

Comparative Study in Early Neonates with Septicemia by Blood Culture, Staining Techniques and C – Reactive Protein (CRP)

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ABSTRACT

Aim: The aim of this study was to compare and evaluate the pathogenic bacteria in neo-natal septicemia by using various diagnostic techniques.

Setting and Design: Our study was designed to evaluate a feasible method to diagnose neonatal septicemia even at primary health centre level.

Materials and Methods: Blood samples were collected aseptically from 70 neonates. The specimens were inoculated into brain heart infusion broth and subcultures were performed with specific media. Antibiotic sensitivity pattern of isolates was studied by Modified Kirby Bauer Disc diffusion technique and differentiate the isolates by staining methods. C-reactive protein (CRP) was evaluated by using standard kit method.

Results: Out of 70 cases of childhood septicemia of age group 1-30 days, 37 had positive CRP, 36 were positive for BCS and blood culture was positive only in 41 cases, where predominant organism being *Klebsiella species* (n=28, 68.29%) followed by *Escherichia coli* (n=4, 9.76%), *Pseudomonas aeruginosa* (n=3, 7.31%), *Proteus mirabilis* (n=2, 4.88%) and Coagulase negative *staphylococcus* (n=4, 9.76%).

Conclusion: Our findings suggest that *Klebsiella species* as an important cause of neonatal septicemia. The isolated organisms were found to be highly sensitive to cefotaxime and amikacin. Hence, these antibiotics can be considered as the first drug of choice for neonatal septicemia.

Keywords: Acridine orange, Buffy coat smear, Gram stain, Septicemia

INTRODUCTION

Septicemia means a severe form of infection in which bacteria multiply and release toxin into the blood stream. Neonatal septicemia is a clinical syndrome occurring in the first 30 days of life characterized by symptomatic systemic illness due to infectious agents [1]. Septicemia arises from various infections, including those of the skin, lungs, abdomen, and urinary tract [2].

Septicemia in newborns remains a significant cause of mortality in developing countries. The fulminant nature of neonatal septicemia and its high mortality rate has always posed a challenge to the skill of a paediatrician. In general, 2% of infants are infected during intra uterine life and 10% during delivery. However, inflammatory lesions were found in about 25% newborn autopsies [1]. Previous studies on neonatal septicemia in south India [3] revealed that neonatal septicemia has contributed for the infant mortality (14.40%).

The newborn infants host resistant mechanism particularly that of preterm infant may be immature and easily overcome by invading organisms. Infection therefore becomes fulminant and causes death within few hours or days. A variety of organisms including bacteria, viruses, fungi and protozoa are the etiological agents. Etiological agents vary in different geographical area [4].

In this study we adopted, Acridine orange which is a fluorochrome stain, particularly for the demonstration of bacteria in blood and binding with the nucleic acid occurs in both living and dead bacteria, also other microorganisms [5]. Bacterial and yeast cells will appear as brilliant orange against a black, light green or yellow background [6]. CRP is an acute phase protein produced by the liver and its interaction with the complement system, in response to inflammation, infection and tissue injury [7]. CRP is useful in detecting the early infection of neonatal septicemia/meningitis and urinary tract infection by using semiquantitative latex-agglutination as a rapid screening method [8]. This CRP test is highly sensitive, but when compared with Buffy coat smear examination, it is easy,

cheap, time saving, rapid and also can be performed in remote centers, where there is a no well equipped laboratory available [9].

This study was undertaken to compare and evaluate the gram stain, acridine orange stain and CRP results with blood culture reports and with each other so as to provide a feasible method to diagnose neonatal septicemia even at primary health centre level. Antibiotic susceptibility of various pathogenic bacteria isolated in neo-natal septicemia were also tested

MATERIALS AND METHODS

Samples were taken from neonates admitted in the sick neonatal ward at Government Rajaji Hospital, attached to Madurai Medical College, Madurai, India, and study was carried out during December - 2013 to September -2014.

