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The Determination of C-Reactive Protein (C-RP) and Tumor Necrosis Factor Alpha (TNF-α) in Sera of Neonates with Sepsis

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Abstract: The concentration of C-Reactive Protein (C-RP) and Tumor Necrosis Factor alpha (TNF-α) were determined in sera of thirty neonates aged 0-30 days with early onset and late onset septicemia between November 2006-August 2007 in Ile-Ife, Nigeria. Determination of the concentration of C-RP and TNF-α as indicators of sepsis for prompt diagnosis of the condition possibly to avert fatality and morbidity in these neonates. Blood samples were intravenously obtained for bacterial culture and ELISA technique of 30 neonates and 10 control subjects. The concentration of C-RP and TNF-α were determined using (ELISA) technique. Bacterial isolates were cultured on selective and differential media from serum samples initially introduced into thioglycolate broth. Samples that showed growth were further studied. Staphylococci were confirmed as *S. aureus* by coagulase production on slide and tube tests using pooled human plasma. Coagulase negative staphylococci were spceciated using carbohydrates. Gram negative rods were characterized using conventional media and antibiotic sensitivity tests of isolates were carried out by the disc diffusion method. The result showed 56.7% (17) of the neonates were categorized as early onset septicemia and 43.3% (13) with late onset condition. The mean C-RP concentration in the sera of neonates with early and late onset septicemia were 10.61±1.9 and 11.25±2.5, respectively compared to controls 2.29±0.89 each. While for TNF-α 38.7±14.9 and 33.01±16.9 for early and late onset sepsis compared to control was 6.8±1.4 pg L^{-1}. The mean C-RP concentration in bacteremic neonates was 10.50±1.9 mg L^{-1} compared with control 2.29±0.89. However, TNF-α mean serum concentration in same subjects was 38.04±14.19 pg L^{-1} compared with 6.8±1.4 pg L^{-1} for g positive organisms. While for gram negative organism the result was 10.55±1.94 mg L^{-1} against 2.29±1.94 mg L^{-1} for control. TNF-α value was 40.68±17.19 for g negative culture and 6.8±1.4 for control. Gram negative rods predominated (56.4%) with *E. coli* being 10% of total g negative rods. Coagulase staphylococci were the single most common isolates recovered. *S. capitis* 12.8% was the predominant staphylococci followed by *S. aureus* 7.7% and *S. saprophiticus* 2.6%. While C-RP and TNF-α are useful markers in early diagnosis of septicemia, elevation in C-RP concentrations may not necessarily indicate infection. With regards to TNF-α, elevation of this protein tend to correlate with infection. The study suggests both molecules are useful for evaluating early sepsis. Antibiotics sensitivity tests showed the bacterial isolates were susceptible mostly to quinolones while cotrimoxazole was the least effective.

Key words: C-RP, TNF-α, concentration, bacterial isolates, antibiotic resistance, infection

INTRODUCTION

Neonatal septicemia remains one the most important causes of morbidity and mortality worldwide despite considerable progress in hygiene, introduction of new vaccines, antimicrobial agents and advanced measures for early diagnosis and treatment (Ako-Nai *et al*., 1999; Chamberlain, 2000). Neonatal infections currently account for about 1.6 million deaths annually in developing countries and sepsis and meningitis are responsible for most of these fatalities (Gotoff, 1996).

Tumor Necrosis Factor alpha (TNF-α) is a key factor of both gram-negative and gram positive bacteria sepsis and has a wide variety of effects due to its ability to

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mediate expression of genes for growth factor and cytokines, transcription factor, receptors, inflammatory mediators and acute-phase proteins. This protein plays a role in host resistance to infection by serving as a immuno-stimulant and mediator of inflammatory response and cytotoxic for tumor (Vaudaux *et al*., 1992). C-RP is an acute phase reactant synthesized in response to inflammatory cytokines and may rise >1000 times during acute phase response. It can fall quickly after efficient elimination of microbial stimulus short half-life of 19 h (Vigushin *et al*., 1993; Ng *et al*., 1997). While bacteriological analysis is useful in diagnosis of septicemia, it is sometimes insufficient in early diagnosis of neonatal septicemia as cultural findings are often incapable of early diagnosis when urgent results are needed in detecting neonatal sepsis. Measurement of TNF-α and C-RP in serum is therefore useful in rapid diagnosis of neonatal sepsis. Resistance to commonly used antimicrobials by bacterial agents involved in septicemia is also emerging constituting an important problem world-wide (Vergnano *et al*., 2005).

