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Isolation and Identification of Bacteria by Blood Culture and their Drug Susceptibility Pattern in Children to Establish the Correlation Between CidIdhood Septicemia with C-Reactive Protein (Crp) levels.

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ISOLATION AND IDENTIFICATION OF BACTERIA BY BLOOD CULTURE AND THEIR DRUG SUSCEPTIBILITY PATTERN IN CHILDREN TO ESTABLISH THE CORRELATION BETWEEN CHILDHOOD SEPTICEMIA WITH C-REACTIVE PROTEIN (CRP) LEVELS.

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SUPERVISOR CERTIFICATE

This is to certify that Md. Al Jahidi Hasan Chowdhury has completed the thesis entitled **"Isolation and Identification of Bacteria by blood culture and their drug susceptibility pattern to establish the correlation between childhood septicemia with C-reactive protein (CRP) levels."** as partial fulfillment of the requirements for the degree of "Master of Philosophy" (M. Phil), in the faculty of Science, University of Rajshahi. It is absolutely based on his own work under my close supervision. His work is genuine and upto the mark.

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DECLARATION

I hereby declare that this thesis entitled **"Isolation and identification of bacteria by blood culture and their drug susceptibility pattern in children to establish the correlation between childhood septicemia with C-reactive protein (CRP) levels."** submitted to the University of Rajshahi, Bangladesh, for the degree of Master of Philosophy in Science are the original research work carried out by me.

The research work was conducted in the department of Microbiology and Paediatric unit, United Hospital Ltd. Gulshan, Dhaka and in the department of Biochemistry & Molecular Biology, University of Rajshahi.

Md. Al Jahidi Hasan chowdhury

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ABSTRACT

Septicemia is clinical state in which bacteria or their products such as toxins are present in blood stream and gives rise to serious systemic symptoms. Microbial invasion of blood stream causes high rate of morbidity and mortality. C-reactive protein (CRP) is an acute phase reactant of hepatic origin synthesized, regulated and secreted under the influence of interleukin-6. CRP levels are useful marker in the diagnostic evaluation of neonates with suspected bacterial infection as well as monitoring the response of therapy.

Suspected septicemic children of 0 day-15 years enrolled in United Hospital, Dhaka were considered for this study. Patients were grouped according to age. Out of 233 cases studied 39 were blood culture positive (22 male & 17 female). Culture proven septicemia was noted in 39 (16.74%) cases, probable septicemia were 136 (58.37%) and non-septicemic febrile patients were 58 (24.90%). Organisms isolated from blood were *S. typhi* 16 (41.03%), *S. paratyphi* A 7 (17.95%), *K. pneumoniae & A. baumannii* 4 (10.26%), *Pseudomonas spp.* 3 (7.69%), *S. marcescens* 2 (5.13%). *S. aureus* 1 (2.56%), *E. coli* 1(2.56%) and *Enterococcus spp.*1 (2.56%).

Blood culture positivity was significantly higher (23.74%, $p < 0.001$) among patients without antimicrobial therapy than with antimicrobial therapy (6.38%). Multidrug resistant *S. typhi was found in* 43.75% cases. *S. typhi* were 75% sensitive to ceftriaxone. *E. coli* and *K. pneumoniae* were highly sensitive to imipenem (100%) but was resistant to amikacin, (100%) amoxyclavonic acid, ciprofloxacin, gentamicin and cefepime. Resistance to gentamicin was observed in *K. pneumoniae* (100%). *A. baumannii* were 75% sensitive to ciprofloxacin & imipenem but resistant (100%) against amoxyclavonic acid. *S. aureus & Enterococcus sp.* sensitive (100%) to vancomycin but resistant (100%) to ampicillin, doxycycline. *Pseudomonas spp.* was resistant (100%) to ceftriaxone, cefixime and imipenem.

In this study, most of the gram negative bacteria showed resistance to commonly used antibiotics.

A total 39 (16.74%) cases were detected as both blood culture and CRP positive, only CRP positive were 136 (58.37%) cases. Both proven septicemia and suspected septicemia cases showed CRP level above the cut-off value (<6 mg/l). Statistically significant difference was found ($p<0.001$) when mean CRP level of proven septicemia group, probable septicemia group and non-septicemic febrile groups were compared with control group. CRP levels were significantly different ($p < 0.001$) among the three study groups.

LIST OF ABBREVIATIONS

%	-	Percentage
^0C	-	Degree centigrade
µg	-	Microgram
>	-	More than
<	-	Less than
AK	-	Amikacin
AMP	-	Ampicillin
ARD	-	Antibiotic Adsorbent Resin
ARDS	-	Adult Respiratory Distress Syndrome
ATCC	-	Americal Type Culture Collection
BA	-	Blood Agar
BHS	-	β-haemolytic streptococcus
BSMMU	-	Bangladesh Sheikh Mujib Medical University
C	-	Complement
CA	-	Chocolate Agar
CEF	-	Cefepime
CFM	-	Cefixime
Cl	-	Chloramphenicol
CN	-	Gentamicin
CRO	-	Ceftriaxone
CIP	-	Ciprofloxacin
CNS	-	Central Nervous System
CDC	-	Centre for Disease control
CFA	-	Colonization Factor Antigen
cfu	-	Colony forming Unit
Cmm	-	Cubic Milimeter
CRP	-	C-Reactive Protein
CNS	-	Coagulase Negative Streptococcus
DIC	-	Disseminated Intravascular Coagulation
DMCH	-	Dhaka Medical College Hospital
DSH	-	Dhaka Shishu Hospital
et al.	-	et alia (and others)
ELISA	-	Enzyme Linked Immuno-sorbant Assay
GBS	-	Group B streptococcus
H_2S	-	Hydrogen Sulphide
H_2O_2	-	Hydrogen Per Oxide
HIV	-	Human immunodeficiency virus
IgM	-	Immunoglobulin M
IS	-	Intermediate Sensitive

IV	-	Intravenous
IL	-	Interleukin
KCN	-	Potassium Cyanide
L	-	Litre
Lbs	-	Pounds
L-C	-	Lytic centrifugation
LPS	-	Lipopolysaccharide
LZD	-	Lynezolid
m	-	Month
MA	-	MacConkey Agar
mg	-	Miligram
ml	-	Mililitre
MHA	-	Muellar Hinton Agar
mmHg	-	milimetre of mercury
MODS	-	Multiple Organ Dysfunction System
MR	-	Methyl Red
MRSA	-	Methicillin resistant Staphylococcus aureus
NA	-	Nutrient Agar
NCCLS	-	National Committee for Clinical Laboratory Standards
NFA	-	Non Fimbrial Antigen
OMP	-	Outer Membrane Protein
o.a.s.i.s.	-	Oxoid Automated Septicemia Invetigation System
P	-	Penicillin
pg	-	Picogram
PAF	-	Platelet Activating Factor
PCR	-	Polymerase Chain Reaction
R	-	Resistant
RU	-	Rajshahi University
S	-	Sensitive
SD	-	Standard Deviation
SXT	-	Cotrimoxazole
SIRS	-	Systemic Inflammatory Response Syndrome
SPS	-	Sodium Polyethanol Sulphate
TSB	-	Tryptic Soy Broth
TSI	-	Triple Sugar Iron
TNF	-	Tumour Necrosis Factor
UHL	-	United Hopital Ltd.
WBC	-	White Blood Cell
VP	-	Voges-Proskauer
V	-	Vancomycin

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Md. Al Jahidi Hasan Chowdhury

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Chapter-1
Introduction

INTRODUCTION

The detection of microorganisms in a patient's blood has great diagnostic and prognostic significance. Blood cultures provide essential information for the evaluation of a variety of diseases like endocarditis, pneumonia, pyrexia of unknown origin and particularly, in patients with suspected sepsis. Many infections in neonatal and pediatric age group can only be established on the basis of etiological agent recovered from blood. A positive blood culture does not necessarily confirm infection, since contamination of blood can occur. The recovery of organisms traditionally considered as pathogens pose no problems in interpretation. However, recovery of organisms such as coagulase negative staphylococci (CoNS), Corynebacterium or Candida spp. is often difficult to interpret. Additional information like the density of bacteremia, number of positive cultures, duration of incubation of the broth to obtain a positive culture, presence of risk factors or an underlying disease is required in order to determine whether infection is truly present.

Sepsis is a systemic inflammatory response to infection. It is an important cause of morbidity and mortality in critically ill patients resulting in frequent diagnostic testing, greater prescription on antibiotics and increased length of hospitalization (Shafazand et al., 2002). This incidence rate of sepsis consistently lies between 50 to 100 cases per 100,000 individuals among industrialized nations (Moss et al., 2004). Although studies from developing nations are still lacking, in most countries one in 10 intensive care unit (ICU) patients have severe sepsis (Zwirble et al., 2004). Although studies from developing nations are still lacking, in most countries 1 in 10 intensive care unit (ICU) patients have severe sepsis (Zwirble et al., 2004).

Microbial invasion of blood stream is common cause of critical illness and has been associated with high rates of morbidity and mortality (Laupland et al., 2004). Approximately 200,000 patients are diagnosed with blood stream infection (BSI) annually in the United States with an estimated mortality of 22 to 29%. Severe sepsis remains a major cause of morbidity and mortality among children (Goldstein et al., 2005). The incidence of neonatal sepsis in India according to the data from national neonatal perinatal database (NNPD, 2002-2003) was 30 per 1000 live births (Sankar et al., 2008). A population based study in U.S. children by Watson and his colleagues, on severe sepsis found more than 42,000 cases in 1995 with a mortality rate of

10.3% (Goldstein et al., 2005). Although this represents a significant improvements over the past few decades, severe sepsis still remains one of the leading causes of death of children worldwide (Goldstein et al., 2005).

Septicemia is the systemic illness caused by spread of microorganisms or their toxins via the blood stream; where bacteremia denotes the presence of viable bacteria in the blood as evidence by positive blood culture (Munford, 2005).

In United States, the septic response is a contributing factor in more than 200,000 deaths per year (Munford, 2005). The bacteremia rate was found to be 8.4% among hospitalized patients suspected of having sepsis in Trinidad and was also associated with a high mortality rate of 15.1% (Orrett et al., 2007). The incidence of laboratory confirmed blood stream infection was 13.9% of hospital admissions in Tanzania (Bhomberg et al., 2007).

The reported incidence of neonatal sepsis varies from 7.1 to 38 per 1000 live births in Asia, from 6.5 to 35 per 1000 live births in Africa and from 3.5 to 8.9 per 1000 live births in South America and the Caribbean. By comparison, rates reported in the United States and Australia range from 1.5 to 3.5 per 1000 for early onset sepsis (EOS) and upto 6 per 1000 live births for late onset sepsis (LOS), a total of 6 to 9 per 1000 for neonatal sepsis. Neonatal infections currently cause about 1.6 million deaths annually in developing countries. Sepsis and meningitis are responsible for most of these deaths (Vergnano et al., 2005).

In Bangladesh neonatal morbidity and mortality due to septicemia is 13-18% (Islam 2001). Study report from Dhaka Shishu hospital revealed that blood culture proven septicemia was 35% (Ahmed et al., 2002). A study in khulna medical college hospital on neonatal infection had shown that septicemia (34.6%) was the commonest major infection (Rasul et al., 2007).

The conditions that predispose an individual to blood stream infection includes the extremes of age, hematological and non–hematological malignancies, diabetes mellitus, renal failure requiring dialysis hepatic cirrhosis immunodeficiency syndromes, severe burns and decubitus ulcers; therapeutic maneuvers associated with an increased risk include intravascular catheters,

surgery and endoscopic procedures; finally certain medications such as glucocorticoids, cytotoxic drugs increases the risk for septicemia (Munford, 2005; Reimer et al., 1997).

The most important group of organisms were Gram positive cocci (57% and enterobacteriace (27%) of all etiologies respectively (Laupland et al., 2004). The spectrum of causative microorganism has shifted from predominantly Gram negative bacteria in late 1970s and 1980s to Gram positive bacteria currently. In addition fungi have emerged as important pathogen. However Gram negative bacteria have gained importance once more in the tertiary care centers (Tanriover et al., 2005). The pathogens most often implicated in neonatal sepsis in developing countries differ from those seen in developed countries. Gram negative organisms are common and mainly represented by Klebsiella sp., Salmonella sp., Escherichia coli, Pseudomonas sp. Of the Gram positive organisms Staphylococcus aureus, coagulase negative Staphylococcus, S. pneumoniae are most common isolated (Vergnano et al., 2005).

The incidence of blood stream infection has increased, new microbial pathogens have been described and the spectrum of the pathogens isolated from blood and their susceptibility to antibiotics has changed. So blood stream infections continue to be one of the most medical problems and subject of many studies (Esel et al., 2003). Despite all the advances in medical practices in recent years, bacteraemia continues to be a serious problem that needs immediate attention and treatment (Nimri et al., 2004).

Neonatal sepsis is a clinical syndrome characterized by systemic signs of infection, accompanied by bacteriemia in the first month of life. Despite major advances in neonatology in the past few decades, bacterial sepsis is still one of the most important causes of morbidity and mortality in this age group worldwide, especially in developing countries. Early detection of bacterial sepsis is difficult for various reasons: firstly, early warning signs and symptoms are often protean and non-specific. Then there is the difficulty of distinguishing the clinical picture of neonatal sepsis from non-infectious causes. Further, microbiological culture results are not usually available until at least 48-72 hours after the specimen reaches the laboratory, and high false-negative rates of culture results may occur. The availability of a laboratory test to accurately and rapidly identify septic neonates would be of great value in improving the outcome of these patients. Equally difficult is the exclusion of infection in infants with suspected sepsis. Early detection of

the absence of infection would decrease the number of children started on antibiotics, shorten the length of hospital stay, and lessen the treatment costs and potential for emergence of resistant organisms.

Several hematological tests [total leukocyte count, total neutrophil count, immature neutrophil count, immature/total neutrophil (I/T) ratio and morphological and degenerative changes in neutrophils] were used for the early and reliable diagnosis of neonatal sepsis in the early and mid 1980's. The non-specific nature of these tests has directed investigators towards finding more specific and earlier increasing infection markers. In the last 25 years, acute phase proteins, complement system components, chemokines, cytokines, adhesion molecules, cell surface markers and combinations of these were investigated for the early and reliable diagnosis of neonatal sepsis. Today, procalcitonin (PCT), C-reactive protein (CRP), interleukin (IL)-6, IL-8, tumor necrosis factor-alpha (TNF-α), and some leukocyte surface antigens (CD11b, CD64) are the most hopeful markers amongst these. In most of the studies, a positive correlation has been shown between these markers and neonatal sepsis. However, quite contradictory results have been reported because of the investigation of patients with different gestational ages and birth weights, use of different sepsis descriptions, study of only small sample groups and variability in the characteristics of the control groups.

Blood culture remains the mainstay of investigation of potential sepsis in infants and children, despite recent advances in the molecular diagnosis of bacterial and fungal sepsis (Buttery, 2002). Isolation of a pathogenic microorganism from blood is a highly specific indicator of blood stream infection and performance of antimicrobial susceptibility testing may assist in the appropriate use of antimicrobial therapy for patients with blood stream infection.

In the last few years, many new techniques have been introduced into the field of early diagnostic process of bacterial infections: (a) Demonstration of indirect evidences of bacterial infection by identifying serological markers of systemic inflammatory response syndrome. Sepsis is considered when there is systemic response to a possible infection, termed as systemic inflammatory response syndrome (SIRS). The quest for surrogate biomarkers to define SIRS has identified several potential candidates. Markers such as C-reactive protein (CRP), procalcitonin, tumor necrosis factor alpha (TNF-α), several interleukins appeared promising. (b) Rapid bacterial culture methods. Blood culture is widely used for the diagnosis of septicemia. However,

in many clinical situations the yield from blood culture is low, positive cultures are obtained from fewer than 30%. Some of the patients with false negative blood culture may have had prior antibiotic treatment or they were not bacterimic at the time of blood collection (Bhatia et al., 2007). Blood culture the gold standard for the diagnosis of septicemia requires up to 48 to 72 hours before the results are known and almost half of the positive samples do not show growth (Bhartiya et al., 2000).

The host response to microbial infection is though to mediate by the release of inflammatory substance that may help the host to eradicate the invading organisms. During the local infection and host response, the release may be confined to the affected tissue, while a systemic infection and host response may be associated with release of inflammatory mediators into the blood stream. Therefore circulating inflammatory mediators have been suggested to be predictive for a systemic infection (Groeneveld et al., 2001).

Invading microbes activate host immune cells including neutrophils and monocytes. Activated phagocytes produce inflammatory cytokines such as tumor necrosis factor-α (TNF- α), interleukin-1β (IL-1β), interleukin-8 (IL-8) and interleukin-6 (IL-6). The release of these mediators into the circulation results in the development of systemic inflammation in adults and children. Interleukin-6 (IL-6) is a pleotropic cytokine involved in many aspects of the inflammatory response. It is an important trigger to hepatocytes for the generation of acute phase proteins including CRP, fibrinogen and serum amyloid A protein (Heney et al., 1992). As interleukin-6 (IL-6) plays a critical role in the induction of C- reactive protein (CRP) synthesis in the liver, it was hypothesized that this cytokine could be detected earlier in blood than CRP during the course of bacterial infection (Buck et al., 1994).