Sample Collection and Selection Criteria of Neonates

Blood Samples was taken from 70 clinically suspected cases of neonatal septicemia. Babies suffering from septicemia selected for this study had one or the other following symptoms described below: refusal of feeds, lethargy, diarrhea, vomiting, poor cry, abdominal distention, fever, respiratory distress, convulsion, hepatomegaly, jaundice, sclerema, boils, umbilical sepsis etc., and exclusion criteria as listed below: Patients with spina bifida, Anencephaly, other neural tube defects and in very sick neonates, after initial stabilization.

C - reactive protein (CRP) Test

CRP was estimated by using standard kit marketed by TULIP Diagnostic P.Ltd., Goa. There are two types of slide methods namely: (1) Qualitative method, (2) Semi quantitative method [10].

(1) Qualitative Method

One drop serum was placed on the glass slide and added one drop of Rhexal CRP latex reagent to the test specimen on the slide.

Using a mixing stick, the serum and Rhelax CRP latex reagent was mixed uniformly over the entire circle. The slide was rocked gently back and forth, observing for agglutination macroscopically after two minutes.

(2) Semi Quantitative Method

Using isotonic saline, serial doubling dilutions of the serum sample was prepared as follows: 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64. Each dilution of the serum sample was pipetted into separate reaction circles. The same procedure as under qualitative method was continued for each dilution.

Buffy Coat Smear Examination

One ml of blood was transferred into sterile wintrobe tube and centrifuged at 2500 rpm for 30 minutes. The plasma was removed and buffy coat was taken for smear preparation [11]. One smear was stained by Gram's technique and other one was stained by Acridine orange. The smears were fixed by methanol.

Gram's staining

The fixed smear were flooded with crystal violet stain for minute and washed with water. The gram's iodine was added and allowed for one minute. After that acetone was added for not more than five seconds and washed with water. Then dilute carbol fuchsin was added and allowed for one minute and washed with water. Finally the smear was air dried and the slide was examined under oil immersion microscope. The gram positive organisms appeared violet in colour and the gram negative organisms pink in colour.

Acridine orange staining

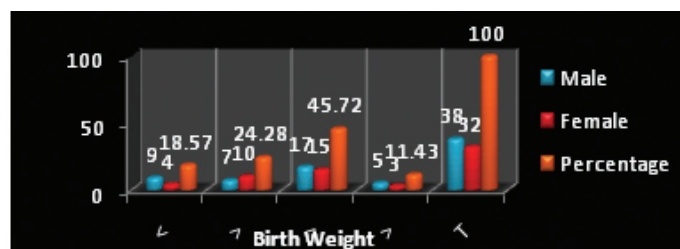
According to Brain Lauer et al., [12], the buffy coat smear was air dried and fixed by methanol. The fixed smears were flooded with acridine orange stain and left for five minutes followed by washing with water. The acridine orange stained smears were examined with incident light fluorescence at 600X magnification and confirmed at 1500X magnification. The bacteria appeared orange or orange red in colour [13].

Blood Culture Methods

Blood collected aseptically was transferred to a culture bottle containing brain heart infusion broth. The culture bottle was incubated at 37°C for 18 to 24 hours, after which sub culturing on the following plates were done using sterile technique. (a) Nutrient agar plate; (b) Blood agar plate; (c) Mac conkey agar plate. Plates were incubated at 37°C for 18 to 24 hours aerobically and growth in the plates observed. The colony size, shape, edge, margin, consistency were noted. Broth was examined for any turbidity, pellicle formation and deposit. The colonies were identified by following techniques; (1) Gram stain-to identify gram positive and gram negative organism. (2) Hanging drop- to find out motile and non motile organism. (3) Oxidase, catalase test- to rule out *Enterobacteriaceae*. (4) Coagulase test to identify *Staphylococcus aureus* and Coagulase negative staphylococci. Member of species was identified by bio chemical reactions using sugar fermentation, Indole, methyl red, voges proskauer, citrate, and urease and hydrogen sulphide production.