The epidemiological data from developing countries regarding neonatal septicemia, compared to that of developed countries shows that the severity, morbidity and mortality rate is higher (Darmstadt *et al*., 2000) hence the importance of early detection in this environment (Adejuyigbe *et al*., 2001; Ako-Nai *et al*., 1990). The study determined the concentration of both C-RP and TNF-α in neonatal septicemia the 1st time this would be done in the environment to aid early diagnosis of sepsis. The bacterial agents involved and sensitivities of the agents to commonly used antimicrobials were also determined to aid (empirical) treatment.

MATERIALS AND METHODS

Study population: The subjects recruited for this study consisted of 30 neonates aged 0-30 days who exhibited symptoms such as hyperthermia, vomiting, refusal to feed, tachypnoea/apnoea and jaundice of early or late septicemia as determined by the consultant pediatrician at the Obafemi Awolowo University Teaching Hospitals Complex (OAUTHC) Ile-Ife, Southwestern, Nigeria. Ten neonates admitted to the intensive care unit for illnesses other than sepsis that did not cause increase in the C-RP protein and neonates born to mothers who had elective caesarian section were used as control subjects for this study. Permission for participation in the study was obtained from parents of these subjects in accordance with the institutional ethics committee guide-line.

Collection of samples: Samples were collected between November 2006 to August, 2007. About 2 mL of venous blood was collected with a sterile 5 mL syringe from each subject into 8 mL of freshly prepared Brain Heart Infusion (BHI) broth and incubated at 35°C initially for 48 h for growth. Serum obtained from each blood sample was stored at -20°C for the determination of concentration of both C-RP and TNF-α.

The C-RP Enzyme-Linked Immunosorbent Assay (ELISA) technique termed quantitative sandwich immunoassay was used. The kits used for the bioassays were obtained from US Biological, Swampscott, Massachusetts 01907, USA. The microtiter plates provided in each kit has been pre-coated with a monoclonal antibody specific for C-RP or TNF-α as the case might be. Standard/serial dilution of samples were then added to appropriate microtitre plate's wells and incubated according to supplier's instructions. The concentration of C-RP or TNF-α was determined with the kit standard provided by the manufacturer (ready to use) assayed alongside the samples to generate a standard curve obtained by plotting the absorbance values versus the corresponding standard values. The concentration of C-RP and TNF-α in patient samples were determined by interpolation from the standard curve (Arnon and Litmanovitz, 2008; Weitkamp and Aschner, 2005). Data was analyzed using the Statistical Package for the Social Sciences (SPSS) version 13 Windows (SPSS, Chicago, IL). Statistical significance was determined by the Fisher's exact test.

Isolation of bacteria: About 2 mL of venous blood was obtained from each neonate and inoculated into freshly prepared brain heart infusion and thioglycolate broth and incubated initially for 48 h. Broth in which growth developed was further analyzed. A loopful of such cultures was streaked on selective and deferential media to isolate bacteria. Discrete bacterial colonies appearing upon Gram reaction were furthered studied. Gram positive cocci in clusters that fermented mannitol on Mannitol Salt Agar (MSA) were confirmed as *Staphylococcus aureus* by their production of coagulase on both slide and tube tests in pooled human plasma and coagulase staphylococci confirmed by standard methods (Schleifer and Kloos, 1975). Gram negative rods were identified based on their reaction in conventional deferential media (Cowan and Steel, 1985).

Antibiotic sensitivity testing: The antibiotic susceptibility tests were carried out by standardised disc-diffusion method (Bauer *et al*., 1966) employing the following disks containing Augmentin (AUG) 30 μg, Amoxicillin (AMX)

25 μg, Erythromycin (ERY) 5 μg, Tetracycline (TET) 10 μg, Cloxacillin (CLO) 5 μg, Gentamicin (GEN) 10 μg, Cotrimoxazole (COT) 25 μg and (NAL) Nalidixic Acid (10 μg chloramphenicol. Mueller Hinton was the plating medium (*S. aureus* ATCC25923 was used as control organism.

RESULTS AND DISCUSSION

The results showed 17 (56.7%) of the septicaemic neonates were categorized as having early onset septicaemia (0-3 days) and 13 (43.3%) had late onset condition (≥4 days). The clinical symptoms presented by the septicaemic neonates were as follows: 20 (66.7%) experienced hyperthermia, 18 (60.4%) experienced vomiting and 15 (50 %) had tachypnoea/apnoea. About 9 (30.0%) of the neonates refused to feed and 6 (20%) were jaundiced.