C-reactive protein (CRP), an excellent marker for established bacterial infection is not useful for early diagnosis (Mathers et al., 1987). Proinflammatory cytokines and CRP should always be measured together to optimize the sensitivity (Axel et al., 2004). Clinically measurement of CRP (late and specific) in combination with IL-6 (early and sensitive) in the first 24 hours of presumed septic episodes have been shown to yield a better sensitivity than either marker alone (Ng PC, 2004).

Only few data is available regarding etiology of septicemia of children and their antimicrobial sensitivity pattern in Bangladesh. In community acquired blood stream infections, the spectrums of microbial pathogens are changing in parallel with the change in their antimicrobial susceptibility patterns. It is of great importance to know the susceptibility profile of the whole range of likely pathogens in order to select appropriate antibiotics (Esel et al., 2003).

Therefore, this study has been designed to identify the etiological agents of septicemia in children and to determine antimicrobial susceptibility pattern and to establish correlation for usefulness of CRP as biomarker for the septicemic children.

Chapter-2
Aims and Objectives

AIMS AND OBJECTIVES

1. To isolate and identify the bacteria from blood culture in suspected cases of septicemia in children.

2. To observe the antimicrobial sensitivity pattern of isolated microorganisms.

3. To estimate C - reactive protein (CRP) level in blood.

4. To establish correlation between septicemic children and CRP level.

Chapter-3
Review of Literature

REVIEW OF LITERATURE

Septicemia

Septicemia is a clinical term used to describe bacteremia with clinical manifestations of severe infection, including chills, fever, malaise, toxicity and hypotension, the extreme form being shock (vandepitte et al., 2003). Recently septicemia has been defined as the systemic disease associated with the presence and persistence of pathogenic microorganisms or their toxins in the blood (Bone, 1991). Septicemia therefore may or may not be associated with positive blood cultures (Reimer et al., 1997). Blood culture yields bacteria approximately 20-40 percent of cases of severe sepsis and 40-70 percent of cases of septic shock (Munford, 2005). Confusion exists and is perpetuated by interchangeable use of the terms "bacteremia", "sepsis" and "septicemia". Bacteremia is the term that simply identifies the viable bacteria in blood. Sepsis is the presence of clinical symptoms of infection in the presence of positive blood cultures. Septicemia is a serious clinical syndrome associated with evidence of acute infection and organ failure related to release of mediators, like cytokines into the circulation (Reimer et al., 1997). In 1992, the American college of chest physicians and the society of critical care medicine outlined definition of sepsis and its related disorder (Munford, 2005).

Bacteremia	:	Presence of bacteria in blood as evidenced by positive blood cultures.
Septicemia	:	Presence of microbes or their toxins in blood.
Systemic Inflammatory response syndrome (SIRS)	:	At least 2 of the following 4 conditions- Oral temperature > 38°C or < 36°C. Respiratory rate >20breaths/min. Heart rate> 90 beats /min. Leukocyte count> 12,000/cmm.or < 4,000/cmm.
Sepsis	:	SIRS that has a proven or suspected microbial etiology.
Severe sepsis	:	Sepsis associated with organ dysfunction, hypo perfusion or hypotension including lactic acidosis, oliguria or acute alternation in mental state.

Septic shock	:	Sepsis induced hypotension (e.g. systolic blood pressure <90 mm Hg or a reduction of > 40 mm Hg from base line) that is unresponsive to fluid resuscitation plus organ dysfunction or perfusion abnormalities as listed above for severe sepsis.
Refractory septic shock	:	Septic shock that lasts for >1 hour and does not respond to fluid or pressor administration.
Multiple organ dysfunction syndrome (MODS)	:	Dysfunction more than one organ, requiring intervention to maintain homeostasis.

Septicemia is not a term which is easy to define precisely. It is used to denote those clinical states in which bacteria are present in the blood stream and give rise to serious systemic symptoms. Septicemia shades on one side into bacteremia in which bacteria are present in the blood stream; on the other side, septicemia shade into pyemia when the patient is desperately ill and multiple abscesses develop under the skin and various internal organs such as liver and/or brain. The suggestion that septicemia and bacteremia are distinguished on the basis of whether or not bacteria multiply in the blood cannot be verified (Philips and Susannah, 1992).

The host reaction for invading microbes involves a rapidly amplifying polyphony of signals and responses that may spread beyond the invaded tissue. Fever or hypothermia, tachycardia and Tachypnoea often herald the onset of sepsis, the systemic inflammatory response to microbial invasion (Munford, 2005). Only when the systemic inflammatory response syndrome (SIRS) is due to infection, it is called sepsis (Noskin and Phair, 1997; Parrillo, 1996). This distinction is crucial; many other conditions such as pulmonary embolism, dissecting or ruptured aortic aneurysm, myocardial infarction, occult hemorrhage, cardiac tamponade, post cardio-pulmonary bypass syndrome, anaphylaxis and drug overdose can result in SIRS and mimic sepsis but they are unrelated to infection (Noskin and Phair, 1997). When accompanied by evidence of hypo perfusion or dysfunction of at least one organ system, this becomes "severe sepsis". Finally, where severe sepsis is accompanied by hypotension or need for vasopressors, despite adequate fluid resuscitation, the term septic shock applies (Lever et al., 2007). As sepsis progresses to

septic shock, the risk of dying increases substantially. Early sepsis is usually reversible, whereas patients with septic shock often succumb despite aggressive therapy (Munford, 2005).

Bacteremia may be transient, intermittent or continuous (Reimer et al., 1997; Chandrasekar and Brown, 1994; Washington and Ilstrup, 1986). Transient bacteremia lasting minutes to hours, is most common and may occur during activities of daily living such as vigorous teeth brushing or difficult bowel movements; these bacteremia usually resolve spontaneously and inconsequential (Chandrasekar and Brown, 1994). Significant transient bacteria may occur after manipulation of infected tissue such as abscesses or furuncles; during certain surgical procedures; when procedures are undertaken that involve contaminated or colonized mucosal surfaces e.g. dental manipulation, cystoscopy or gastrointestinal endoscopy; and predictably, at the onset of acute infections such as pneumoniae, meningitis, septic arthritis and acute haematogenous osteomylitis (Reimer et al,. 1997; Aronson and Bor, 1987). Intermittent bacteria are that which occurs, clears and then recurs in the same patient due to the same microorganism (Reimer et al., 1997). Classifically this type of bacteria is associated with undrained closed space infections of the respiratory or urinary and infected intravenous lines (Reimer et al., 1997; Chandrasekar and Brown, 1994). Continuous bacteremia is characteristic of infective endocarditis as well as other endovascular infections such as supportive thrombophlebitis, infective mycotic aneurysms. It also occurs early in the course of brucellosis and typhoid fever (Reimer et al., 1997; Aronson and Bor, 1987). Bacteremia may also be categorized as unimicrobial or polymicrobial (6 to 18% of episodes) and the term "break through bacteremia" has been used to describe the occurrence of bacteremia in patients receiving appropriate therapy for the microorganisms that is grown from the blood (Reimer et al., 1997).

PATHOPHYSIOLOGY

Invasion of blood by microorganisms usually occurs by one of the two mechanisms: drainage from the primary focuses of infection via the lymphatic system to the blood stream or direct entry from needles (e.g. in intravascular drug users) or other contaminated intravascular devices such as catheters or graft material (Russel JA, 2006; Munford RS, 2005; Reimer et al., 1991 and Bone RC, 1991). The presence of represents either the failure of an individual's host defenses to localize an infection at its primary site or the failure of a physician to remove, drain or otherwise

sterilize that focuses. Ordinarily, host defenses respond promptly to a sudden influx of microorganisms, particularly by efficient phagocytosis by macrophages of the mononuclear phagocytic system that help clear the blood within minutes to hours. Clearance may be less efficient when microorganisms are encapsulated, or it may be enhanced if the host has antibodies specific for the infecting organism. Clearance of blood stream is not always successful; septicemia develops in individuals whose host defense mechanisms either is too impaired to respond efficiently or is simply overwhelmed.

Host mechanism for sensing microbes

Animals have exquisitely sensitive mechanisms for recognizing and responding to conserved microbial molecules. Lipid A is bioactive center of LPS of all Gram negative bacteria found in nature. A host protein, LPS binding protein or LBP binds lipid A and transfers LPS to CD14 on the surface of monocytes, macrophages and neutrophils. LPS and CD14 then interact with toll-like receptor TLR4 and MD2 to form a molecular complex that transduces the LPS signal to the interior of the cell. This signal rapidly triggers the production and release of mediators such as tumor necrosis factor (TNF) α that amplify the LPS signal and transmit it to other cells and tissues. Bacterial peptidoglycan, lipoteichoic acids, DNA, certain polysaccharides and fimbriae elicit responses that are similar to those induced by LPS; whereas some of these molecules also bind CD14, they interact with several different TLRs. Other molecular pattern recognition proteins that are important for sensing microbial invasion include complement (principally alternative pathway), mannose-binding lectin, bacterial permeability increasing protein and C-reactive protein.

Local and systemic host responses to invading microbes

Recognition of microbial molecules by tissue phagocytes triggers the production and/or release of numerous host molecules (cytokines, chemokines, prostaglandins, leukotrienes and others) that increase blood flow to the infected tissue, enhances the permeability of the local blood vessels, recruits neutrophils to the site of infection and elicits pain. These phenomenons are familiar elements of local inflammation, the body's major innate immune mechanism for eliminating microbial invaders. Systemic responses are activated by neutral and/or humoral communication with the hypothalamus and brainstem.

Parapathogenesis

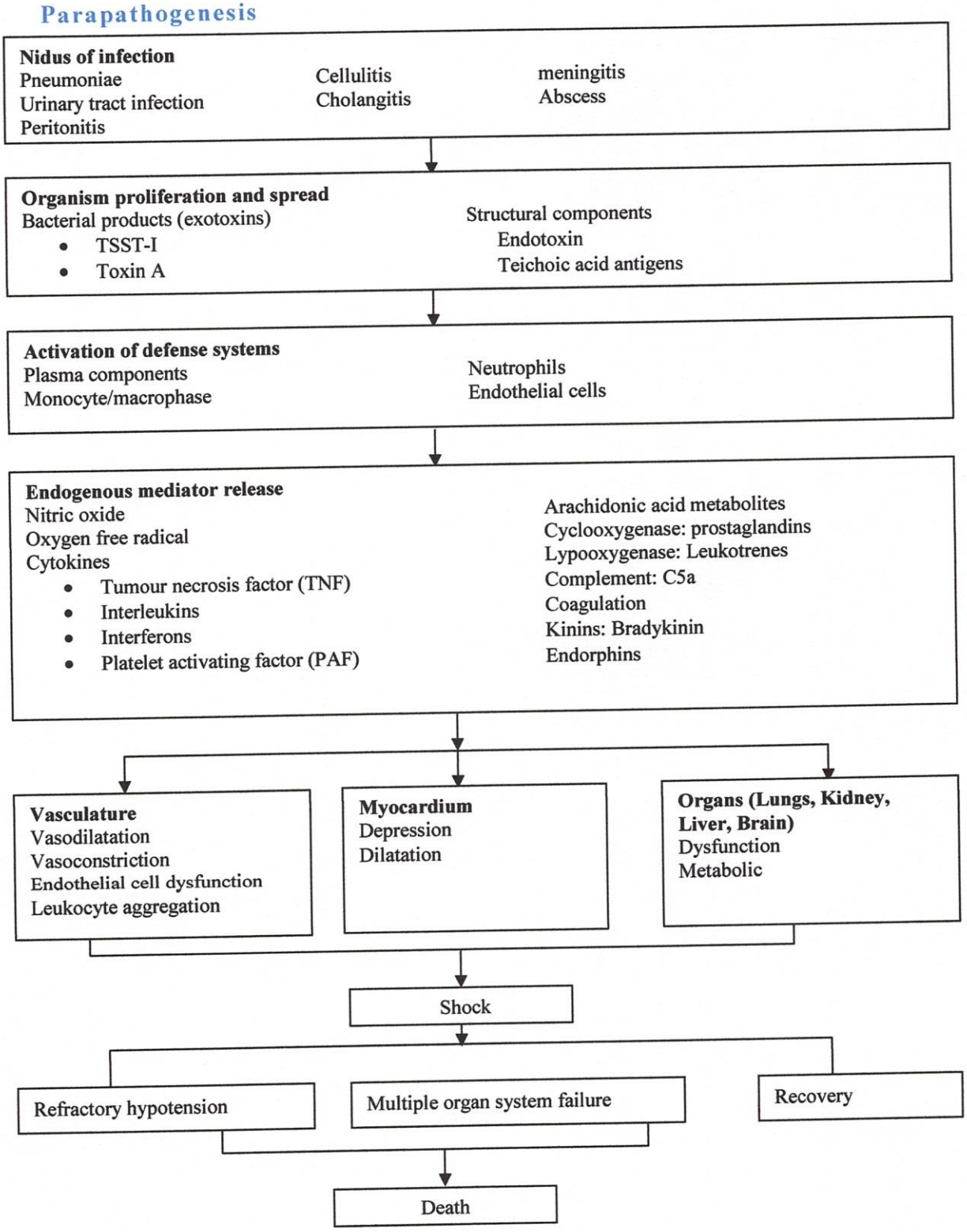


Figure-1: Pathogenesis of sepsis and septic shock (Adapted from Parrillo, 2004).

Cytokines and other mediators

Cytokine can exert endocrine, paracrine and autocrine effects. TNF-α stimulates leukocytes and vascular endothelial cells to release other cytokines to express cell-surface molecules that enhance neutrophil-endothelial adhesion at sites of infection and to increase prostaglandin and leukotrene production. Although TNF-α is a central mediator, it is only one of the many pro-inflammatory molecules that contribute to many innate host defense. Chemokines, most prominently IL-8 attracts circulating neutrophils to the infection site. IL-1β exhibits many of the same activities as TNF-α. TNF-α, IL-1β, interferon-γ, IL-12 and other cytokines probably interact synergistically with one another and with additional mediators.

Coagulation factors

Intravascular thrombosis, a hallmark of local inflammatory response, may wall off invading microbes and prevent infection and inflammation from spreading to other tissue. Intravascular fibrin deposition, thrombosis and DIC can also be important features of systemic response. IL-6 and other mediators promote intravascular coagulation initially by inducing the blood monocytes and vascular endothelial cells to express tissue factor. It binds to factor VIIa to form an active complex that can convert factors X and IX to enzymatically active forms. The result of the activation of both extrinsic and intrinsic clotting pathways, culminating in the generation of fibrin. Clotting is also favored by impaired function of the protein C- protein S inhibitory pathway and depletion of anti thrombin and protein C, while fibrinolysis is prevented by increased plasma levels of plasminogen activator inhibitor 1. Thus there may be a striking propensity of intravascular fibrin deposition, thrombosis and bleeding. Contact system activation occurs during sepsis but contributes more to the development of hypotension than DIC.

Complement system

Microbial LPS activates the complement system, principally the alternative pathway of activation, leading to widespread deposition of complement complexes in the microcirculation. During the early stages of sepsis, complement is an important defense mechanism, helping to clear invading bacteria. However, complement activation ultimately contributes to and amplifies the ischemia-reperfusion injury during the late stages. Complement split products (C3a and C5a)

enhance cytokine production, elicit leukocyte chemotaxis and activation, promote reactive oxygen species generation and adhesion molecule expression.

Endothelial injury

Wide spread vascular endothelial injury is the major mechanism for multi organ dysfunction. Stimuli such as TNF-α induce vascular endothelial cells to produce and release cytokines, procoagulant molecules, platelet activating factor (PAF), nitric oxide and other mediators. In addition, regulated cell adhesion molecules promote the adherence of neutrophils to endothelial cells. While these responses can attract phagocytes to infected sites and activate their antimicrobial arsenals, endothelial cell activation can also promote increased vascular permeability, micro vascular thrombosis, DIC and hypotension.

Tissue oxygenation may be diminished as the numbers of functional capillaries are reduced by luminal obstruction due to swollen endothelial cells, decreased deformability of circulating erythrocytes, leukocytes-platelet-fibrin thrombi or compression by edema fluid. Changes in vascular tones and endothelial permeability leads to extra vascular plasma escape (capillary leak), which enhances the viscosity, decreases the flow rate and hydrostatic pressure of blood. All these can lead to tissue hypo-perfusion, hypoxia and ischemia that further develop into organ failure and death.

Causes of bacteremia

- Numerous localized infections are often accompanied by bacteremia: meningitis, pneumoniae, pyelonephritis, osteo myelitis, arthritis, cholecystitis, peritonitis, enterocolitis, traumatic or surgical wound infections, bedsores etc.
- Bacteremia is a feature of some infectious diseases like brucellosis, leptospirosis and typhoid fever.
- Persistent bacteremia is a feature of endovascular infections, e.g. endocarditis, infected aneurysm and thrombophlebitis.
- Transient bacteremia can arise from various surgical manipulations but usually resolves spontaneously in healthy subjects.