Kirby Bauer- disc diffusion technique

After isolation of the organism antimicrobial sensitivity test was done to select the antimicrobial drugs. Disc diffusion technique was used to identify the zone inhibition of bacterial strains [14]. In this study we used ampicillin, Gentamycin, co-trimoxazole, cefatoxime and amikacin for all isolates and in addition cloxacillin for gram positive agents.



[Table/Fig-1]: Birth weight of the neonates in relation to sex



[Table/Fig-2]: Age and sex distribution in 70 neonates

Clinical Features	Numbers*	Percentage
Gastro intestinal tract symptoms		
Refusal of feeds	50	71.42
Vomiting	12	17.4
Diarrhoea	8	11.42
Abdominal distension	9	12.85
Hepatomegaly	42	60
Jaundice	8	11.42
Upper respiratory tract symptoms		
Respiratory distress	34	48.57
Central nervous system symptoms		
Lethargy	33	47.14
Poor cry	32	47.71
Convulsion	4	5.71
Other manifestations		
Fever	13	18.57
Skin manifestation	1	1.43

[Table/Fig-3]: Clinical features of neonates with septicemia

* Many newborn had more than one manifestation

RESULTS

In the present study 70 cases of neonatal septicemia were taken. There were 38 males (54.29%) and 32 females (45.71%) percentage respectively. So it is inferred that males are more affected by septicemia than females. The breakup details in relation to sex, birth weight and age are given in [Table/Fig-1,2] respectively. From [Table/Fig-3], it could be inferred that out of 70 cases studied for septicemia, most of the infants 62 (88.57%) belonged to lower birth weight of less than 2.5 kg. The clinical symptoms and signs of these 70 neonates with septicemia are furnished in the [Table/Fig-3].

From 41 samples one or other bacteria was isolated. It was inferred that among the organisms isolated *Klebsiella pneumonia* ranked first accounting for 68.29% followed by *Escherichia coli* and Coagulase negative *staphylococcus* (9.75% each). The antimicrobial sensitivity was furnished in [Table/Fig-4].

Isolation of microbes is the gold standard test in patients with septicemia. In this study, the gram stain, acridine orange stain and CRP results were compared with blood culture reports and with each other. It was found that 37 culture positive cases had elevated CRP, 36 culture positive cases had positive response to gram staining, 39 of the 41 culture positive cases were also positive for acridine

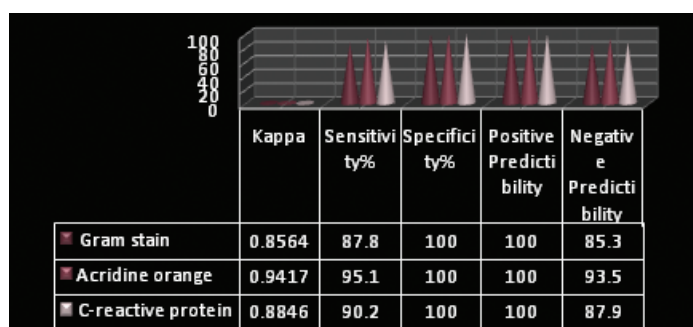
Drugs	<i>Klebsiella pneumoniae</i>		<i>Escherichia coli</i>		<i>Proteus</i>		<i>Pseudomonas aeruginosa</i>		Coagulase -ve staphylococcus	
	S	R	S	R	S	R	S	R	S	R
Ampicillin	12	16	3	1	1	1	1	2	1	3
	42.90%	57.10%	75%	25%	50%	50%	33.30%	66.70%	25%	75%
Gentamycin	21	7	3	1	2	0	2	1	2	2
	75%	25%	75%	35%	100%	---	66.70%	33.30%	50%	50%
Cefatoxime	26	2	3	1	2	0	2	1	4	0
	92.90%	7.10%	75%	25%	100%	---	66.70%	33.30%	100%	---
Amikacin	28	0	4	0	2	0	3	0	4	0
	100%	---	100%	---	100%	---	100%	---	100%	---
Cotrimoxazole	15	13	2	2	1	1	1	2	0	4
	53.60%	46.40%	50%	50%	50%	50%	33.30%	66.70%	---	100%
Cloxacilin	---	---	---	---	---	---	---	4	0	---
								100%	---	