Of the 30 blood cultures analyzed, 26 (86.7%) grew microbes and 4 (13.3%) were bacteria free. Altogether, 40 bacterial isolates cultured. About 57.5% of the isolates were gram negative rods and 45.5% were gram positive organisms of which gram positive staphylococci were 33.3% made up of coagulase negative staphylococci CONS (25.6%) and pathogenic *S. aureus* (7.7%). *Bacillus* sp. constituted 10.3%. Overall coagulase negative staphylococci were the single most predominant bacteria cultured. Enteric rods constituted (45.5%) with *E. coli* (10.3%) (Table 1).

The mean serum concentration of C-RP and TNF-α in the neonates were determined and compared in early and late on-set sepsis. The mean serum concentration of C-RP of neonates with early onset and late onset sepsis is shown in Table 2: 10.61±1.9 mg L^{-1} against control samples 2.29±0.89 mg L^{-1} for early onset and 11.09±2.5 mg L^{-1} against 2.29±0.89 for control for late onset sepsis. However, the mean serum concentration of TNF-α in neonates with early onset sepsis was 38.7±1.49 pg L^{-1} against late onset sepsis 33.07±6. 9 pg L^{-1} (Table 3).

The result showed the mean serum concentration for C-RP and TNF-α in sera of neonates with early onset sepsis that were bacteria free was 12.00±2.92 mg L^{-1} against control 2.29± mg L^{-1} and 20.54±10.29 pg L^{-1} and 6.8±0.4 pg L^{-1} for control, respectively. While the mean value for C-RP and TNF-α for neonates with early onset septicaemia from whom gram negative bacteria were cultured was 10.55±1.94 pg L^{-1} against control 2.29±0.89 pg L^{-1}. In contrast, the results showed the mean serum concentration for TNF-α was 40.68±17.19 pg L^{-1} against 6.8±1.4 pg L^{-1} for control.

Neonatal septicemia is one of the most common causes of admission into neonatal care units in hospitals. Prompt diagnosis of septicemia in serious sequalae caused the condition. Determination of the concentration of C-RP and TNF- have been used as indicators of sepsis in developed countries but sparingly used method in most developing countries because of cost and availability of the kits hence the majority of pediatricians rely on bacteriological analyses which are sometimes insufficient for prompt diagnosis as cultural findings are often incapable for early detection when urgent results are needed in neonatal sepsis. The study was undertaken to determine the concentration of C-RP and TNF-α in detecting sepsis among neonates, characterize the bacterial isolates responsible and the susceptibility of the antibiotics used in the treatment of this condition at a tertiary health institution in Ile-Ife, South-Western, Nigeria.

The study showed that the mean serum concentration of C-RP of neonates with early onset and late onset septicaemia was significantly elevated compared with control sera but that the degree of elevation between early onset and late onset septicaemia is statistically insignificant. The mean serum concentration of TNF-α was also significantly elevated in which both early and late onset septicaemia compared

Table 1: Frequency of bacterial isolated from blood of neonates with sepsis

Organism	Frequency (%)
Staphaylococci	13 (33.3)
Coagulase positive staphylococcus	-
Staphylococcus aureus	3 (7.7)
Coagulase negative Staphylococcus	
Staphylococcus saprophyticus	1 (2.6)
Staphylococcus capitis	5 (12.8)
Staphylococcus epidermidis	2 (5.1)
Staphylococcus hominis	2 (5.1)
Bacillus	4 (10.3)
Bacillus cereus	2 (5.1)
Bacillus subtilis	2 (5.1)
Gram negative rods	
Edwardsiella sp.	2 (5.1)
Shigella sonnei	2 (5.1)
Serratia maracescens	2 (5.1)
Proteus rettgeri	2 (5.1)
Enterobacter sp.	2 (5.1)
Escherichia coli	4 (10.3)
Salmonella sp.	2 (5.1)
Klebsiella pneumoniae	2 (5.1)
Citrobacter freundii	2 (5.1)
Pseudomonas aeruginosa	2 (5.1)
Proteus mirabilis	1 (2.6)
Total	40 (100.0)

Table 2: Mean CRP concentration in neonates with early and late onset septicaemia compared with control

Onset	Parameter	Subject (n = 30)	Control (n = 10)	t-value	p-value
Early	CRP mg L^{-1}	10.61±1.9	2.29±0.89	2.74	0.61
Late	CRP mg L^{-1}	11.09±2.5	2.29±0.89	3.14	0.51

Table 3: Mean TNF-α concentration in early and late onset septicaemia in neonates compared with control

Onset	Parameter	Subject (n = 30)	Control (n = 10)	t-value	p-value
Early	TNF-α pg L^{-1}	38.7±14.90	6.8±1.4	2.77	0.96
Late	TNF-α pg L^{-1}	33.01±16.9	6.8±1.4	1.72	0.97

also to control sera. The degree of elevation in early onset septicaemia was however, slightly higher than early onset septicaemia.