- Bacteremia may result from the iatrogenic introduction of microorganisms by the intravenous route: through contaminated intravenous fluids, catheters or needle-puncture sites.
- Bacteremia may develop in the users of intravenous drugs.
- Bacteremia may occur in immunosuppressed subjects, including those with human immunodeficiency virus/ the acquired immunodeficiency syndrome (HIV/AIDS). They are often caused by "opportunistic" microorganisms and may have serious consequences (Vandepitte et al., 2003).

Epidemiology

Blood stream infection cause significant morbidity and mortality worldwide and are common among the most common health care associated infections (Orrett et al., 2007; Karlowsky et al., 2004). Increasing rates of antimicrobial resistance, changing pattern of antimicrobial usages and the wide application of new medical technologies (e.g. indwelling catheter and other devices) may change the epidemiology and outcome of blood stream infection. It is therefore important to continually review and update the epidemiology. (Diekema et al., 2003).

Incidence

To establish the true incidence of septicemia is difficult because it is not a reportable disease. It is a conventional practice to express the frequency of bacteremia infections as number of case per 1000 hospital admissions (Philips and Susannah, 1992). In USA, each year approximately 750,000 cases of severe sepsis occur of which 435,000 develop septic shock (Rudis et al., 2005). A very large epidemiological study of up to 6 million peoples in USA gives an incidence of 3 per 1000 populations per year (Lever et al., 2007). The incidence was highest in infants 5.16 per 1000, fall dramatically in old children 0.20/1000 in 10-14 years (Watson et al., 2002). European observations of blood stream infections from the 1980s and 1990s had estimated incidence rates to be between 76.5 and 153 per 100, 000. (Uslan et al., 2007).

The incidence of community acquired bacteremia in Africa is several folds higher than in industrialized countries. In a population based study in Kenya, blood cultures taken from all

children admitted to the hospital showed that the minimum estimated incidence of community acquired bacteremia per 100,000 children was 1457 in infants, 1080 in children younger than 2 years and 505 in those under 5 years of age. A report from Calabar, Nigeria showed an incidence of septicemia in children was 45.9% (Meremikwu et al., 2005).

The reported incidence of neonatal sepsis varies from 7.1 to 38 per 1000 live births in Asia, from 6.5 to 23 per 1000 live births in Africa and from 3.5 to 8.9 per 1000 live births in South America and the Caribbean. By comparison, rates reported in the United States and Australia range from 1.5 to 3.5 per 1000 EOS sepsis and up to 6 per 1000 live births for LOS sepsis, a total of 6-9 per 1000 for neonatal sepsis (Vergnano et al., 2005). Kurume university school of medicine, Fukuka, Japan noted that approximately 2% of all hospitalized patients have had sepsis (Oda et al., 2000). In Vientiane, Laos clinically significant isolates were obtained from11.2% of adults, 9.9% of children and 8.2% of infants in 2006. 2006).

A study from India, on neonatal septicemia had shown an incidence of 26.3% (Monga et al., 1986). The incidence of neonatal sepsis according to the data from Neonatal Perinatal Database (NNPD, 2002-2003) was 30 per 1000 lives births (Sankar et al., 2008). A study carried out in Microbiology department, Gandhi hospital, Secunderabad, India reported incidence of septicemia in paediatric patient was 18.7% (Murty et al., 2007). A study in Khulna Medical College, Bangladesh reported that septicemia (34.6%) was the commonest major infections among neonates (Rasul et al., 2006). A study in Dhaka Shishu Hospital reported 35% neonatal septicemia cases (Ahmed et al., 2002). Although there is no definite study on incidence, etiology with their antibiogram, various predisposing factors and prognosis of septicemia in Bangladesh, this would certainly be much higher in comparison to develop countries (Hossain and Rashid, 1989).

Incidence by age

The age distribution in bimodal, with peaks in children under 10 years and the elderly (Raymond et al., 2006). Several studies limited to Gram negative bacteremia have demonstrated an increase in the incidence with increasing age (Uslan et al., 2007). However, in developing countries like

Kenya and Nigeria, septicemia is seen most often among neonates and young children (Berkly et al., 2005; Alusa et al., 1997).

Incidence by season

A study on the population-adjusted seasonal incidence rates for sepsis and severe sepsis for the United States from 1979-2002 showed that there was a statistically significant changes in the rates of sepsis and severe sepsis across seasons. The average season incidence rate of sepsis increased 16.5% from a low of 41.7 cases per 100,000 populations in the fall to a high of 48.6 cases per 100,000 in winter (Danai et al., 2007). Similarly, seasonal rates for severe sepsis statistically increased 17.7% from fall to winter. The greatest change in sepsis incidence occurred with respiratory sources, increasing 40% during the winter compared with fall. In contrast to respiratory sources of sepsis, the highest rates of genitourinary-related sepsis were noted in the summer, 10.9 cases per 100,000; compared with a nadir spring incidence of 9.9 cases per 100,000. Sepsis originating in the gastrointestinal system, skin and soft tissues and other sources on infections were not seasonally affected. Seasonal changes in sepsis incidence vary according to geographic region.

Incidence by place of acquisition

Septicemia occurs in both community and hospital acquired infections. If the positive blood cultures were drawn more than 48 hours after the patient had been admitted to the hospital, or if the patient had been recently discharged from the hospital, the infection was classified as 'hospital-acquired'. If the cultures were drawn within the first 48 hours after admission to the hospital, the infection was classified as 'community-acquired' (Raymond et al., 2006; Gray, 2004; Esel et al., 2003; Weinstein et al., 1997). Community infections commonly associated with blood stream invasions include pneumonia, pyelonephritis, meningitis, associated with perforating injuries of abdominal and thoracic viscera and lesions that obstruct the gastrointestinal and genitourinary tract, patients with skin and soft tissue infections specially with diabetes mellitus, malnutrition and HIV infection, patients on corticosteroid or other immunosuppressive therapy are also at increased risk. On the other hand, 5-7% of all nosocomial infections results in bacteremia (Noskin and Phair, 1997).

In USA, nearly half of the cases of septicemia were reported to be nosocomial or hospital acquired (Weinstein et al., 1997). A study in Birmingham children's hospital, UK reported that 48.2% episodes were hospital acquired (Gray, 2004). The majority of episodes of bacteremia (73.4%) were found to be acquired in hospital in a study in a Turkish university hospital (Esel et al., 2003).The proportion of nosocomial blood stream infections has been increasing worldwide in recent years (Esel et al., 2003). The frequency of hospital-acquired bloodstream infections, their epidemiology and the invading microorganisms have changed in parallel with the evolution in medical care, particularly with the emergence of an increasingly ill and Immunocompromized population of hospitalized patients who are often heavily dependent on medical support and indwelling devices (Karchmer et al., 2000; Yinnon et al., 1997). Although a wide range of organisms can cause community-acquired bloodstream infections, three species, *Echerichia coli, staphylococcus aureus and Streptococcus pneumoniae* were responsible for majority of the cases (Laupland et al., 2006; Esel et al., 2003). Hospital-acquired bloodstream infections were more often due to coagulase negative Staphylococci, Enterobacteriaceae other than *E. coli, Pseudomonas,* other aerobic Gram negative bacilli and *C. albicans* (Raymond et al., 2006).

Sources of septicemia

Although almost any localized infection can disseminate to the blood stream, systemic studies on bacteremia have shown that the most primary foci are intravascular devices (catheters primarily), the respiratory tract, the genitourinary tract, various intra-abdominal sites, skin and soft tissue infection, bone and joint infections, surgical wound infection, central nervous system and iatrogenic sources (Weinstein et al., 1997; Reimer et al., 1997). Despite the availability of increasingly sophisticated imaging techniques, the sources for nearly one-forth of episodes could not be determined. The increased incidence of intravascular catheters as a source of bacteremia has paralleled the increased use of long-term central and peripheral lines for chemotherapy access and for parenteral nutrition. Concomitantly, "sticky" organisms of low virulence, such as coagulase negative Staphylococci, which are capable of adhering to the surfaces of catheter materials, have become more prominent as etiologic agents of bacteremia.

Table-I

Septicemia in previously healthy persons.

Site of origin	Usual pathogen(s)
Urinary tract	Aerobic Gram negative rods (90%). Aerobic Gram positive cocci (10%).
Skin	Gram-positive cocci.
Respiratory tract	*Streptococcus pneumoniae*
Abdominal: Gall bladder	Aerobic Gram negative rods. *Streptococcus faecalis*
Bowel perforation	Aerobic Gram negative rods.
Pelvic inflammatory disease	*Neisseria gonorrhoeae* Mixed anaerobes.

(Adapted from Finch et al., 2005; Murphy, 1988)

Table-II

Septicemia in hospitalized patients.

Clinical problem	Usual pathogen(s)
Urinary catheter	*E. coli, Klebsiella, Proteus, Serratia, Pseudomonas.*
Intravenous catheter	*S. aureus and S. epidermidis, Klebsiella, Pseudomonas, Candida albicans.*
Peritoneal catheter	*S. epidermidis.*
Post surgery: Wound infection	*S. aureus, E. coli, Pseudomonas,* anaerobes *(depending on site).*
Deep infection	Depends on anatomical location.
Burns	Gram positive cocci, *Pseudomonas, Candida albicans.*
Immunocompromized patients	Any of the above.

(Adapted from Finch et al., 2005)

Etiology

Almost any bacterium known to man can cause septicemia on occasion. All septicemias start some kind of infected focus. If that focus is obvious clinically, the septicemia is regarded as 'secondary'. Almost any organism may cause a secondary septicemia. Pneumococcus, following its usual path of nasopharyngeal colonization to pneumoniae to sepsis, is an example. The essence of secondary septicemia is that some circumstance allows the generation of large local population of organisms. From the local lesion, organisms are fed into the bloodstream.

Primary septicemias are those without a clinically obvious focus of infection. Of course, there always a portal of entry, but it may be subtle. Meningococcal nasopharyngitis, trivial staphylococcul folliculitis, or the intestinal ulcers, which develop in people of chemotherapy, are examples. Since there is no large local population, the organisms have to be capable of maintaining themselves in the bloodstream. In one kind of primary septicemia; high grade capsulated pathogens infect reasonably normal people. In the other kind, organisms of the low virulence invade persons whose defenses have been abrogated by disease or its treatment (Murphy, 1988).

The microbiology of bacteremia is constantly in evolution. Historically in the pre-antibiotic era, the pyogenic Gram positive cocci: *Streptococcus pneumoniae, S. aureus* and *Streptococcus pyogens* were the common causes of bacteremia. In the late 1950s nosocomial bacteremia due to Gram negative bacilli were increasingly recognized. The organisms most frequently isolated from blood cultures were: *Escherichia coli, Serratia marcescens, Proteus, Klebsiella, Enterobacter spp. and Pseudomonas aeruginosa.* In 1970's, a renewed interest in the anaerobic bacteriology resulted in the recognition of bacteremia due to bacteroids spp. since 1980's the Gram positive organisms including *S. aureus* (MRSA) and *S. epidermidis* and other coagulase negative Staphylococcus are being frequently isolated. Enterococci have emerged as an important nosocomial bloodstream pathogen with increasing resistance to antimicrobial agents (Noskin and Phair, 1997).

Table-III

Common etiological agents of bacteremia

Gram negative organisms	Gram positive organisms
Escherichia coli	*Staphylococcus aureus*
Klebsiella spp.	*Staphylococcus epidermidis*
Enterobacter spp.	α-haemolytic (Viridans) Streptococci
Proteus spp.	*Streptococcus pneumoniae*
Salmonella typhi.	*Enterococcus faecalis*
Salmonella spp. other than *S. typhi.*	*Streptococcus pyogens*
Pseudomonas aeruginosa	*Streptococcus agalactiae*
Neisseria meningitides	*Listeria monocytogenes*
Haemophilus influenzae	*Clostridium perfringens*
Bacteroids fragilis (anaerobe)	*Peptostreptococcus spp. (anaerobe.)*
Brucella spp.	
Burkholderia (Pseudomonas)	
Pseudomallei (in certain areas)	

(Adapted from Vandepitte et al., 2003)

The spectrum of microorganisms that invade the bloodstream has been systemically evaluated in three large studies in Australia and Germany in the time period 1983-1985, 1991-1992 and 2000-2001. *E. coli and S. aureus* together made almost 50% of all blood culture isolates and this overall picture was constant over the years. These studies revealed coagulase negative Staphylococcus (CONS) as third common cause of bacteremia. Other important interval changes are the proportionate increase in Enterococcal bacteremias (Erika et al., 2004). The ascendance of CONS in this group has created increased interpretative difficulties for clinicians, since the great majority of CONS (85%) continue to represent contamination rather than true bacteremia (Weinstein et al., 1997). In children, the microorganisms causing bacteremia are similar to those in adults, but there are several important differences. A paediatric list would be headed by Pneumococcus and *Haemophilus influenzae* and a neonatal one by Gram-negative rods and group B Streptococci.

Table-IV

Rank order of microorganisms in blood stream infections.

Rank	Total	Nosocomial	Community acquired
1.	*E. coli*	*S. aureus*	*E. coli*
2.	*S. aureus*	coagulase negative Staphylococci	*S. aureus*
3.	coagulase negative Staphylococci	*E. coli*	*Klebsiella* spp.
4.	*Klebsiella* spp.	*Enterococcus* spp.	*β-haemolytic streptococci*
5.	*β-haemolytic streptococci*	*Klebsiella* spp.	Viridans streptococci
6.	Polymicrobials	*Pseudomonas* spp.	*S. pneumoniae*
7.	Viridans streptococci	*Candida* spp.	Polymicrobials
8.	*S. pneumoniae*	Anaerobe	coagulase negative Staphylococci
9.	*Enterococcus* spp.	Polymicrobials	Anaerobe
10.	Anaerobe	*β-haemolytic streptococci*	*Enterococcus* spp.
11.	*Pseudomonas spp.*	Viridans streptococci	*Pseudomonas* spp.
12.	*Candida* spp.	*S. pneumoniae*	*Candida* spp.
13.	Others	Others	Others

(Adapted from Uslan et al., 2007)

Predisposing factors for bacteremia

The conditions that predispose an individual to bloodstream infection include not only age and underlying diseases but also medications and procedures whose primary purposes are maintenance of restoration of health. There is increased risk at the extremes of age; premature infants are especially at risk for bacteremia. A list of illness that are associated with an increased risk of bloodstream infection would include hematologic and non-hematologic malignancies;

diabetes mellitus; renal failure including dialysis; hepatic cirrhosis; immune deficiency syndromes and conditions associated with the loss of normal skin barriers such as serious burns and decubitus ulcers (Reimer et al., 1997). Therapeutic maneuvers associated with an increased risk of bacteremia include procedures as benign as placement of intravascular catheters as well as all surgery of all types but specially involving the bowel and genitourinary tract and endoscopic procedures of the genitourinary and lower gastrointestinal tracts. Finally, certain medications such as corticosteroids alter cell mediated immune function and increase of the bloodstream infection with obligate intracellular microorganisms, whereas others agents such as cytotoxic drugs used for chemotherapy predictably cause granulocytopenia and increase the risk for septicemia due to pyogenic bacteria and fungi.

Table-V

Conditions that may predispose to infections with positive blood cultures .

Microbial isolate	**Condition**
Gram-negative bacilli	Diabetes mellitus Lympho proliferative diseases Cirrhosis of liver Invasive procedures or devices Neutropenia Indwelling urinary catheter Diverticulitis, perforated viscus
Gram-positive bacteria	Intravascular catheters Indwelling mechanical devices Burns Neutropenia Infection with super antigen producing *Streptococcus pyogens*

Prognosis and outcome of septicemia

The outcome of bacteria depends on many factors; overall mortality has ranged in between 20 and 50% in large studies (Reimer et al., 1997). Data from different parts of the world revealed a considerable range of mortality rate. One Australian study demonstrated a 31.1% mortality rate, whereas a French registry revealed a mortality rate of 42% in severe sepsis. On the other hand, the mortality of severe sepsis was reported to be 55% by a Polish group and 54% mortality in severe sepsis and septic shock was reported in a Russian study (Tanriover et., al 2005). Various mortality figures due to neonatal sepsis have been reported from Asian and European centers ranging from 14 to 32% and 15 to 33% respectively (Movahedian et al., 2006).

Weinstein et al. 2003 found that several variables were independent predictors of increased mortality. Poor prognostic findings included: (Tanriover et., al 2005; Weinstein et al., 1997).

- Advanced age (>40 years).
- Nosocomial bacteremia.
- An Enterococcal, Gram negative or fungal etiology.
- Underlying disease conditions like cirrhosis of liver, malignancy, renal failure or the presence of multiple underlying problems.
- A primary focus in the respiratory tract, skin (including burns), surgical wound, abscess or an unknown source.
- Presence of septic shock.
- Lack of febrile response to shock.

Table-VI

High and low risk variables associated with death from bacteremia and fungemia (Weinstein et al., 1983).