[Table/Fig-4]: Results of antimicrobial sensitivity of the organisms isolated

Investigation	Clinical Septicemia Neonates	
	Positive (%)	Negative (%)
Blood culture	41(58.57)	29(41.42)
Serum CRP	37(52.85)	33(47.14)
B.C.S.Gram stain	36(51.42)	34(48.57)
B.C.S. acridine orange stain	39(55.71)	31(44.28)

[Table/Fig-5]: Results of blood culture, serum C-reactive protein and buffy coat smear by gram stain and acridine orange stain

Note- B. C.S- Buffy Coat Smear.



[Table/Fig-6]: Result of statistical analysis

orange. The details are provided in the [Table/Fig-5]. The sensitivity and specificity of the staining technique and CRP in comparison with blood culture was analyzed statistically (correlation) and results were presented in [Table/Fig-6]. Positive and Negative predictability studies to find the association between blood culture and staining as well as CRP did not reveal any superiority of these staining or serology over blood culture.

DISCUSSION

In this study bacteriological agents were isolated from 41 of the 70 patients. Gram negative bacteria were isolated in 37 (90.24%). Among them *Pseudomonas aeruginosa* was isolated in 3 (7.31%), *Proteus* genus in 2 (4.88%), *Escherichia coli* in 4 (9.76%) and *Klebsiella* in 28 (68.29%), gram positive organism was isolated in another 4 (9.76%) cases and it was Coagulase negative staphylococcus. Thus in this study 90.24% of neonates were affected by gram negative organisms and 9.76% by gram positive organisms. This report is supported by the study of Vaidya et al., [15].

Klebsiella was the predominant organism (68.29%) in this study, which also correlates with the study of Bhutta et al., [16] and Daoud et al., [17] in whose studies *Klebsiella* was the commonest etiological agent for the neonatal septicemia. Sanghvi and Tvdehope [18] reported that *Group B streptococcus* contributed to 52% of septicemia in developed countries, but in this study there was no isolation of *Group B streptococcus*.

In this study, the gram negative organisms like *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* were highly sensitive to cefatoxime and amikacin. Gentamycin was moderately sensitive to both gram positive and gram negative organism. Coagulase negative staphylococcus isolated in this study was sensitive to amikacin, gentamycin and cloxacillin. Gupta et al., [19] also reported coagulase negative staphylococcus was sensitive to amikacin. This finding also coexist with the study of Vaidya et al., [15] who described that the gram negative organisms were highly sensitive to cefatoxime and moderately sensitive to gentamycin and kanamycin.

Jha et al., [20] studied the out of 150 cases of childhood septicemia of age group 0-14 years, 83 had positive (CRP >6g/ml), 70 were positive for BCCS and blood culture was positive only in 83 cases, where predominant organism being *Klebsiella* species followed by *Staphylococcus* species. CRP test showed 100.0% sensitivity and 87.30% specificity, where BCCS showed 76.5% sensitivity and 91.2% specificity. This study also related to our finding.

Rajendra et al., [21] studied the 200 cases, from that 95 (47.5%) were blood culture positive. Gram negative isolates were 70.53% and Gram positive 29.47%. Buffy coat had specificity of 86.1% and sensitivity of 55.3%. CRP test had 87.37% sensitivity and 71.43% specificity. Usefulness of buffy coat smear, Gram positive and Gram negative isolates, during present study is also identical. In this study, Buffy coat had specificity of 100% and sensitivity of 87.8%.

