spp. Resistance (n=170)	E.coli resistance (n=155)	Resistance in Enterobacteriaceae (%)
1	Ampicillin	*	96 (61.9%)	61.9
2	Cefuroxime	106 (62.3%)	95 (61.29%)	61.8
3	Ceftazidime	91 (53.5%)	81 (52.25%)	52.9
4	Cefotaxime	91 (53.5%)	81 (52.25%)	52.9
5	Cefepime	88 (51.7%)	79 (50.9%)	51.3
6	Ampicillin-sulbactam	79 (46.4%)	70 (45.16%)	45.8
7	Gentamycin	56 (32.9%)	47 (30.32%)	31.7
8	Amikacin	49 (28.8%)	40 (25.8%)	27.3
9	Tetracycline	46 (27.05%)	39 (25.16%)	26.2
10	Co-trimoxazole	59 (34.7%)	52 (33.54%)	34.1
11	Ciprofolxacin	44 (25.8%)	37 (23.8%)	24.9
12	Levofloxacin	41 (24.1%)	34 (21.9%)	23.1
13	Imipenem	2 (1.17%)	1 (0.6%)	0.92

Note: (*)Indicate intrinsic resistance

Antibiotics susceptibility pattern of Non fermenter organisms:

Acinetobacter spp. (n=25) demonstrated resistance to piperacillin, ticarcillin (8(32.0%) each), ceftazidime 7(28%), ciprofloxacin 6(24%), (levofloxacin, Gentamycin, (5(20%)each)) & less

resistance to (cefepime, Amikacin, cefepime-tazobactam, (3(12%)each)), and no resistance was found against Imipenem.

P. aeruginosa(n=85) demonstrated high level of resistance to (piperacillin, ticarcillin (61(71.7%) each)), (ciprofloxacin, levofloxacin, ceftazidime (59(69.4%)each)), (aztreonam, Gentamycin, Netilmicin (46(54.11%)each)) & less resistance to (cefepime, Amikacin, cefepime-tazobactam, (21(24.7%)each)) while most were sensitive to imipenem 80(94.11%). All Gram-negative organisms were sensitive to colistin 100%.

Candida spp.(n=115) were highly resistant to azole group ranging from 60% to 90% and less resistant to Voriconazole 41(35.6%) & Nystatin 35 (30.4%) while most were sensitive to Amphotericin B (97.3%).

ESBL & AmpC producer: Among 155 *E. coli*, 46(29.6%) were ESBL & 57(36.7%) were AmpC producer. Among 170 *Klebsiellapneumoniae*, 33(19.4%) were ESBL & 37(21.7%) were AmpC producer.

Carbapenemase produced in 3(0.8%) Enterobacteriaceae detected by the modified Hodge test. Metallo-beta lactamases identified by CDST representing class A, B, and Class D beta-lactamases.

Metallo-beta lactamases production in 5(5.88%) *P. aeruginosa* identified by CDST representing class B beta-lactamases.

Multidrug resistance organism: Gram-negative rods: Pan-antibiotic resistance found in 8(1.83%) isolates: 1 *E. coli*, 2 *Klebsiella pneumoniae* & 5 *Pseudomonas aeruginosa*.

Discussion

We have compared our study with various other studies on the following basis:

1. The incidence & microbial pattern
2. Associated risk factors
3. AntibioGram of isolates

1) The incidence & microbial pattern

The incidence of neonatal septicemia found in the present study was 26.47%. The other study, by Yadav N.S. et al,⁴ was (16.9%), by Gohel K et al,⁵ was (9.2%), by Vrishali Muley et al, India⁶ was (26.6%), by Hitesh Assudani et al, India⁸ was (35.34%), by Bhat M. et al, India⁹ was (48.3%), by Roy I. et al, India¹⁰ was (47.5%), by Sharma et al, India¹¹ was (56%).

The microbial pattern in the various study are the following: The present study found *Klebsiella pneumoniae* (25%) as the most common isolate, followed by *E. coli* (22.9%). The Vrishali Muley et al, India⁶ found *Klebsiellapneumoniae* (35.04%) as the most common isolate, followed by *Staphylococcus aureus* (22.9%). Bhat M. et al, India⁹ found *Klebsiellapneumoniae* (34.88%) as the most common isolate, followed by *Staphylococcus aureus* (32.5%).

The present study found 17.03% *Candida* spp. while others like Banerjee et al, India¹⁴ found 27.5%, and Chander J et al, India⁷ found 29.4%

The predominance of *K. pneumoniae* as the causative agent of neonatal sepsis may be due to the harshness of the organism to survive in a hospital environment and the selective pressure of antimicrobial agents. So that resistant organisms lead to colonize and proliferate in neonates⁶.

2) Associated risk factors

The sex-wise distribution: The study of Jimba Jatsho et al¹ was found 52.3% in males and 47.7% in females, Jyoti Sonawane et al, India¹² was found 64.6% in males, and 35.39% in females, Hitesh Assudani et al, India⁸ was found 76.7% in males and 23.28% in females. The present study found 57.7% in males and 42.2% in females.

The male infants confer less immunological protection compared to females. The reason behind such sex difference is X chromosome contains a gene involved in the function of the thymus or synthesis of immunoglobulins¹.

The age of onset in a different study: The present study found 59.4% EONS and 40.5% LONS. Our results were comparable with the study by Jimba Jatsho et al¹ found 54.5% EONS and 45.5% LONS, Bhat M. et al, India⁹ found 58.13% EONS and 41.8% LONS, and Hitesh Assudani et al, India⁸ found 56.03% EONS and 43.9% LONS.