	Category	
Variable	**High-risk**	**Low-risk**
Demographic:		
Age	>40 years	<40 years
Sex	Male	Female
Hospital service	Medicine, Surgery, Transplant	Obstetrics, Gynecology
Place of infection	Hospital (Nosocomial)	Community
Predisposing factors	Cirrhosis, hematologic and other malignancy, >predisposing factor	None, corticosteroids, trauma, surgery, renal failure, diabetes mellitus.
Source of infection	Respiratory tract, abscess, skin, surgical wound, unknown.	Genitourinary tract, iv catheter, central nervous system, foley catheter.
Clinical:		
Microorganism	Enterococci, Gram negative cocci, fungi	Gram-positive bacteria (except Enterococci)
Leucocyte count	<4,000/µl	≥ 4,000/ µl
Absolute Granulocyte count	<1000/ µl	≥ 1000/ µl
Hypotension	Present	Absent
Temperature	<38°C	≥38°C

Indirect evidences of bacterial infection by identifying serological markers

a) Cytokines: Bacterial cell wall constituents such as lipopolysaccharide (LPS), peptidoglycans, and lipoteichoic acid are particularly responsible for the deleterious effects of sepsis. An LPS-activated macrophage becomes metabolically active and produces intracellular stores of oxygen free radicals and other microbicidal agents and secretes inflammatory mediators like interleukins, TNF-α, gamma interferon, granulocyte macrophage colony stimulating factors (GN_CSF), platelet activating factors (PAF), chemokines, eicosanoids and anaphylatoxins. Measurement of these cytokines can be taken as fingerprints of sepsis.

b) Procalcitonin (PCT): PCT is relatively new marker that has been associated with inflammation and sepsis. The PCT plasma level in healthy individuals is low; usually bellow 0.1ng/ml. The level has been shown to rise with severity of sepsis.

c) C-reactive protein (CRP): The acute phase protein CRP is released from the liver in response to Proinflammatory cytokines and thought to recruit monocytes in early infection. Sensitivity and specificity of CRP levels in older children has been poor; however, in newborns CRP specificity average 90%.

d) Cell surface receptors (neutrophils CD64 and CD 11): Neutrophil CD64 expression quantitation provides improved diagnostic detection of infection/sepsis compared with the standard diagnostic tests used in current medical practice with a sensitivity of 87.9% and specificity of 71.2%. Another cell surface receptor CD11 is also found to be unregulated during infection.

e) **Immediate tests for detection of bacterial antigens, e.g.** Limulus Amoebocyte Lysate (LAL), Gas liquid chromatography (GLC) and Counter immunoelectrophoresis (CIE). These non culture methods are rapid but less sensitive.

Review of Blood culture

Indeed, because invasion of bloodstream represents one of the most important sequel of infection, blood cultures have become critically important and frequently performed tests in the clinical microbiology laboratories (Reimer et al., 1997). Although several other assays and methods are available to detect the evidence presence of bacteria in blood, the blood culture remains the standard method for detecting bacteremia (Connell et al., 2007). Since septicemia is thought of as a life threatening emergency, the culture of blood is mandatory in every case of suspected septicemia (Spencer, 1988).

Test description

In order to detect clinically significant bacteremia, a patient's blood must be sampled at one or more points in time and then incubated in culture media; bacterial growth is detected by various techniques, a specific organism(s) identified and antimicrobial susceptibility tested. A single sampling is termed a blood culture. Frequently several samples are collected, thereby constituting a blood culture series (Aronson, 1987).

Patients to whom blood cultures are appropriate

- Patients with symptoms/signs suggestive of microorganisms in blood i.e. fever, chills/rigor and tachycardia. (Chandrasekar and Brown, 1994).
- Patients with fever or hypotension not explained by non-infectious causes.
- In the absence of fever:
  - Patients with local infection, e.g. pneumoniae, meningitis and acute osteomyelitis.
  - Children or elderly with sudden failure to thrive.
  - Elderly patients with deterioration from base line status e.g. confusion, frequent falls.
  - Patients with renal insufficiency and unexplained leucocytosis or altered mentation.

- Immunocompromized patients looking ill or with unexplained pulmonary, renal or hepatic dysfunction.

- Patients receiving antibiotic therapy for documented bloodstream infection (blood cultures are needed to confirm clearance of microorganisms from blood).

Diagnostic strategies for blood cultures

A. Blood culture collection

i) Number of cultures

For adult patients, two or three blood cultures per septic episode should be obtained separated by intervals of approximately 1 hour or less if treatment cannot be delayed. This number is based on several factors. One studies from USA in 1983 had shown that more than 95% of episode of bacteremia were detected when two or three blood cultures were drawn. Second, routinely obtaining more than one blood culture per septic episodes has the added benefit of ensuring that adequate volumes of blood are cultured. Third, it helps physicians distinguish between clinically important and contaminated microorganisms, since the proportion of positive cultures is crucial in interpreting blood culture results. In children, raising the number of blood culture to two to three bottles does increase the yield (Vandepitte et al., 2003; Reimer et al., 1997; Collee et al., 1996).

ii) Timing of cultures

Whenever possible, blood culture should be taken before antibiotics are administered. A shower of bacteria into the blood precedes a temperature spike or chill approximately 30-60 minutes before the symptoms. Ideally a blood culture must be obtained about 1 hour before the onset of chill and fever but it is rarely possible. Hence, blood should be cultured as early as possible in the course of febrile episode.

iii.) Collection procedure

Contamination of blood culture at some stage of sampling or subsequent processing is not only a great waste of time and laboratory workload, moreover it may put the patient at risk. The skin of the patient or the operator is the most likely source of contamination. Whenever possible, blood should be collected by venipuncture of peripheral veins and not via indwelling vascular catheters. After a likely site of venipuncture has been selected, a tourniquet is applied to the limb and the vein is palpated. The tourniquet is then released and the site of venipuncture is then cleansed and disinfected with 70% alcohol followed by 2% iodine in a circular fashion for a final diameter of 5 to 6 cm. Since the antiseptic effect of iodine is time dependent, the easiest way to ensure adequate disinfection is to allow the solution to completely dry (usually 1 to 3 min.) before venipuncture. The tourniquet is then reapplied, the venipuncture is then made and blood is withdrawn. The preferred method is to use a 21-gauge needle with a syringe that will hold 5 to 10 ml more blood than is needed. Changing the sampling needle, replacing it with a sterile one and disinfection of the top of the blood culture container prior to inoculation of sampled blood will further reduce the chances of contamination.

B. Principles of laboratory detection

i) Volume of blood cultured

Culturing adequate volumes of blood improves microbial recovery for both adult and paediatric patients. Indeed, the volume of blood cultured is the most important variable in optimizing the microbial recovery for adult patients. This is because the number of microorganisms present in the blood in adults is small, typically fewer than 10 CFU/ml and less than 1 CFU/ml. For adult, each additional milliliter of blood for blood culture increases the recovery by upto 3%. Although the microorganisms present per milliliter of blood drawn from infant and small children is larger, typically greater than 100 CFU/ml and often greater than 1,000 CFU/ml, increasing the volume of cultured still increases microbial recovery. Therefore, it is important to collect reasonable quantity of blood: at least 10 ml per venipuncture for adults; 2-5 ml may suffice for children; for infants and neonates, 1-2 ml is often the most that can be obtained.

(ii) Ratio of blood to broth

Diluting blood into broth to ratios of greater than 1:5 increases microbial recovery, probably by diluting antimicrobial agents and natural inhibitory factors in the blood to sub inhibitory concentrations. Furthermore, diluting blood helps prevent clotting, one of the reasons that overfilling of blood culture bottles is discouraged. Maximum sensitivity was reported when the blood volume was 10-20% of the total medium volume.

iii) Media

Many media have been used for the isolation of aerobic organisms from the blood including nutrient broth, trypticase soy broth, Brain heart infusion broth, Columbia broth, thiol broth etc. Of all the media used three are most popular: Trypticase soy broth, Brain heart infusion broth, Columbia broth. Media used for anaerobes include cooked meat blood culture media and thioglycolate media.

iv) Anticoagulants

The use of 0.025 to 0.05% sodium polyanethol sulfonate (SPS) as an anticoagulant is recommended. SPS also inhibits lysozyme, inactivates clinically achievable concentration of some aminoglycoside and polymyxin antibiotics, inhibits part of the complement cascade and inhibits phagocytosis. SPS is not without its drawbacks; however, it has been shown to inhibit the growth of *Neisseria* spp. and Peptostreptococcus anaerobius. Supplementing the blood culture bottle with 1.2% gelatin partially counteracts the bacterial growth inhibitory effect of SPS. A similar compound, sodium amylosulfate (SAS) has been evaluated as a blood culture anticoagulant. But use of SAS results in lower recovery rate of Staphylococci and members of Bacteroidaceae. Other common anticoagulants such as Heparin, EDTA and sodium citrate should never be used in the broth blood culture media or to collect blood cultures (one notable exception being the Lysis-centrifugation method).

v) Antimicrobial agent removal

Various approaches are used to inactivate or neutralize the antimicrobials if already present in blood. Penicillinase or broad spectrum β lactamases may be added to the broth to inactivate penicillin; or components of thiol complex in the thiol broth can also inactivate penicillin. New approaches to antibiotic neutralization include devices containing antibiotic adsorbent resins (ARD, Marion Laboratories; BACTEC 16B, Johnston Laboratories) used with manual blood culture systems. Several manufacturers have marketed blood culture media containing antibiotic adsorbent resins. Such media include BacT/Alert FAN media (Organon Teknika). Another approach to neutralization on antibiotics is using lysis-centrifugation system, by which red and white blood cells are lysed, microorganisms are concentrated by centrifugation and the concentrate is cultured on media free of antibiotic. Studies of the lysis filtration and lysis centrifugation have demonstrated significantly increased and faster yields of bacteria and fungi from the blood of septicemia patients, including those receiving antimicrobial therapy at the time blood was obtained for culture.

vi) Bottle headspace atmosphere

For automated systems, most commercial blood culture bottles are manufactured with a carefully controlled atmosphere in the bottle headspace. Aerobic bottles generally contain ambient atmosphere to which different amounts of carbon di oxide have been added. Anaerobic bottles typically contain an atmosphere composed of carbon di oxide and nitrogen. Blood culture bottles manufactured for manual systems contain headspace atmospheres similar to those used with automated systems. One important difference is that aerobic bottles should be transiently vented to room air (to increase the oxygen content) prior to inoculation. In particular, *P. aeruginosa* and pathogenic yeasts are strictly aerobic and are unlikely to grow to detectable levels in blood culture bottles containing insufficient oxygen.

vii) Bottle agitation

Several clinical studies have documented that agitating bottles increase the microbial recovery from aerobic culture bottles. Agitation is thought to improve microbial recovery by increasing the oxygen concentration in the broth medium.

viii) Duration of incubation

Generally all significant aerobic and anaerobic isolates are detected in culture within seven days of incubation. However, in special instances incubation should be continued for 14 days, so that organisms such as candida, fastidious Gram negative rods, Hemophilus spp., Cardiabacterium and Brucella spp. might be isolated.

ix) Temperature of incubation

Once incubated, blood culture bottle should never be cooled below room temperature. Blood culture bottle can be temporarily held at room temperature without adversely affecting microbial recovery. Blood culture bottles (both conventional and automated systems) should be incubated at 35 to 37°C.

x) Examination of conventional blood culture bottles

Conventional blood culture bottles are examined macroscopically for growth twice for the first day and once daily thereafter. Since macroscopic detection of microbial growth becomes difficult after 2 to 3 days of incubation (most inoculated blood hemolyzes by then) and since macroscopic evidence of growth may be absent, bottles processed with manual blood culture systems should be incubated for 7 days with terminal subcultures. Growth is evidence by: a floccular deposit on the top of the blood layer, uniform or surface turbidity, hemolysis, coagulation of the broth, a surface pellicle, production of gas and white grains on the surface or deep in the blood layer. Regardless of the media used, the inspection of the bottle alone cannot be relied upon to detect all positive blood cultures; only 65% of positive cultures will be detected when bottles are only inspected for turbidity. Whenever visible growth appears, a smear should be prepared with a small amount of broth removed from the bottle aseptically and should be stained by Gram's method. Other staining procedures, such as acridine orange method, permit

detection of bacteria at 10^4 organisms/ml, which is ten fold more sensitive than Gram stain and is sensitive as blind sub culturing.

xi) Subcultures

In manual systems, blind subcultures are performed at an arbitrary time during the incubation cycle; usually at 24 to 48 hour after the culture was drawn and in the absence of macroscopic or other evidence of growth. Terminal subcultures are performed at the end of incubation period in the absence of objectives evidence of microbial growth. It is recommended that blood culture should be routinely sub cultured at 24 hours, 48 hours, 7 day and once before discarding at 14^{th} day if the culture appears negative. For automated blood culture systems, anaerobic blind subcultures are necessary.

Guidelines to improve blood culture yield

- Skin should be adequately disinfected prior to blood culture.
- Avoid singular blood culture sets; two or three sets (each set consist of aerobic and anaerobic bottles) should be obtained from different venipuncture sites in a 24 hours period.
- At least 10 ml. of blood should be drawn per culture bottle.
- Blood culture samples drawn from vascular catheters should be paired with a peripheral blood culture sample to help distinguish contaminants from true pathogens (Shafazand et al., 2002).
- Various culture media and collecting systems should be used in accordance with the clinical scenario and with the advice of microbiology laboratory.
- Whenever possible, blood should be drawn prior to initiation of antibiotics. In the presence of antibiotics, drawing culture samples when antibiotic concentrations have reached trough levels may improve yield.

True bacteria vs. contaminated blood cultures

All positive blood cultures are not clinically significant even in ideal conditions of sampling and processing of blood. 3-5% of blood cultures 'grow contaminants' originating from skin (*S.*

epidermidis, Propionibacterium acnes, Clostridium spp. diptheroids) or from environment (*Acinetobacter* spp., Bacillus spp.). Such organisms, however, may occasionally behave as pathogens and even cause endocarditis. Contamination of blood culture can be avoided by meticulous skin preparation and by adherence to strict aseptic procedures for inoculation and sub inoculation (Vandepitte et al., 2003; Shafazand et al., 2002).

While there are no standardized criteria, several observations can help guide the clinician in the determining the clinical significance of positive blood culture results:

Suggestive of true bacteremia:

- If the same organism grows in two bottles of the same blood specimen.
- If the same organism grows in cultures from more than one specimen.
- If the growth is rapid (within 48 hours).
- If different isolates of one species show the same biotypes and antimicrobial susceptibility profiles.

Suggestive of contaminated culture:

- Prolonged incubation period before growth of organism.
- Lack of reproducibility in subsequent cultures.
- Culture yields multiple organisms except in immunocompromised and neutropenic patients or in presence of intra-abdominal infections.
- Patient's clinical condition is not suggestive of sepsis.
- Growth of skin flora, e.g. Coagulase negative Staphylococci, Diptheroids and Bacillus species.

All culture results should be reported to the clinician, including the presumed contaminants. However, for the later no antibiogram need be performed and appropriate mention should be made on the report slip, e.g. *Propionibacterium acnes (skin commensal), Staphylococcus epidermidis* (probable contaminant). It is to the advantage of all concerned to establish good communication between physicians and laboratory personnel.

Blood culture systems

A. Manual detection system

(i) Conventional or traditional system

The simplest blood culture system consists of bottles filled with broth medium and with a partial vacuum in the headspace. Broth (50ml) for blood culture is generally dispensed in a special flat or round bottle of about 100-120ml capacity and fitted with a screw cap with a central hole giving access to a rubber washer seal. The bottle cap and the exposed part of the washer are protected by foil, which is removed immediately before the blood is inoculated by inserting the syringe needle through the washer. Tryptic or trypticase soy, supplemented peptone, brain-heart infusion, Columbia or Brucella broth is used for recovery of aerobes and facultative anaerobes; thioglycolate or anaerobic heart infusion broth is used for isolation of anaerobes. An adequate space above the broth ensures that the blood is not injected under undue pressure and that some air is still available for strict aerobes. To convert such bottles to aerobic bottles, the oxygen concentration is increased by transiently venting bottles to room air after they have been inoculated with blood. Unvented bottles remain relatively anaerobic. After inoculation, the bottles are incubated and are examined daily for seven days for macroscopic evidence of growth. Such evidence consists of haemolysis, turbidity, gas production, "Chocolatization" of the blood and the presence of visible colonies or a layer of growth on the fluid meniscus or a combination of these. Blind or terminal subculture is needed to reliably recover pathogenic microorganisms from this type of bottles. These systems have the advantage of being inexpensive with regard to reagents but they are relatively labor-intensive (Reimer et al., 1997; Collee and Marr, 1996)

(ii) Derivatives of "Castenada bottles

This manual blood culture system consists of a biphasic medium blood culture bottle containing broth to which an agar paddle is attached. Subcultures can easily and repeatedly perform by tipping the bottle and allowing the blood broth mixture to flow over the agar slant. Commercial versions include Septi-Chek and Opticult (Becton-Dickinson). These bottles have the advantages of not requiring venting, inoculating subcultures automatically, providing an alternative means of detecting microbial growth via the agar media and providing early isolated colonies from which

final identification and susceptibility procedures can be performed. However, it is less practical and time consuming.