In this present study, showing the Sensitivity and specificity and positive predictive value of CRP are 90.2% 100% and 100% respectively. The report was coincided to the Sucilathangam et al., [22] which is 50%, 69% and 38.8% respectively.

Kumar [23] studied 22 cases were bacteriologically positive. Most common isolated organism was *Escherichia coli* followed by *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus mirabilis*, Group B *Streptococcus* and *Streptococcus fecalis*. This study endorsed by the present work and common organisms isolated was *Klebsiella* species followed by *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and Coagulase negative *Staphylococcus*.

In this study, we inferred that out of 70 cases Studied for septicemia, most of the infants 62 (88.57%) belonged to lower birth weight of less than 2.5 kg. The clinical presentation of sepsis, 41% cases were blood culture positive and 29% cases were blood culture negative. This finding correlated with the findings of Desai et al., [24] studies, Out of 60 total cases of proven neonatal sepsis was higher in low birth weight (≤ 2500 gms) infants (65%). Amongst 100 neonates with clinical presentation of neonatal sepsis, 52% cases were blood culture positive and 28% cases were blood culture negative.

CONCLUSION

In this present study, male neonates (54.29%) were more affected than female neonates (45.71%). Accordingly, we conclude that serum CRP and Buffy Coat Smear Examination is simple method for diagnosis of childhood septicemia. These tests are also helpful to rule out child infection and their treatment can be started by clinician as soon as possible within one hour, which is not only in tertiary care centers but also in remote area primary health care centers, unlike conventional methods that take upto seven days for blood culture for diagnosis of infection and septicemia. The isolated organisms were found to be highly sensitive to cefotaxime and amikacin. Hence, these antibiotics can be considered as the first drug of choice for neonatal septicemia.