While this observation is interesting, Peltola showed elevation in concentration of these molecules in sera of subjects screened compared with controls, the difference in the level in of subjects in early and late septicaemia was statistically insignificant. Some investigators (Peltola and Holmberg, 1983) have reported that slight elevation in the concentration of C-RP in serum do not eliminate the possibility of bacteremia consequently moderate elevation of C-RP tend to be common in patients with contaminated blood cultures and those with bacteremia. It has been suggested that it is only when C-RP concentration in serum is >10 mg dL^{-1} and if other causes of marked elevation of C-RP are eliminated is C-RP concentration in serum could be a relatively specific indicator of infection. This observation agrees with the finding in which the mean serum concentration for bacteremic samples were 10 mg L^{-1} for C-RP. However, some investigators have argued that elevation of C-RP concentrations is neither completely sensitive nor specific for detecting infections in patients with bacteremia (Peltola and Holmberg (1983), Weitkamp and Aschner (2005), Bone (1991). Altogether the mean serum concentration TNF-α and C-RP levels were significantly higher in patients groups compared with the control group in the study (Table 2 and 3). The relatively small number of subjects in this group is a limitation to draw a meaningful conclusion (Mattsson *et al*., 1994).

The results of bacterial aetiology of neonatal sepsis seem not to have changed at all since reported by Ako-nai in the same environment. While their study showed *S. aureus* as the predominant gram positive isolate, overall, gram negative rods predominated. In contrast, the study revealed gram negative rods predominated (56.4%) with *E. coli* (10.3%) being the most common gram negative rod. Coagulase negative staphylococci were the predominant gram positive cocci *Staphylococcus capitis* (12.8%) was dominant followed by *S. aureus* (7.7%) and *S. saprophicus* (2.6%). This finding is similar to that reported by Rodrigo (2002) in Sri Lanka where the leading bacteria were coagulase negative staphylococci. In contrast in Pakistan, gram negative rods were the predominant organisms (Anwer *et al*., 2000). However, studies from the United States, reported Group B streptococci as the predominant organism cultured (Schrag *et al*., 2002). Interestingly in Ile-Ife where this study was carried out, gram negative organisms remain the predominant isolates cultured underscoring the fact that the incidence and distribution of bacterial agents in septicemia vary from one geographic locality to another (Ako-Nai *et al*., 1990).

Table 4: Antibiotic sensitivity pattern of isolates from blood samples

Antibiotics	No. of isolates tested	No. of resistant (%)
Penicillins	40	-
Amoxicillin	-	12 (30.0)
Cloxacillin	-	6 (15.0)
Tetracycline	40	-
Tetracycline	-	6 (15.0)
Macrolides	40	-
Erythromycin	-	4 (10.0)
Nitrofurans	40	-
Nitrofurantoin	-	5 (12.5)
Chloramphenicol	40	-
Chloramphenicol	-	2 (5.0)
Other β-lactam	40	-
Augmentin	-	11 (27.5)
Trimethoprim	40	-
Cotrimaxozole	-	13 (32.5)
Aminoglycoside	40	-
Gentamicin	-	4 (10.0)
Quinolones	40	-
Nalidixic acid	-	3 (7.5)
Ofloxacin	-	0 (0.0)

The diagnosis of neonatal septicaemia is difficult to establish based on clinical criteria alone hence empirical treatment should not be delayed because of high mortality (Van den Hoogen *et al*., 2010; Kocabas *et al*., 2007). Prompt administration of antimicrobials is therefore crucial to the resolution of the condition. Cotrimoxazole and penicillins remain the least effective antibiotics as 32.5% of isolates encountered were resistant to cotrimaxazole, 30% to penicillin and 15% to amoxicillin in this study. All the 40 bacterial isolates encountered were however, sensitive to ofloxacin underscoring the efficacy of this antibiotic in the event of an epidemic in this environment (Table 4).

CONCLUSION

This study showed the mean serum concentrations of C-RP and TNF-α in neonates with early onset and late onset septicaemia were significantly elevated compared with control sera but that the degree of elevation between early onset and late onset septicaemia was statistically insignificant. Similarly, the degree of elevation of TNF-α in neonates with early onset sepsis was marginally higher than late onset septicaemia. Both molecules are also sensitive indicators of neonatal sepsis.

The study also showed the pattern of bacterial etiology of neonatal septicaemia at this centre have not changed much in the last 10 years. The study also underscores the in-effectiveness of commonly used anti-bacterial agents thus creating a challenge for choice of appropriate use of most of these antibiotics in this environment in the event of an epidemic which is of epidemiological importance in the control of diseases caused by these agents.

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