(iii) Oxoid signal blood culture system

This manual blood culture system consists of an aerobic blood culture bottle only. After the bottle is inoculated, a "signal" device, which consists of a long needle in a plastic sleeve is attached to the bottle. As the microorganisms grow and liberate gasses into the headspace atmosphere, the pressure in the bottle increases, forcing the blood-broth mixture upwards through the needle into the reservoir at the top of the plastic sleeve. Hence, in addition to other types of macroscopic evidence of growth, signal utilizes a manometric method. However, it will detect non-gas producing organisms.

(iv) Concentration technique

(a) Lysis filtration:

Formed elements of blood are lysed by a lytic agent and the lysed blood is passed through a membrane filter. The filter is then placed on the surface of the agar plate. Isolated colonies are available after 24 to 48 hours of incubation. Membrane filtration system, however, remains cumbersome, expensive and time consuming. There is no commercially available system based on this technique.

(b) Lysis centrifugation system

A different type of manual system one that is not broth based, is lysis centrifugation. Commercially available version is the Isolator (Wampole laboratories, Cranbury, N.J.). Commercially Lysis centrifugation system involves a tube containing saponin that lyse blood cells, SPS, which prevents coagulation and polypropylene glycol, a foam retarding agent. Once blood is drawn into the tube, rapid lysis of red and white cells occurs, which allows the recovery of viable phagocytised microorganisms. The contents of the tube are then centrifuged to concentrate any organisms that might be present. After centrifugation, the supernatant is discarded and the centrifuged material is cultured on antibiotic free solid media. Lysis

centrifugation system is superior to conventional broth or biphasic broth cultures in terms of speed of detection of positive culture. Positive cultures with lysis centrifugation system occur as colonies on agar plates and therefore this system offers potential advantages in the time required to identify and determine antimicrobial susceptibility of the isolated organisms. A significant disadvantage of this system is high contamination rate and it is a labor-intensive method.

The widespread use of automated blood culture systems has not totally eliminated manual systems, since these systems are simple to use, require no instrumentation other than an incubator and are the least expensive method to use.

(B) Automated detection systems

For rapid detection of growth of bacteria and to make blood culture processing more efficient, several manufacturers have developed and marketed a number of automated blood culture systems. These systems employ equipment that automatically detects early sign of bacterial growth in a special blood culture bottle such as a change in electrical impedance or release of radioactive CO_2 or non radioactive gas.

One such system is based on the colorimetric detection of CO_2 produced during microbial growth. A CO_2 sensor is bonded to the bottom of each blood culture bottle and is separated from the broth medium by a membrane that is impermeable to most ions and to components of the media and blood but freely permeable to CO_2. Inoculated bottles are placed in cells in the instrument, which provide continuous rocking of both aerobic and anaerobic bottles. If bacteria are present, they generate CO_2, which is released into the broth medium; the pH then decreases, causing the sensor to change the color from green to yellow. Color changes are monitored once every 10 minutes by colorimetric detector.

A second continuous monitoring system is based on fluorescent technology. Bonded to the base of each vial is a CO_2 sensor that is impermeable to ions, medium components and blood but freely permeable to CO_2. CO_2 generated by microorganisms decreases the pH of the medium. The subsequent decrease in pH increase the fluorescence output of the sensor, changing the

signal transmitted to the optical and electronic component of the instrument. The computer generates growth curves and data are analyzed according to growth algorithms.

A third system detects growths of organisms in broth by measuring gas consumption and / or gas production. Each inoculated vial is fitted with a disposable connector that contains a recessed needle. The needle penetrates the bottle stopper and connects the bottle headspace to the sensor probe. The sensor monitors the changes within the headspace in the consumption and / or production of all gases (CO_2, N_2 and H_2) by growing organisms and creates data points internally in the computer.

Commercial automated blood culture systems currently in use with their manufacturers, principles of detection of microbial growth and monitoring systems are listed in table-VII.

Table-VII

Commercial automated blood culture systems.

System	Manufacturer	Microbial detection	growth	Continuous monitoring
BACTEC 460	Becton-Dickinson	Radiometric detection	CO_2	No
BACTEC 660/730/860	Becton-Dickinson	Infra-red detection	CO_2	No
Bact/Alert	Organon Teknika	Colorimetric detection	CO_2	Yes
BACTEC 9240	Becton-Dickinson	Fluorescent detection	CO_2	Yes
Vital	Bio Merieux Vitek	Fluorescent detection	CO_2	Yes
ESP	Difco	Manometric		Yes
Bio Argos	Diagnostic Pasteur	Infra-red detection	CO_2	No
o.a.s.i.s.	(Oxoid Unipath automated septicemia investigation system)	Manometric		Yes

Advantages of the most recent continuous monitoring blood culture systems are many folds – earlier detection of the microbial growth, uses 10 ml standard blood inocula, decreased laboratory workload i.e. blind and terminal subculture are not necessary, no need for radioactive waste disposal (which was the hazard of earlier BACTEC series), no need for separate incubator, agitator or gas supply, no possibilities of pseudobacteremia and decreased false positive instrument signals. But the main disadvantages are the cost both capital and maintenance, limited medium types with some systems and the size of certain instruments (Reimer et al., 1997).

C-reactive protein (CRP)

Historical background

C-reactive protein (CRP) was first discovered in 1930 by Tillet and Francis at Rockefeller institute of medical research, who identified a substance in the sera of patients acutely infected with Pneumococcal pneumoniae that formed a precipitate when combined with polysaccharide C of *Streptococcus pneumoniae*. Subsequently it was found that this reaction was not unique to pneumococcal pnuemoniae but could be found with a variety of other acute infections (Ablij et al., 2002; Clyne et al., 1999). In 1933, Rachel Welsh found strongly positive precipitation tests with gram negative microorganisms. Avery and Macleod in1941 characterized the C- reactive material as a protein which required calcium ion for its reaction with C-polysaccharide and introduce term 'acute phase' to refer to serum from patients of acutely ill with infectious disease and containing the C-reactive protein (CRP).

Loofstrom, 1944 independently described a non-specific capsular swelling reaction of some strains of Pneumococci when mixed with any inflamed sera and subsequently showed that substance responsible was CRP. He detected CRP in non-infectious as well as infectious conditions and a acute phase reaction, in which the concentration of plasma protein increases is now recognized as general and non infective inflammatory processes, cellular and / or tissue necrosis and malignant neoplasm (Pepys 1981).

CRP – Structure and binding sites

It is a pentameric protein consisting of five noncovalently bonded identical subunits (protomers). Each subunit consists of 206 amino acids in a single polypeptide chain with a molecular weight 23000Da. The two cysteine residues at positions 36 and 78 are involved in a disulfide bond. Each subunit has the ability to bind to two calcium ions so that the calcium dependent specific binding of a ligand is possible. The most avid ligand is phosphocholine (PCH), a constituent of the phospholipids of cell membranes and plasma lipoproteins. Phosphocholine is universal for most

eukaryotic organisms. Fraction C of cell wall also contains Phosphocholines. Other ligands are nuclear constituents: histones, chromatin and small nuclear ribonucleoproteins (snRNPs). Therefore, CRP plays an important role in the clearance and processing of nuclear antigens, thus preventing autoimmune responses to nuclear material. A calcium independent binding exists for cationic polymers (lysine and arginine-rich proteins). The role of this binding property may be that it has modulatory effects on the inflammatory process because most polycations are secreted by neutrophils (Volanakis, 2001).

Biosynthesis and kinetics of CRP

The major site of CRP synthesis is the hepatocyte. Under physiological circumstances, human CRP is a protein with a median serum concentration of 0.8 mg/l. the human CRP gene is located on the long arm of chromosome1. Plasma CRP is mainly regulated at the transcriptional level induced by IL-6. In vitro CRP mRNA transcription is dramatically up regulated by IL-6. This response is greatly enhanced IL-1β. This synergistic phenomenon occurs due to the regulation of CRP synthesis at the transitional level by IL-1β. After transcription, CRP mRNA is translated to protomers. In the endoplasmic reticulum five protomers are assembled to one cyclic pentamer which is either secreted or stored. (Marnell et al., 2005; Ablij et al., 2002).

CRP – An acute phase reactants

Acute phase reactants are complex multifunctional group of substances that comprised of complement components, coagulation proteins, protease inhibitors, metal binding proteins, CRP and others whose plasma concentration increases (positive acute-phase proteins) or decreases (negative acute-phase proteins) in response to tissue injury or inflammation. The magnitude of the increase varies from about 50% in case of ceruloplasmin and several complement components to as much as 1000-fold in case of C-reactive protein and serum amyloid-A. The biggest concentration of CRP has been reported in patient with acute bacterial infection (James, 1990).

C-reactive protein (CRP) has long been recognized as a key player in the innate response to pathogenic infection and has been suggested to play a possible role in linking innate and acquired immune responses.

CRP – response and half life

After the onset of inflammation or acute tissue injury, CRP synthesis increases within 4 to 6 hours, doubling every 8 hours and peak at 36 to 50 hours (Pepys, 1981, Steel 1994). The plasma level is as much as 0.4gm/L. within 24-48 hours. CRP levels remain elevated with ongoing inflammation and tissue destruction but with resolution it declines rapidly, because of relatively short half life of 17 hours (Young et al., 1991; Pepys and Baltz, 1983).

Prognostic value

CRP is increased during active inflammation and tissue injury and dropped rapidly as the inflammation subsides. Most acute phase proteins have half lives of 2-4 days but CRP has a half life of 17 hours. For this reason, CRP has both diagnostic and prognostic value. It is also a sensitive indicator for monitoring the efficacy of antimicrobial therapy (BenGershom et al., 1986).

Laboratory methods for measuring CRP

Methods for measuring CRP were originally based on the ability of the serum to precipitate the C-fraction polysaccharide (Quellung reaction) followed in later years by semi quantitative latex agglutination assays. After biochemical characterization of CRP in 1978 and development of monoclonal antibody a wide array of sensitive, quantitative immunological methods for measurement evolved.

Presently, accurate and rapid quantitative measures of CRP are obtained using laser nephelometry, rate immunonephelometry or turbidimetry and enzyme immunoassay (Clyne et al., 1999). Nephelometry measures light scattering from aggregates of CRP with specific antibodies

providing accurate and reproducible results within 15-30 minutes with an analytical sensitivity of 0.04mg/L (Young et al., 1991). A laser nephelometer makes it possible to measure CRP concentration in a short time and it requires only small amount of serum or CSF (approximately 20µl), with this equipment repeated measurement of CRP can be performed easily (Kawamura et al., 1995).

Chapter-4
Materials & Methodology

MATERIALS AND METHODOLOGY

Study population and sample size

In this study, sample size were in total 273, among which 233 were clinically suspected septicemia cases and 40 were healthy controls in age group 0 day to 15 years. These samples were taken consecutively.

Place of study

The study was carried out in United Hospital Ltd. (UHL), Gulshan, Dhaka and the department of Biochemistry & Molecular Biology, Rajshahi University during the period of January 2010 to December 2010. The patients were selected from the department of paediatrics unit of United Hospital Ltd.

Selection of patients and controls

A) Case

Patients were selected according to the following clinical features and laboratory findings as follows:

Temperature	: > 38 degree C or	< 36 degree C
Tachypnoea	: Respiratory rate	> 40/min. in children
Tachycardia	: Heart rate	> normal limit for the age of child.
Leukocyte count	: Leukocytosis	> 15,000/cmm
	Leukopenia	< 4000/cmm

At least two of the above four conditions with one or more of the followings, with or without localized site of infection will be considered as suspected septicemia case.

- Fever with chills and rigor
- Convulsion
- Vomiting
- Abdominal distension.
- Poor feeding
- Skin septic spots
- Jaundice
- Hypotension

- Hepato-splenomegaly

Control

Children of paediatrics unit who don't have any features of septicemia.

Exclusion criteria

- Neonatal asphyxia
- Haemorrhagic disease
- Congenital malformation
- Those parents who are not interested to take part in this study.

Study patients

The study patients were grouped (age range 0-15 years):

A. According to clinical features, Blood culture result and CRP concentration in blood (Bont et al., 1994).

1. Patients with clinical suspicion of sepsis, positive blood culture and CRP concentration > 6 mg/l were included in culture proven group.
2. Patients with clinical suspicion of sepsis but without bacteriological confirmation and CRP>6 mg/l were included in probable septicemia group.
3. Patients with clinical suspicion of sepsis but without bacteriological confirmation and CRP<6 mg/l were included in non-septicemia febrile group.
4. The control group comprised age and sex matched children without history of fever or any other inflammatory and immunological illness for last 3 months.

B. According to antibiotic therapy

1. Patients with anti-microbial therapy

Patients who were receiving antimicrobial agents within 48 hours of taking blood samples for culture.

2. Patients without anti-microbial therapy

Who did not receive antimicrobial agents within 48 hours of taking blood sample.

Samples

Specimen was only blood. Blood sample was taken from the patients.

Methods of collection of blood samples

In all cases the parents were informed and their written consent was taken before collection of sample.

A single sample of venous blood (Anticubital/Femoral/scalp vein, preferably anticubital) was collected from each patient with sterile disposable syringe and needle after disinfection of the selected venipuncture site with 70% ethyl alcohol in a expanding circular scrubs from the center to the periphery of the needle insertion site followed by 2% tincture of iodine which was allowed to dry for one minute (Collee and Marr, 1996; Chandrasekar & Brown, 1994; and Chessbrough, 1985). If the first attempt at drawing blood was unsuccessful, a new needle set was used for each successful attempt. At least 4 ml of blood from each neonate and 6-10 ml from other paediatric patients were collected from single venipuncture. After removing the syringe and needle from venipuncture site, the sampling needle was discarded and replaced by fresh sterile needle. The top of the rubber stopper of the blood culture receptacle was disinfected with 70% ethyl alcohol and 3 ml of blood was injected into the blood vial containing resin for neonates and 6-8ml for paediatrict. The remaining blood was collected in a clean test tube for estimation of CRP level. Immediate after introduction of blood, the blood cultures vials were agitated gently to mix the blood with the resin. These procedures were performed at the bedside of patient.

The blood culture vials and the test tubes with blood were then transported immediately to the laboratory in the department of Microbiology for culture and test tube to the department of Biochemistry for estimation of CRP level. All the blood culture were done aerobically and few were anaerobically at 37 degree celsius.

Laboratory procedure

For serum analysis specimens were processed as soon as they were transported to the laboratory.

Serum separation

2-3 ml of blood in at test tube were centrifuged at 4,000 rpm for ten minutes. Serum was taken by using micropipette for estimation of CRP concentration.

Blood culture Method

In this study, Blood culture was analyzed by the instrument BACTEC 9120 series which is automated system following FAN (Fastidious antibiotic neutralization) method. This fluorescent instrument is designed for the rapid detection of bacteria and fungi in clinical blood cultures. Samples were drawn from patients and directly injected into the BACTEC blood culture vials at the site of collection. The blood culture vials are incubated inside the instrument at 37oC and are agitated for maximum recovery of organisms automatically by the instrument at particular interval.

When microorganisms are present, they metabolize nutrients in the culture medium, releasing carbon dioxide into the medium. A dye in the sensor reacts with CO2. This modulates the amount of light that is absorbed by a fluorescent in the sensor. The instrument's photo detectors measure the level of fluorescence, which corresponds to the amount of CO2 released by organisms. Positive cultures are immediately flagged by an indicator on the front of the instrument and displayed on the monitor. Then positive vials pull them from the instrument and a sterile syringe is used to collect blood from the vials. One drop of blood is poured on each of Blood agar media and MacConKey agar media then to inoculate it on the media and put them into the incubator at 35-37oC. for 18-24 hours for confirmation of results and for isolation and identification of the organism.

Culture media used in the study

1. Blood agar media (BA)
2. MacConKey agar media (MA)
3. Muellar Hinton agar media (MHA)
4. Chocolate agar media (CA)
5. Saboroid Dextrose agar media (SDA)

Preparation and composition of the media are given in appendix-B.

Identification of the organism

The organisms were identified by their colony morphology, staining characters, pigment production, motility and other relevant biochemical tests as per standard methods (Sonnenwirth and Jarret 1980; Collee et al., 1996). Appendix-C.

Antibiotic susceptibility tests

All the isolates obtained by culture were tested for antimicrobial susceptibility by disc diffusion method against different antimicrobial agents (Bauer et al., 1966).

Antimicrobials used for susceptibility tests

Isolated Gram negative bacteria were tested against Amikacin (AK), Amoxyclavonic acid (AMC), Ceftriaxone (CRO), Ceftazidime (CAZ), Co-trimoxazole (SXT), Ciprofloxacin (CIP), Cefepime (FEP), Cefixime (CFM), Gentamicin (CN), Netilmicin (NET) and Imipenem (IMP). Salmonella spp. was tested against Ceftriaxone (CRO), Co-trimoxazole (SXT), Ciprofloxacin (CIP), Cefepime (FEP), Cefixime (CFM), Chloramphenicol (C), Azithromycin (AZM) and Ampicillin (AMP). For Pseudomonas spp. Piperacillin/Tazobactam (TZP) were added alone with above mentioned discs. Gram positive bacteria were tested against Ampicillin (AMP), Co-trimoxazole (SXT), Ciprofloxacin (CIP), Cefepime (FEP), Cefixime (CFM), Penecillin-G (P), Doxycycline (DO), Lynezolid (LZD), Oxacillin (OX) and Vancomycin (VA).