REFERENCES

- [1] John P. Cloherty, Eric C. Eichenwald, Anne R. Hansen, Ann R. Stark/Jay P. Goldsmith .Manual of Neonatal Care. Lippincott Manual Series. 7th Ed. Wolters Kluwer Health. 2012; 1024.
- [2] Medline plus Medical Encyclopedia. Sepsis. U.S. National Library of Medicine. National Institutes of Health. Available at <http://www.nlm.nih.gov/medlineplus/ency/article/000666>.
- [3] Kurien A Kuruvilla, Swati P, Mary J and Atanu Kumar J. Bacterial profile of sepsis in a neonatal unit in south India. *Indian pediatrics*. 1998; 35: 851-58.
- [4] Asindi A Asindi, Eric I Archibong, Nivedita B Mannan. Neonatal septicemia. *Saudi Medical Journal*. 1999; 20: 942-46.
- [5] Caksen H, Uksel S Y, Ursatozturk M K, Umerkan B S and Coskun A. Use of acridine orange leukocyte Cytospin test in diagnosis of neonatal sepsis. *Journal of Paediatrics and Child Health*. 2001; 37(5): 523.
- [6] Koneman EW, Allen SD, Janda WM, Schreckenberger PC and Winn WC. Color Atlas and Textbook of Diagnostic Microbiology. Fifth Edition. Lippincott Williams & Wilkins. Philadelphia, PA, USA. *Lippincott-Raven Publishers*. 1997; 149-151.
- [7] CRP highly accurate testing for the detection of inflammation and infection (www.randox.com/powerline).
- [8] Setal B Chauhan, Viren Vagharia, Bimal B Chauhan. CRP in early diagnosis of neonatal septicemia. *National Journal of Medical Research*. 2012; 2(3): 276-78.
- [9] Rosan R, Delemon R.A. CRP level in the child hood septicemia. *Journal Prenatal*. 1998; 18: 138-41.
- [10] Kashyap B, Jhamb R, Shukla Das, Berry N, Kaur IR. Correlation of Cryptococcal antigen assay with CRP as serum and urine biomarker in cryptococcal meningitis: *Experience in a Tertiary Hospital*. 2013; 1(3): 64.
- [11] Brooks GF, Pribble AH, Beaty HN. Early diagnosis of bacteremia by buffy coat examinations. *Archives Internal Medicine*. 1973; 132(5): 673-75.
- [12] Lauer BA, Barth Reller L, and Mirrett S. Comparison of acridine orange and gram stain for detection of microorganisms in CSF and other clinical specimens. *Journal of Clinical Microbiology*. 1981; 14(2): 201-05.
- [13] Fazii P, Ciancaglioni E, and Storza GR. Differential fluorescent staining method for detection of bacteria in blood cultures, cerebrospinal fluid and other clinical specimens. *European Journal of Clinical Microbiology and Infectious Disease*. 2002; 21: 373-78.
- [14] Jorgensen JH, Turnidge JD, Washington JA, Murray PR, Baron EJ, Pfalter MA, et al. Antibacterial susceptibility tests: dilution and disk diffusion methods. Manual of clinical microbiology. 7th ed. Washington, D.C.: *American Society for Microbiology*. 1999; 1526-43.
- [15] Vaidya V, Bhava S, Hegde V, and Pandit AN. Neonatal septicemia a reappraisal with special reference to the use of cefotaxime. *Indian Paediatrics*. 1991; 28(11): 1265-70.
- [16] Bhutta ZA, Naquvi SH, Muzaffar T and Farooqui BJ. Neonatal sepsis in Pakistan. Presentation and pathogens. *Acta.pediatrica. Scand*. 1999; 80(6-7): 596-601.
- [17] Daoud AS, Abuekteish F, Obeidat A, el Nassir Z and al-Rimawi H. The changing face of neonatal septicemia. *Annals of Tropical Pediatrics*. 1995; 15(1): 93-96.
- [18] Sanghvi KP and Tvdehope DI. Neonatal bacterial sepsis in a neonatal intensive care unit- a five year analysis. *Journal of Paediatrics and Child Health*. 1996; 32: 333-38.
- [19] Gupta AK, Anand NK, Man Mohan, Lamba IMS. Coagulase negative septicemia in new born. *Indian Journal of Paediatrics*. 1991; 28: 124-48.
- [20] Jha BK, Singh YI, Mahadevmurthy S, Chaudhary NK. Septicemia detection by blood buffy coat smears in primary health care centers. *Journal of College of Medical Sciences-Nepal*. 2011; 7(1): 19-23.
- [21] Rajendra Prasad BPM, Basavaraj KN, Antony B. Rapid diagnosis of neonatal septicemia by buffy coat smear examination and C- reactive protein test in correlation with blood culture. *International Journal of Biological and Medical Research*. 2012; 3(2): 1658-61.
- [22] Sucilathangam G, Amuthavalli K, Velvizhi G, Ashihabegum MA, Jeyamurugan T, Palaniappan N. Early diagnostic markers for neonatal sepsis: comparing Procalcitonin (PCT) and CRP. *Journal of Clinical and Diagnostic Research*. 2012;6(4): 627-31.
- [23] Kumar B. Evaluation of serum CRP in diagnosis and prognosis of neonatal septicemia. *Webmed Central Pediatrics*. 2013;4:1-18.
- [24] Desai P, Shah AN, Pandya T, Desai P and Pandya T. CRP, immature to total neutrophil ratio and micro ESR in early diagnosis of neonatal sepsis. *International Journal of Biomedical and Advance Research*. 2014;5(8):364-66.

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Date of Submission: **Dec 7, 2014**

Date of Peer Review: **Jan 29, 2015**

Date of Acceptance: **Feb 02, 2015**

Date of Publishing: **Mar 01, 2015**

FINANCIAL OR OTHER COMPETING INTERESTS: None.