3) AntibioGram of isolates

Imipenem was the most sensitive drug with < 6 % resistance. All Gram-negative isolates demonstrated resistance to tetracycline was 27%, co-trimoxazole was 34%, Ampicillin-sulbactam was 46%, cefotaxime was 52.8%, and ampicillin was 61.9%.

ESBL producer identified in 29.6% of *E. coli* & 19.4% of *K. pneumoniae*. AmpC beta-lactamases producer identified in 36.7% of *E. coli* & 21.7% of *K. pneumoniae*. The study found resistance for tetracycline was 14%, Co-trimoxazole was 47.2%, for gentamicin was 47.2%, ciprofloxacin was 26.3%, penicillin was 77.2%. Methicillin-resistant found in *Staphylococcus aureus* was 22.7%.

The study of Hitesh Assudani et al, India⁸ found ESBL producer *E. coli* was 0.0% & *K. pneumoniae* was 37.50 %. Chandel DS et al¹³ found ESBL producer *E. coli* was 33.33% & *K. pneumoniae* was 30.97 %. Vrishali Muley et al, India⁶ noted ESBL producer *E. coli* was 25.0 % & *K. pneumoniae* was 29.4 %.

The study of Salamat, Sonia et al¹⁶ found AmpC beta-lactamases producer among gram negative isolates were 22% among them *E. coli* was 9.9% & *K. pneumoniae* was 5.7 %.

The study of Ahmad Mohamed Khadija et al¹⁵ found ESBL producer 23% and AmpC beta-lactamases producer gram negative isolates were 59%. Similar results found in present study AmpC beta-lactamases producer is higher than ESBL producer.

Extensive use of invasive procedures and third generation cephalosporins should be restricted to avoid the emergence of AmpC beta-lactamases in neonates. Moreover, indiscriminate use of broad spectrum cephalosporins should be the cause of the emergence of AmpC beta-lactamases producing bacteria¹⁶.

The study by Vrishali Muley et al, India⁶ found the following resistance patterns: in gram-negative isolates by co-trimoxazole, cefotaxime, ampicillin 50% to 73%, in Gram-positive isolates by co-trimoxazole 54.5%, ciprofloxacin 44.5%, penicillin 72.7%, gentamicin 44.5%, and MRSA 18.1%.

The study by Yadav N.S. et, al⁴ noted: most effective antibiotic against Gram-positive bacteria was found to be gentamicin (93%) followed by amikacin (89%) and ofloxacin (85%). Among *K. pneumoniae* and *E. coli* isolates, amikacin, gentamicin, meropenem, and imipenem were found to be 100% sensitive. Ampicillin, cefotaxime, and ceftazidime were found to be 100% resistive among *K. pneumoniae* whereas amoxicillin was resistant towards all *E. coli* isolates. Ampicillin showed the highest resistivity 78% among Gram-positive and 91% among Gram-negative isolates.

The study by Salamat, Sonia et al¹⁶ noted cefoxitin, gentamicin and amikacin resistance among gram negative organisms were 90-99% whereas imipenem was 100 % sensitive.

The study by Gohel K et, al⁵ noted: *Staphylococcus aureus* isolates exhibited resistance of oxacillin 70.6%, tetracycline 10%, vancomycin 21.6%. *Enterococcus* isolates exhibited resistance of tetracycline and vancomycin is respectively 33% and 40%. In gram-negative isolates exhibited resistance of amoxicillin, ceftazidime, ciprofloxacin 92% while Cefotaxime + clavulanate, Piperacillin + tazobactam exhibited 50% and tetracycline shows 72% to 84% resistance.

Ampicillin and amoxicillin which have been revealed as ineffective drugs in present study might be due to emergence of antimicrobial resistance genes in bacteria and inappropriate use of antibiotics prior to hospitalization of neonatal cases. Tetracycline are more sensitive in GPC rather than GNB. The initial empiric antibiotic use must therefore be a combination of drugs to cover for the prevalent bacterial organisms in that locality⁴ as combination in prophylaxis reduce the emergence of antimicrobial resistance. Therefore, the knowledge of the etiological organisms of neonatal sepsis and their antibiotic susceptibility profile and reviewing prophylaxis policy is necessary for effective therapeutic intervention.

Conclusion

Based on the above findings following recommendations are given:

- To decrease the emergence of multidrug-resistant organisms, healthcare workers should be routinely screened for the presence of multidrug-resistant bacteria, and their education regarding hand hygiene and convenient aseptic techniques should be encouraged along with proper periodic implementation and monitoring of Antibiotics stewardship program and role of Hospital infection control committees.
- To Follow strict antimicrobial prophylaxis guidelines. In the present study, aminoglycosides, tetracycline, fluoroquinolones, and voriconazole are the better preferred. So, rather than choosing higher-level antibiotics, advise waiting for antibiotic sensitivity results.

Abbreviations

AmpC= AmpC β -lactamases, CBC= Complete Blood Count, CDC= Centre for Disease Control & Prevention, CLSI= Clinical and Laboratory Standards Institute, ESBL= Extended spectrum β -lactamases, ESR= Erythrocyte Sedimentation Rate, LIS= Laboratory Information System, LRF= Lab Request Form, MBL= Metallo β -lactamases, MHA= Mueller Hinton Agar, MIC= Minimum Inhibitory Concentration, MRSA= Methicillin resistant Staphylococci, EONS= Early-onset neonatal sepsis, LONS= Late-onset neonatal sepsis, UTI = Urinary tract infection.

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