Antibiotic disc

All the discs used in this study were Oxoid Ltd, UK which is commercially available.

Preservation of the discs

All the discs were kept at 2-8^{o}C. Prior to use, the containers were left at room temperature for about half an hour to minimize condensation resulting from the warm air reaching the cold container. The concentrations of the antimicrobial agent of each disc are given in the Table-VIII.

Quality Control

The discs from each batch were standardized by testing against reference strains of *E. coli* ATCC 25922, *staph. aureus* 25923 and zones of inhibition were tested with standard value (Table-VIII).

Table-VIII

Diameter of clear zone produced by control strains against standard antibiotics.

Antimicrobial agents	**Disc potency µg/disc**	**Diameter of Zone of inhibition (mm)**	
		S. aureus (ATCC 25923)	*E. coli* (ATCC 29522)
Amoxycillin/Clavulanic acid	30		
Penicillin G	10 IU	26-37	-
Ampicillin	10	27-35	16-22
Ceftriaxone	30	22-28	29-35
Ceftazidime	30	16-20	25-32
Ciprofloxacin	5	22-30	30-40
Gentamicin	10	19-27	19-26
Chloramphenicol	30	19-26	21-27
Co-trimoxazole	25	24-32	24-32
Amikacin	30	20-26	19-26
Imipenem	10	-	26-32
Vancomycin	30	17-21	-
Oxacillin	1	18-24	-
Doxycycline	30	23-29	18-24
Azithromycin	15	21-26	-
Cefixime	5	-	23-27
Cefepime	30	23-29	31-37
Linezolid	30	25-32	-

(Adapted from NCCLS, 2001)

Media for antibiotic susceptibility test

Muellar-Hinton agar media were used for antimicrobial susceptibility testing for all the isolated bacteria (Bauer et al., 1966).

Drug Sensitivity test

A loopful of confluent growth of the test organisms was taken with a sterile wire loop from a pure culture in a tube containing a sterile normal saline (Vandepitte et al., 1991). Turbidity of the organisms in the tube was adjusted by adding more bacteria or more saline to a turbidity equivalent to that of one half of McFarland standard (Appendix-E) which approximately corresponds to 1.5×10^8 organisms/ml (Sonnenwirth and Jarret, 1980). Within 15 minutes after standardization of inoculums, a sterile cotton swab was immersed into the bacterial suspension. The excess suspension was removed by rotating the swab with firm pressure against the inner side of the tube above the fluid level. The plates were dried in an incubator at 37°C for 30 minutes before use. The swab was then streaked evenly on the surface of freshly prepared media in three different planes (by rotating the plate 60°each time) to get a uniform distribution of inoculums. The plates were left at room temperature for 10-15 minutes with lid closed to allow the inoculums to dry. The antimicrobial sensitivity discs were then placed on the inoculated surface by sterile fine forceps 15 mm away from the edge of the Petridish and having 20-25 mm gap between the discs. The plates were then inverted and incubated at 37°C for 18-24 hours.

Reading of the sensitivity test

Each plate was examined after overnight incubation (18-24 hours) and diameter of the complete zone of inhibition were measured in mm with the help of scale placed on the under surface of the petridish without opening the lid. Zone of inhibition was measured in two directions at right angles to each other through the center of the disc and average of the two reading was taken (Washington, 1985).

Biochemical Test

- **Principle of the test**

Microgen™ GnA + B-ID system was used to identification Enterobacteriaceae and an extensive range of oxidase-positive Gram negative Bacilli. This system employs 12

standardised biochemical substrates in microwells to identify the Enterobacteriaceae which had been selected on the basis of computer analysis. The dehydrated substrates in each well are reconstituted with a saline suspension of the organism to be identified. When individual substrate is metabolized, a color change occurs during incubation or after addition reagents. The permutation of metabolized substrates can be interpreted using the Microgen identification system software to identify the test organism.

- **Inoculation and Incubation**

Emulsify a single colony from an 18-24 hour culture in 3 ml sterile 0.85% normal saline for the GN A micro well test strip. Carefully peel back the adhesive tape sealing the microwell test strip. Using a sterile Pasteur pipette, add 3-4 drops of the bacterial suspension to each well of the strip. After inoculation, overlay well 1, 2, 3 and 9 (GN A strip) with 3-4 drops of mineral oil. Seal the top of the microwell test strip with adhesive tape and incubated 35-37oC. Test strip are read after 18-24 hours incubation for organism.

Procedure

Record all positive reactants with the aid of the color chart and record the results of the form provided. Add 2 drops of Kovac's reagent to well 8. Red color indicates positive result and records it after 60 seconds. Add 1 drop of VP 1 reagent and 1 drop of VP 2 reagent to well 10. Pink/red color indicates positive result and record it after 15-30 minutes. Add one drop of TDA reagent to well 12. Cherry red color indicates positive result and records it after 60 seconds. The sum of the positive reactions for each triplet forms a single digit of the octal code that is used to identify the isolate through Microgen Identification system software.

Interpretation of the zone size

Zone of inhibition produced by each drug were considered into 3 susceptibility categories namely sensitive (S), Intermediate sensitive (IS) and resistant (R) according to NCCLS, guide line, 2001 (Table-IX).

Table-IX

Interpretation chart of zone sizes for determination of antimicrobial susceptibility by disc diffusion method. (NCCLS, 2001).

Antimicrobial agents	**Disc potency (µg/disc)**	**Diameter of clear zone of inhibition (mm)**		
		Resistant (R)	Intermediate (I)	Sensitive (S)
Penicillin G	10 IU	≤20	21-28	≥29
Ampicillin	10			
For Gram negative enterics		≤13	14-16	≥17
Staphylococcus		≤28	-	≥29
Co-trimoxazole	25	≤10	11-15	≥16
Ceftriaxone	30	≤13	14-20	≥21
Ceftazidime	30	≤ 14	15-17	≥18
Cloxacillin	5	≤9	10-13	≥14
Gentamicin	10	≤12	13-14	≥15
Chloramphenicol	30	≤12	13-17	≥18
Ciprofloxacin	5	≤15	16-20	≥21
Amikacin	30	≤14	15-16	≥17
Imipenem	10	≤13	14-15	≥16
Vancomycin	30	-	-	≥15
Oxacillin	1	≤10	11-12	≥13
Azithromycin		≤13	14-17	≥18
Lynezolid	30	-	-	≥21
Amoxycillin/ Clavulanic acid	30	≤19	-	≥20
Doxycycline	30	≤12	13-15	≥16
Cefepime	30	≤14	15-17	≥18
Cefixime	5	≤15	16-18	≥19

Estimation of CRP

Both qualitative and Quantitative methods were used to determine the level of CRP.

Qualitative estimation of CRP level

Principle

The CRP test is based on the serological reaction between human C-reactive protein (CRP) of a patient specimen and corresponding anti-human CRP antibodies bound to latex particles. The positive reaction is indicated by a distinctly visible agglutination of the latex particles in the test cell of the slide.

Kit components

Omega Diagnostics Ltd., United Kingdom.

1. CRP latex reagent
2. Control serum positive
3. Control serum positive
4. Buffer
5. Stirrers
6. Pipette
7. Agglutination slide.

Test procedure

Eppendorp tube was used for Qualitative detection of CRP for each sample.

1. Kit reagent was allowed to come at room temperature.
2. One drop (50 µl) of patient's serum was transferred to the test circle on the slide.
3. Mixed the drops using the disposable stirrer ensuring coverage of the test circle with the mixture.
4. The slide was placed on an automated rotator at 100 r.p.m. for 2 minutes and observed for agglutination.

Interpretation of results

Positive results: Positive result was indicated by the generating of an agglutinated pattern within 2 minutes of mixing the latex with the test sample (serum), showing clearly visible clumping of latex particles.

Negative results: The latex did not agglutinate and the milky appearance remains substantially unchanged throughout the test.

Quality control: (visual inspection) The latex suspension were always inspected for aggregation as they were dropped into the test card and if there was evidence of clumping before addition of the test sample, the suspension was not used.

Positive control procedure

Step 1 – Placed one drop of test latex in one circle in a reaction card.

Step 2 – Dispensed 1 drop of positive control.

Step 3 – Contents were mixed using a mixing stick and discarded it for safe disposal.

Step 4 – The card was rocked manually for 2 minutes. After 2 minutes, definite agglutination was visible in the latex.

Negative control procedure

Step 1 – Placed one drop of test latex in one circle in a reaction card.

Step 2 – Dispensed 1 drop of positive control.

Step 3 – Contents were mixed using a mixing stick and discarded it for safe disposal.

Step 4 – The card was rocked manually for 2 minutes. After 2 minutes, there was no agglutination.

Quantitative estimation of CRP level

CRP was measured by the auto biochemical analyzer former name **OLYMPUS AU 640** (presently ***BECKMAN COULTER*).**

Contents, Reagent Composition

1. Tris buffer (pH 7.5) : 80 mmol/L
2. Sodium chloride : 125 mmol/L

3. Polyethylene glycol 6000 : 1.5% w/v
4. Goat anti-CRP antibodies : 0.6 g/L
5. Preservative

Reagent 1 & 2 is togetherly called reagent R_1 and
Reagent 3 & 4 is togetherly called reagent R_2.

Principle

When a sample is mixed with R1 buffer and R2 antiserum solution, CRP reacts specifically with anti-human CRP antibodies to yield insoluble aggregates. The absorbance of these aggregates is proportional to the concentration in the sample.

Reagent preparation: The reagents are ready to use and can be placed directly on board the instrument.

Storage and stability: The reagents are stable, unopened up to the stated expiry date when stored at 2-8 degree celsius. Once open, reagents stored on board the instrument are stable for 90 days.

Specimen: Serum and heparinised plasma: Stable in serum and plasma for 2 months when stored at 2-8 degree celsius and 11 days when stored at 15-25 degree celsius.

Calibration: Olympus serum protein Multi-calibrator Cat no. 0DR3021

Quality control: Olympus ITA Control sera ODC0014, ODCOO15, ODCOO16 by this Olympus system may be used.

Data and statistical analysis

All the data collected were compiled and analyzed in a tabulated form (appendix – A). Statistical analysis was performed by SPSS for windows 12.0

Chapter-5
Results

RESULTS

This study covered a total number of 233 diagnosed septicemia cases in children out of which 123 were male and 110 were female. Mean age was 3.84 ± 4.36 years. The highest number 71 (30.47%) of patients were 0 to 1 month of age followed by 47 (20.60%) from 1 month to 1 year, 44 (18.88%) from 1 year to 5 years of age group. Then 5 years to 10 years and 10 years to 15 years age group, there were 40 (17.17%) and 30 (12.88%) patients respectively in each group (Table-X).

The highest rate of blood culture positively 10 (25%) were found among patients of 5 years to <10 years age group followed by 9 (30%) in 10 years to <15 years age group. On the other hand, lowest rate of blood culture positivity 5 (10.42%) were found between 1 month to <1 year age group (Figure-2).

Table XI shows that out of 233 patients, blood culture were positive in 39 (16.74%) study cases. Out of 123 male patients, blood culture were positive in 22 (17.89%) and out of 110 female patient's blood culture were positive in 17 (15.45%) patients. No significant difference was observed in between sex groups ($P>0.05$).

A total 39 strains of bacteria were isolated. Among them, *S. typhi* 16 (41.03%) was the most common predominantly isolated bacteria followed by *S. paratyphi* A 7 (17.95%). *K. pneumoniae* and *A. baumannii* there were same 4 (10.26%) in each bacteria. Furthermore, *Pseudomonas* spp. 3 (7.69%), *S. marcescens* 2 (5.13%). *S. aureus* 1 (2.56%), *E.coli* 1 (2.56%) and *Enterococcus* spp. 1 (2.56%) were isolated. (Figure-3).

Table-X

Distribution of study population according to age and sex. (n=233)

Age group	Male	Female	Total
0 day – 1 month	38	33	71 (30.47%)
1 month – <1 year.	27	21	48 (20.60%)
1 year – <5 years.	21	23	44 (18.88%)
5 years - < 10 yrs.	22	18	40 (17.17%)
10 years - <15 years.	15	15	30 (12.88%)
Total	123 (52.79%)	110 (47.21%)	233 (100%)

Figures within parentheses indicate percentages.

(Mean age = 3.84 ± 4.36 years.)

Figure-2:

Blood culture positivity among different age groups of children. (n= 233)

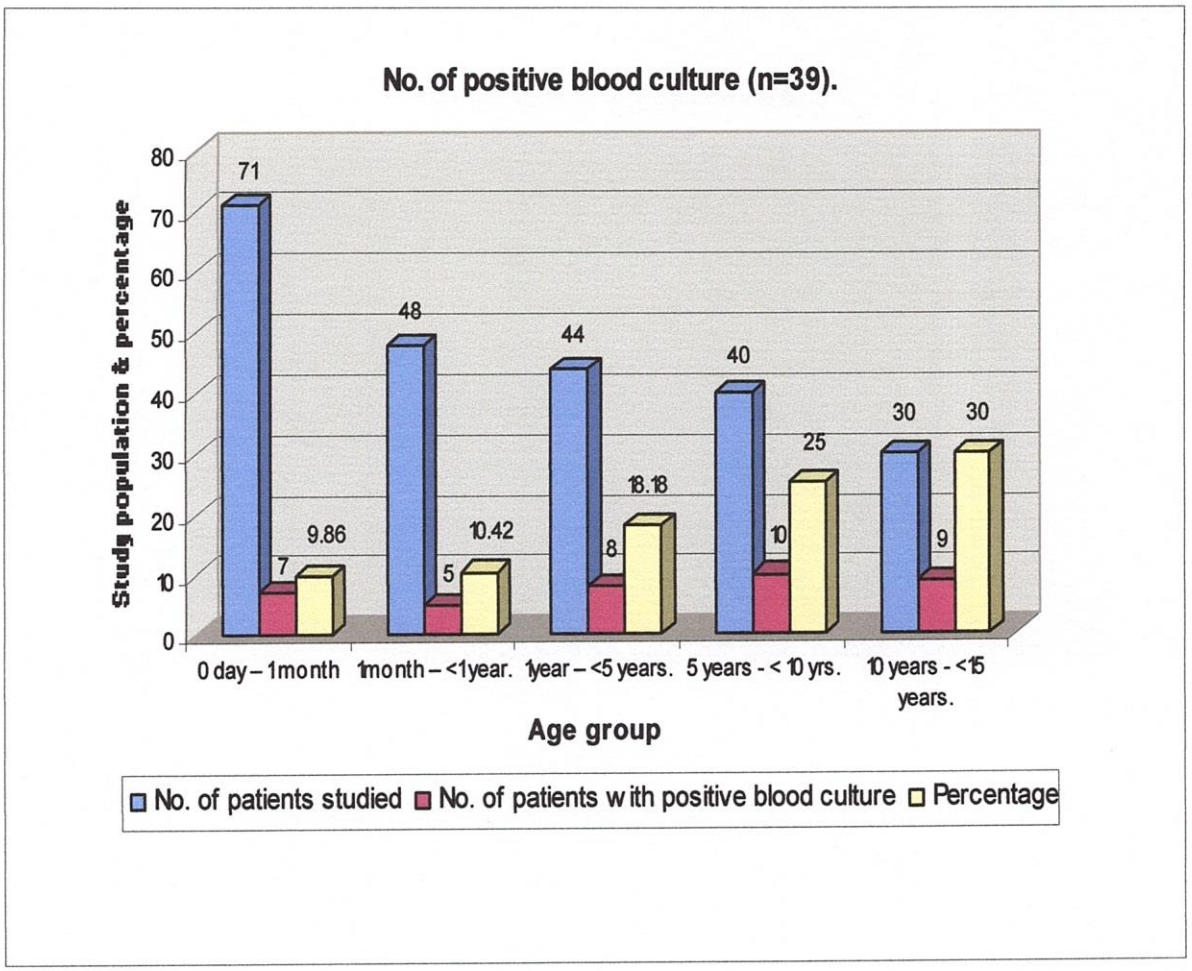


Table-XI

Distribution of Blood culture results according to sex. (n=233)

Sex groups	Blood culture positive	Blood culture negative	Total
Male	22 (17.89%)	101 (52.11%)	123 (52.79%)
Female	17 (15.45%)	93 (84.55%)	110 (47.21%)
Total	**39 (16.74%)**	**194 (83.26%)**	**233 (100%)**

Figures within parentheses indicate percentages. (P>0.05). (No significant difference between sex groups. *p*- values are from one way ANOVA test.)

Figure-3:

Distribution of bacteria isolated from blood culture. (n=39)

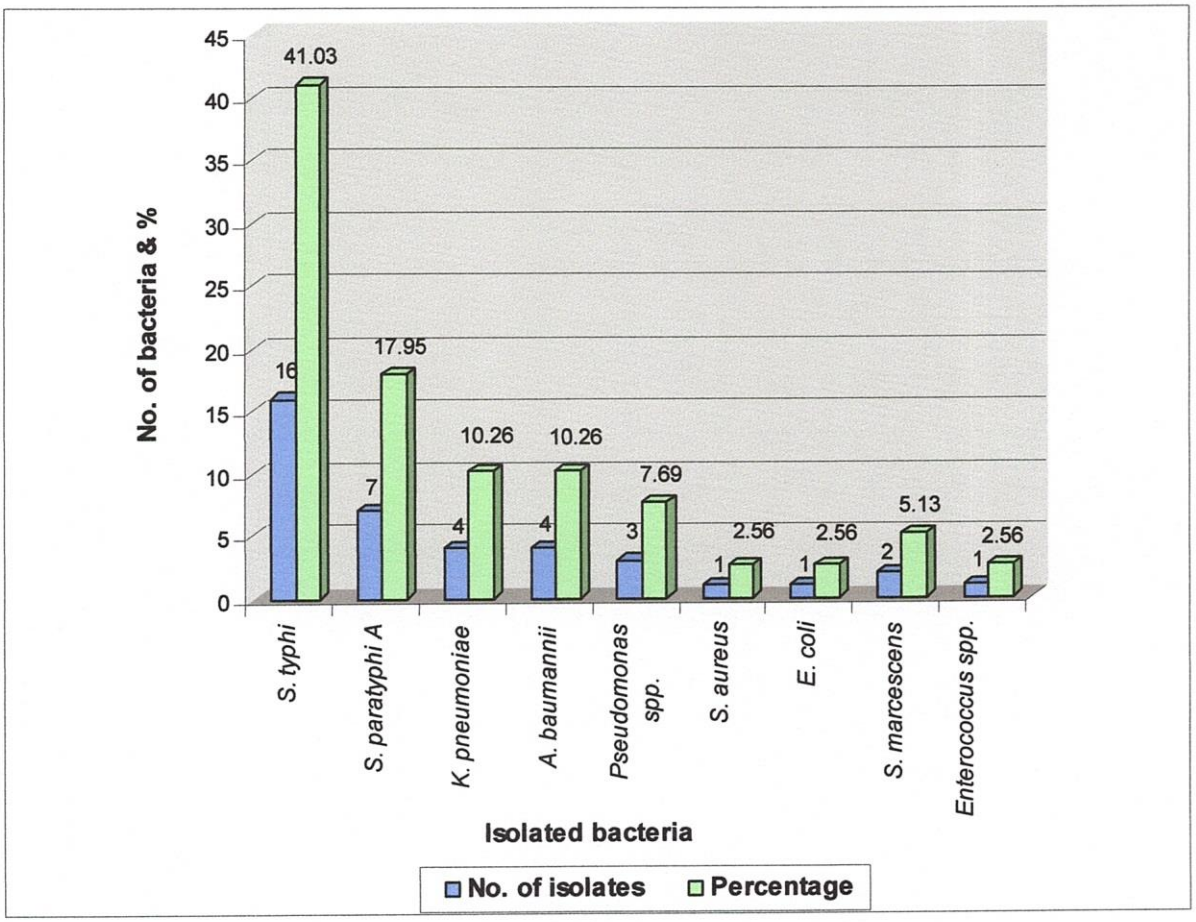


Table-XII

Blood culture positivity among patients with or without antimicrobial therapy. (n =233)

Categories of patients	No. of patients	No. of positive blood culture.	No. of negative blood culture
Without antimicrobial	139	33 (23.74%)	106 (76.26%)
With antimicrobial	94	6 (6.38%)	88 (93.62%)
Total	**233**	**39 (16.74%)**	**194 (83.26%)**

(*P*<0.001. Significantly higher positivity rate among patients without antimicrobial therapy. *p*- values are from one way ANOVA test.)

Out of 233 blood samples cultured, 139 were from patients without antimicrobial therapy and 94 were with antimicrobial therapy. Patients, without antimicrobial therapy, blood culture were positive in 33 (23.74%) and with antimicrobial therapy was positive in 6 (6.38%) patients. The difference was statistically significant (P<0.001) (Table-XII).

Table–XII shows the antibiogram of different bacterial isolates of Enterobacteriace from blood culture. *S. typhi* were 75% sensitive to ceftriaxone followed by ciprofloxacin and cefepime 62.50% each. Susceptibility to ampicillin (56.25%) was reported. On the other hand, resistance were observed to cotrimoxazole (56.25%), chloramphenicol (50%) and cefixime (43.75%). 75% highly resistance was noted to azithromycin.

Bacterial isolates of *S. paratyphi* A showed 71.43% sensitivity to ceftriaxone and cefepime each. Sensitivity to ciprofloxacin and cefixime were 57.14% each where as resistance to ampicillin, chloramphenicol and cotrimoxazole were 57.14%, 42.86% and 57.14% respectively. Finally, 85.71% highly resistivity was found against azithromycin.

K. pneumoniae isolates in this study were found 100% sensitive to imipenem, 75% to amikacin and cefepime to 50%. On the other hand, 100% resistance was observed against gentamicin. *K. pneumoniae* showed 75% resistance against Ceftriaxone, cotrimoxazole and ciprofloxacin. 50% resistivity was found against amoxyclavonic acid.

E. coli were sensitive (100%) to Ceftriaxone and imipenem but were 100% resistant to amikacin, amoxyclavonic acid ciprofloxacine, gentamicin, Cefepime and netilmycin.
S. marcescens were 100% sensitive to cotrimoxazole, cefepime and imipenem whereas 100% resistant to cefixime and gentamicin. 50% resistant were observed against ceftriaxone and ciprofloxacin.

Table-XIV disclosed that isolates *of Pseudomonas* spp. were 100% sensitive to amikacin and piperacillin-tazobactam but 100% each resistant to ceftriaxone, cotrimoxazole, imipenem and cefixime. Ciprofloxacin and cefepime showed 66.67% sensitivity each. However, 66.67%, 33.33% resistances were found against ceftazidime and gentamicin respectively.

A. baumannii were 75% sensitive to ciprofloxacin & imipenem each and 50% each sensitive to amikacin, ceftriaxone and cotrimoxazole. Resistance were found 100% against amoxyclavonic acid and 75% to ceftazidime, gentamicin and cefepime.

It appears from Table-XV that *S. aureus was* 100% sensitive to lynezolid, oxacillin and vancomycin. On the contrary, 100% resistance were revealed to ampicillin, cefepime, cefixime, doxycycline and penicillin.

Enterococcus sp. were observed 100% sensitive to cefepime, lynezolid and vancomycin but 100% resistant to ampicillin, ciprofloxacin, cefixime doxycycline, and penicillin.

Table-XIII

Sensitivity pattern of isolated Enterobacteriaceae against different antimicrobial agents.

Antimicrobial agent	Sensitivity pattern	Name of bacterial isolates				
		S. typhi (n= 16)	*S. paratyphi* A (n=07)	*Klebsiella pneumoniae.* (n=04)	*E. coli* (n=01)	*S. marcescens (n=02)*
AMC	S IS R	-	-	1 (25.00%) 1 (25.00%) 2 (50.00%)	0 (00) 0 (00) 1 (100%)	0 (00) 1 (50.00%) 1 (50.00%)
AMP	S IS R	9 (56.25%) 0 (00) 7 (43.75%)	3 (42.86%) 0 (00) 4 (57.14%)	-	-	-
AZM	S IS R	3 (18.75%) 1 (6.25%) 12 (75.00%)	1 (14.29%) 0 (00) 6 (85.71))	-	-	-
CRO	S IS R	12 (75%) 0 (00) 4 (25%)	5 (71.43%) 0 (00) 2 (28.57%)	1 (25.00%) 0 (00) 3 (75.00%)	1 (100%) 0 (00) 0 (00)	0 (00) 1 (50.00%) 1 (50.00%)
C	S IS R	8 (50.00%) 0 (00) 8 (50.00%)	3 (42.85%) 1 (14.29%) 3 (42.86%)	-	-	-
SXT	S IS R	7 (43.75%) 0 (00) 9 (56.25%)	3 (42.86%) 0 (00) 4 (57.14%)	0 (00) 1 (25.00%) 3 (75.00%)	0 (00) 1 (100%) 0 (00)	2 (100%) 0 (00) 0 (00)
CIP	S IS R	10 (62.50%) 2 (12.50%) 4 (25.00%)	4 (57.14%) 0 (00) 3 (42.86%)	1 (25.00%) 0 (00) 3 (75.00%)	0 (00) 0 (00) 1 (100%)	0 (00) 1 (50.00%) 1 (50.00%)
FEP	S IS R	10 (62.50%) 0 (00) 6 (37.50%)	5 (71.43%) 0 (00) 2 (28.57%)	2 (50.00%) 0 (00) 2 (50.00%)	0 (00) 0 (00) 1 (100%)	2 (100%) 0 (00) 0 (00)
CFM	S IS R	8 (50.00%) 1 6.25% 7 (43.75%)	4 (57.14%) 0 (00) 3 (42.86%)	1 (25.00%) 0 (00) 3 (75.00%)	0 (00) 1 (100%) 0 (00)	0 (00) 0 (00) 2 (100%)
AK	S IS R	-	-	3 (75.00%) 0 (00) 1 (25.00%)	0 (00) 0 (00) 1 (100%)	1 (50.00%) 1 (50.00%) 0 (00)
CN	S IS R	-	-	0 (00) 0 (00) 4 (100%)	0 (00) 0 (00) 1 (100%)	0 (00) 0 (00) 2 (100%)
IPM	S IS R	-	-	4 (100%) 0 (00) 0 (00)	1 (100%) 0 (00) 0 (00)	2 (100%) 0 (00) 0 (00)
NET	S IS R	-	-	1 (25.00%) 1 (25.00%) 2 (50.00%)	0 (00) 0 (00) 1 (100%)	1 (50.00%) 0 (00) 1 (50.00%)

AMC- Amoxyclavonic acid, AMP- Ampicillin, AZM-Azithromycin, CRO-Ceftriaxone, C-Chloramphenicol, SXT-Cotrimoxazole, CIP- Ciprofloxacin, FEP- Cefepime, CFM- Cefixime, AK- Amikacin, CN- Gentamicin, IPM- Imipenem, NET- Netilmicin.

S-Sensitive, IS- Intermediate sensitive, R- Resistant.

Table-XIV

Sensitivity pattern of *Pseudomonas spp.* and *A. baumannii against* different antimicrobial agents.

Name of Antimicrobial agent	Sensitivity pattern	Name of bacterial isolates	
		***Pseudomonas* spp. (n= 03)**	***Acinetobacter baumannii* (n= 04)**
AMC	S IS R	-	0 (00) 0 (00) 4 (100%)
AK	S IS R	3 (100%) 0 (00) 0 (00)	2 (50.00%) 0 (00) 2 (50.00%)
CRO	S IS R	0 (00) 0 (00) 3 (100%)	2 (50.00%) 1 (25.00%) 1 (25.00%)
CAZ	S IS R	0 (00) 1 (33.33%) 2 (66.67%)	0 (00) 1 (25.00%) 3 (75.00%)
SXT	S IS R	0 (00) 0 (00) 3 (100%)	2 (50.00%) 1 (25.00%) 1 (25.00%)
CIP	S IS R	2 (66.67%) 1 (33.33%) 0 (00)	3 (75.00%) 0 (00) 1 (25.00%)
FEP	S IS R	2 (66.67%) 0 (00) 1 (33.33%)	1 (25.00%) 0 (00) 3 (75.00%)
CFM	S IS R	0 (00) 0 (00) 3 (100%)	-
CN	S IS R	1 (33.33%) 1 (33.33%) 1 (33.33%)	1 (25.00%) 0 (00) 3 (75.00%)
IPM	S IS R	0 (00) 0 (00) 3 (100%)	3 (75.00%) 0 (00) 1 (25.00%)
NET	S IS R	-	-
TZP	S IS R	3 (100%) 0 (00) 0 (00)	-

S-Sensitive, IS- Intermediate sensitive, R- Resistance.

AMC- Amoxyclavonic acid, CRO-Ceftriaxone, SXT-Cotrimoxazole, ATM- Aztreonam, CIP-Ciprofloxacin, FEP- Cefepime, CFM- Cefixime, AK- Amikacin, CN- Gentamicin, IPM-Imipenem, NET- Netilmicin, TZP- Piperacillin-Tazobactam

Table-XV

Sensitivity pattern of *S. aureus* and *Enterococcus* spp. against different antimicrobial agents.

Name of antimicrobial agent	Sensitivity pattern	Name of bacterial isolates	
		S. aureus (n=01)	*Enterococcus* spp. (n= 01)
AMP	S IS R	0 (00) 0 (00) 1 (100%)	0 (00) 0 (00) 1 (100%)
SXT	S IS R	0 (00) 1 (100%) 0 (00)	0 (00) 1 (100%) 0 (00)
CIP	S IS R	0 (00) 1 (100%) 0 (00)	0 (00) 0 (00) 1 (100%)
FEP	S IS R	0 (00) 0 (00) 1 (100%)	1 (100%) 0 (00) 0 (00)
CFM	S IS R	0 (00) 0 (00) 1 (100%)	0 (00) 0 (00) 1 (100%)
DO	S IS R	0 (00) 0 (00) 1 (100%)	0 (00) 0 (00) 1 (100%)
LZD	S IS R	1 (100%) 0 (00) 0 (00)	1 (100%) 0 (00) 0 (00)
OX	S IS R	1 (100%) 0 (00) 0 (00)	-
P	S IS R	0 (00) 0 (00) 1 (100%)	0 (00) 0 (00) 1 (100%)
VA	S IS R	1 (100%) 0 (00) 0 (00)	1 (100%) 0 (00) 0 (00)

AMC- Amoxyclavonic acid, AMP- Ampicillin, SXT-Cotrimoxazole, CIP- Ciprofloxacin, FEP- Cefepime, CFM- Cefixime, DO- Doxycycline, LZD- Linezolid, OX- Oxacillin, P-Penicillin, VA- Vancomycin.

S-Sensitive, IS- Intermediate sensitive, R- Resistant.

Figure-IV showed the results of CRP among study population. Out of 233 cases of clinically diagnosed septicemia in children 175 (75.11%) were CRP positive and 58 (24.89%) were CRP negative.

Out of 233 suspected septicemia cases in children both blood culture and CRP were positive in 39 (16.74%) patients, only CRP were positive in 136 (58.37%) cases and both blood culture and CRP level were negative in 58 (24.89%) cases (Figure-V).

Figure- 4:

CRP level in blood among study population. (n=233)

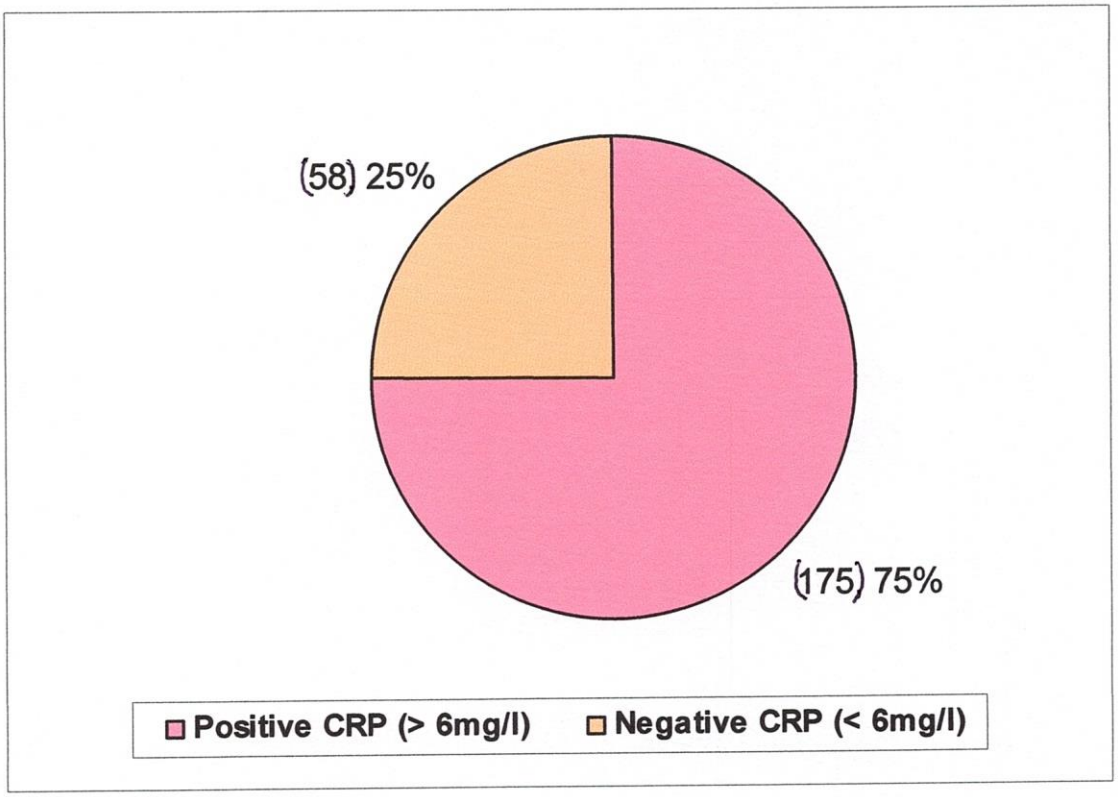
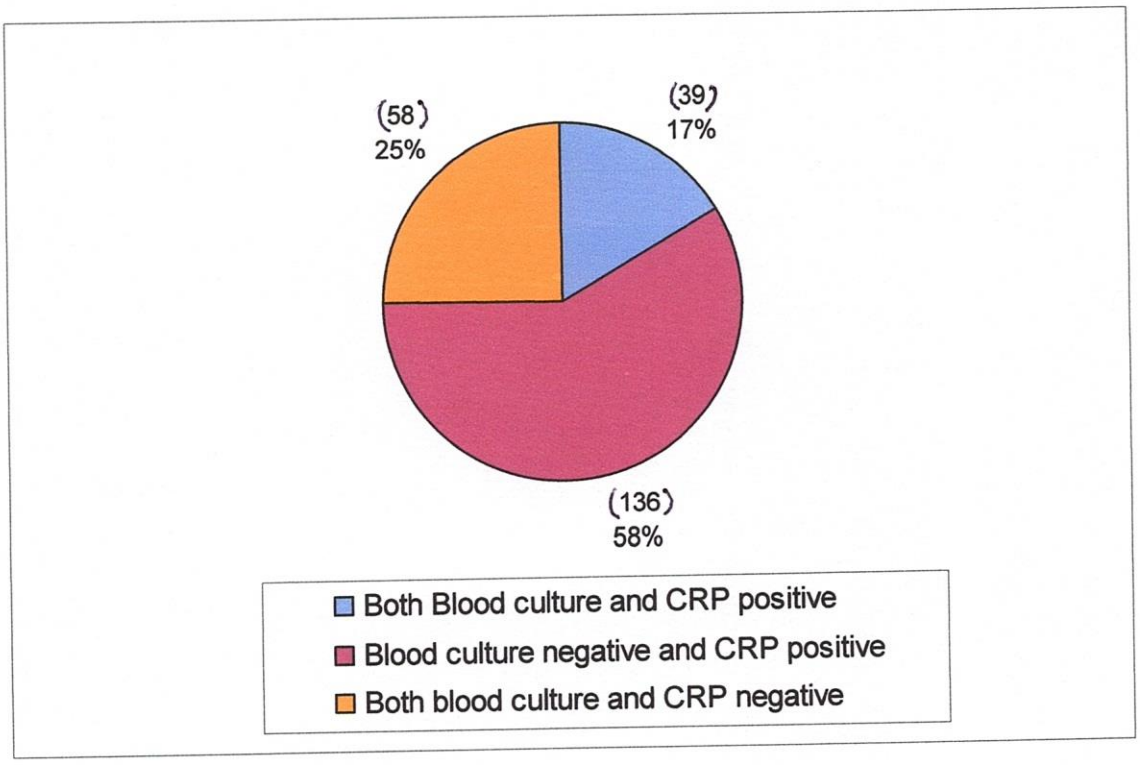


Figure- 5:

Relation between blood culture and CRP level among suspected septicemic children (n=233).



On the basis of blood culture test, CRP estimation and clinical signs of septicemia, the study cases and controls were categorized into four groups. The result showed that 39 (14.28%) were blood culture proven septicemia (both blood culture and CRP positive), 136 (49.82%) were probable septicemia (Blood culture negative, CRP positive), 58 (21.25%) were non-septicemic febrile patient (both blood culture and CRP negative) and 40(14.65%) were control group (Table- XVI).

It appears from Table- XVII that mean serum CRP values (mg/l) of blood culture proven septicemia group, probable septicemia group, non-septicemic febrile group and control group were 70.42, 34.05, 3.08 and 0.98 respectively. Statistically significant differences were found ($p<0.001$) when mean CRP level of proven septicemia group, probable septicemia group and non-septicemic febrile group each compared with control group.

The average age (mean ± SD) of the culture proven septicemia, probable septicemia, non-septicemic febrile and control groups were 2098.23 ± 1860.09, 1093.75 ± 1484.79, 1011.44 ± 1427.67 and 1631.8 ± 1681.04 days, respectively Table- XVII. There were no significant differences observed among study groups with respect to age.

Table- XVIII shows that the mean value of CRP in culture proven septicemia, probable septicemia and non-septicemic febrile group was 70.42, 34.05 and 3.08 mg/L respectively. The concentrations of serum CRP were significantly ($p < 0.001$) different among three study group.

Table-XVI

Categorization of patients in different groups. (n= 273)

Study groups	No. of patients	Percentage
Culture proven septicemia (Both Blood culture and CRP positive).	39	14.29
Probable septicemia (Only CRP positive).	136	49.82
Non-septicemic febrile patient (Both blood culture and CRP negative).	58	21.24
Control	40	14.65
Total	**273**	**100**

(Control 40 subjects for baseline C-reactive protein (CRP) study were included in the study population.)

Table-XVII

CRP levels among different groups of study cases.

Parameters	Study different group				*P*-value
	Culture proven Septicemia	Probable Septicemia	Non-septicemic febrile group	Control group	
No. of subjects	39	136	58	40	
Male/female					
Age (days)	2098.23 ± 1860.09	1093.75 ± 1484.79	1011.44 ± 1427.67	1631.8 ± 1681.04	0.810 [a], 0.358 [b], 0.306 [c]
CRP (mg/L)	70.42 ± 43.25	34.05 ± 18.95	3.08 ± 1.29	0.98 ± 0.45	< 0.001 [a], < 0.001 [b], < 0.001 [c]

[a] *p*-value comparison between control and Culture proven septicemia;

[b] *p*-value comparison between control and Probable Septicemia;

[c] *p*-value comparison between control and Non-septicemic febrile group.

Culture proven septicemia and Probable Septicemia.

p-value consider significant at $p < 0.05$. *p*- values are from one way ANOVA test.

Data are presented as mean ± SD.

Statistically significant difference ($p<0.001$) was found when mean CRP level of proven septicemia group, probable septicemia group and non-septicemic febrile group each compared with control group.

There was no statistically significance found in age comparison between control and study population.

Table-XVIII

Comparison of CRP level among the study groups

Parameters	Study subjects group			*P*-value
	Culture proven Septicemia	Probable Septicemia	Non-septicemic febrile group	
CRP (mg/L)	70.42 ± 43.25	34.05 ± 18.95	3.08 ± 1.29	$< 0.001^{a}$, $< 0.001^{b}$, $< 0.001^{c}$

[a] *p*-value comparison between Culture proven septicemia and Probable Septicemia ;

[b] *p*-value comparison between Culture proven septicemia and Non-septicemic febrile group;

[c] *p*-value comparison between Probable Septicemia and Non-septicemic febrile group.

p-value consider significant at $p < 0.05$.

Data are presented as mean ± SD.

The concentrations of serum CRP were significantly ($p < 0.001$) different among three study group.

Chapter-6
Discussion

DISCUSSION

Septicemia is a clinical state in which bacteria are present in blood stream and gives rise to serious systemic symptoms (Murphy, 1988). Sepsis among children is a significant health problem and is a leading cause of death of children worldwide (Watson et al., 2005). Detection of bacteria in blood is critically important because septicemia has mortality as high as 50% (Washington and Ilstrup, 1986). Early initiation of treatment with appropriate antimicrobial agents is essential to reduce mortality (Weinstein et al., 1983; Kreger et al., 1980). Blood culture remains the ideal method for the diagnosis and treatment of patients with suspected septicemia (Spencer, 1988; Pierce and Murray, 1986).

However, in many critical situations, the yield from blood culture is low and it requires 48-72 hours before the results are known. In the last few years, many new techniques have been introduced into the field of early diagnostic process of bacterial infections. Demonstration of indirect evidences of bacterial infections by identifying serological markers is one of them. C-reactive protein (CRP) has been used as a marker of infection for many years. High levels of IL-6 have been associated with severe inflammation and sepsis. It has a central role in inducing the synthesis of acute-phase proteins such as CRP. This makes IL-6 an interesting molecule to evaluate in the early phase of infection and sepsis (Gaini et al., 2006).

Evaluation of cytokines as early markers for sepsis has been well documented and reviewed. In the present study, we could not measure IL-6 level during the course of bacterial infection of children due to our resource limitations.

In this study, blood samples from 233 children with clinically suspected septicemia were cultured, of which 39 (16.74%) yielded growth of aerobic bacteria. Similarly, Fahmida (2005) reported 21% and Dewanjee (2000) reported 26.08% of septicemia among paediatric age groups in BSMMU. Saha et al., (1992) in Bangladesh isolated aerobic bacteria from blood in 23.5% cases of septicemia in children. Murty et al., (2007) in India showed 18.7% blood culture positivity among paediatric populations presented with bacteremia. Among blood culture positive cases 22 (17.89%) were male and 17(15.45%) female. No significant difference ($P > 0.05$) was observed in the rate of blood culture positivity male and female groups. Similar results were also reported by Dewanjee (2000) and Ahmed (1991) in Bangladesh, and Alausa et al., (1977) in Nigeria.

In the present study the highest number, 10 (25.64%) of septicemia cases were detected in the age group 5 years - <10 years followed by 9 (23.08%) in the age group 10 years - <15 years. Similarly, Meremikwu et al. (2005) in Nigeria found 41% cases in age group 6 years – 10 years and 25.8% in age group 11 years – 18 years. Oda et al. (2000) in Japan found 23.36% cases in age group 7 years – 12yrs. In the present study, 8(20.51%) cases were in age group 1 year - <5 years, which is similar to Hill et al. (2007) in West Africa who found 24.7% septicemia cases in 2 yrs – 6 yrs. age group. Then 5(12.82%) cases were found in 1 month - <1yr age group in the present study. Similarly one study showed 8.2% cases were found in this age group in 2006 in Vientiane, Laos. Berkly et al. (2005) in Kenya found 6.9% cases in 2 months – 12 months age group. In the present study, 7(17.95%) were isolated in age group 0 day - <1 month. This result is closely related to Begum et al. (2007) in Bangladesh who found 10.14% cases in this age group in Dhaka Medical college hospital.12.1% cases in 7 days -59 days age group were reported by Berkly et al. (2005) in Kenya, which is similar to this present study.

Here total 39 bacterial strains were isolated from 39 study patients. Among them *S. typhi* (41.03%) was the most frequently isolated bacteria, followed by *S. paratyphi* A (17.95%). *K. pneumoniae* and *A. baumannii* were (10.26%) in each. Next to *Pseudomonas* spp. (7.69%), *S. marcescens* (5.13%), *S. aureus* (2.56%), *E. coli* (2.56%), and finally *Enterococcus* sp. (2.56%) were revealed in our study. Our results are in agreement with those of Fahmida (2005) from BSMMU in Bangladesh, Sharma et al. (2006) in Nepal, Cheng et al. (1991) in Hongkong and Blomberg et al. (2007) in Tanzania. In a study in Dhaka Medical College, Bangladesh, Begum et al. (2007) found 22.03% *Salmonella* spp. isolates from blood culture in neonates. Brooks et al. (2005) in Bangladesh also found *S. typhi* as the common pathogen (75.4%) isolated from blood culture. Another study from Nigeria showed 15.9% isolation rates of *Salmonella* species from blood culture (Abucejo and Capeding, 2001). Septicemia due to Salmonella was predominant in children of Bangladesh because of living in unhygienic conditions, lack of sanitation facilities and taking of unhygienic foods and polluted water. In contrast to our findings, Ahmed et al. (2002) in Bangladesh, from Dhaka Shishu Hospital revealed that the principal organisms were *E. coli* (30%) followed by *Klebsiella* spp., S. *aureus, pseudomonas* spp., *Streptococcus* spp. and *Acinetobacter* spp. Weinstein et al. (1996) from U.S.A. reported the most common blood stream isolates were *S. aureus, K. pneumoniae, E. coli,* Coagulase negative Staphylococcus and *Pseudomonas* spp. These different findings in USA may be due to striking geographical difference, increased

use of invasive procedures, extensive surgery, intravascular devices and increase in the number of Immunocompromized persons. The isolation frequency of different etiological agents of septicemia may however, vary from country to country, from hospital to hospital and from one community to another (Kurruvilla 1988; Khatua et al., 1986).

In our study, 6.38% of patients showed positive blood culture among the patients already on antimicrobial therapy and 23.74% among the patients without antimicrobial therapy. Blood culture positivity rate was significantly higher ($p<0.001$) among the group without antimicrobial therapy. Our result was similar to Murty et al. (2007) in India who found 8.45% positive blood cultures in antibiotic user group and 38.89% in antibiotic non-user group. The findings are also similar to Dewanjee (2000) in Bangladesh who found in his study blood culture positivity in patients with and without antimicrobials were 7.5% and 29.17% respectively.

Early initiation of appropriate antimicrobial therapy is critically important for reducing mortality among patients with blood stream infections, the initiation of such therapy is almost always empirical, frequent emergence of resistance among bacteria makes empirical therapy decisions more difficult (Diekema et al., 1999). An accurate identification and up-to-date sensitivity pattern of causative organism is extremely necessary for successful treatment of severe infections like septicemia (Miles and Amyes, 1996).

In this study, 75% isolates of *salmonella typhi* were susceptible to ceftriaxone while resistance to ampicillin, cotrimoxazole and chloramphenicol were 43.75%, 56.25% and 50% respectively. Among them 43.75% *S. typhi* isolates were multi drug resistant (MDR) but single drug resistant to ampicillin was (43.75%), cotrimoxazole (56.25%) and chloramphenicol (50.00%). Chuang et al. 2008 reported 41.2% MDR *S. typhi* in a surveillance study on antimicrobial resistance of *S. typhi* in seven Asian countries during 2002-2004. Another report in 1997 from in Viet Nam found 63% and Rahaman et al. (2006) in Bangladesh reported 42% MDR *S. typhi.* In our study, isolated *S. typhi* were 25% resistant to ciprofloxacin. Fahmida (2005) at BSMMU, Bangladesh found 28.57% ciprofloxacin resistant *S. typhi.* Capoor et al. (2007) in India reported that instances of ciprofloxacin resistant *S. typhi* increased from 0.6 to 15.2% over a five year period extending from 2001 to 2005. Resistance to fluroquinolones has been reported in other studies where widespread availability and uncontrolled use of antibiotics including quinolones are common (Rupali et

al., 2004). The developments of antibiotic resistance in *S. typhi* pose considerable threat of increased mortality and morbidity to many communities of the world. Almost similar susceptibility pattern of *S. paratyphi* A were noticed when compared with *S. typhi*. They were 71.43% sensitive to ceftriaxone and cefepime but resistant to ampicillin, cotrimoxazole (57.14%) and to chloramphenicol (42.86%). Among them, MDR rate was 42.86%.

K. pneumoniae isolates in this study were 100% sensitive to imipenem and 75% to amikacin but 100% resistant to gentamicin, 75% resistant to cotrimoxazole, ciprofloxacin, ceftriaxone and cefixime each. These findings are almost similar to Dewanjee (2000) at BSMMU, Bangladesh.

Ceftriaxone and imipenem were the most effective (100%) antimicrobial agent against *E. coli.* However, isolates were 100% resistant to ciprofloxacin, Cefepime, amikacin, gentamicin, and netilmicin. Isolated *S. marcescens* were 100% sensitive to imipenem, cotrimoxazole and cefepime but 100% resistant to gentamicin and cefixime.

In our study, *Pseudomonas* spp. was 100% sensitive to amikacin and piperacillin-tazobactam but 100% resistant to ceftriaxone, cefixime, cotrimoxazole and imipenem. Ciprofloxacin and cefepime showed 66.67% sensitivity whereas 66.67% and 33.33% resistance to ceftazidime and gentamicin respectively. Almost similar sensitivity pattern was reported by Blomberg et al. (2007) in Tanzania. Lower sensitivity pattern of gentamicin was reported by Monga et al. (1986) from India 22% and by Bisbe et al. (1988) in USA 48%.

For *A. baumannii*, 75% sensitivity were noticed to ciprofloxacin & imipenem each and 50% to both amikacin and Cotrimoxazole. Resistance were observed 100% against amoxyclavonic acid where ceftazidime, cefepime and gentamicin showed 75% each. In Tanzania, similar results were reported by Blomberg et al. (2007). *S. aureus* isolates were 100% sensitive to oxacillin which was similar to reported by Dewanjee (2000) and Fahmida (2005) in Bangladesh. On the other hand, the strain was reported 100% resistant to ampicillin, cefepime, doxycycline and penicillin.

In our study, study population, *Enterococcus* sp. gram positive bacteria was also isolated which were found to the 100% sensitive to cefepime, lynezolid, and vancomycin but 100% resistant to ampicillin, ciprofloxacin and penicillin.

The antimicrobial sensitivity patterns in our study revealed an alarming trend that most of the gram negative bacteria isolated from blood culture showed resistance to commonly used antibiotics such as amikacin, cefepime, cotrimoxazole, cefixime, ciprofloxacin and gentamicin. These drugs are most commonly employed in combination on empirical therapy in suspected cases of septicemia before blood culture and sensitivity results are available. Indiscriminate use of antimicrobial therapy in patients with blood stream infections increases mortality (Harbarth et al. 2001, Ibrahim et al. 2000).

In our present study, pattern of antibiotic sensitivity is slightly different from other studies. The sensitivity pattern to various antimicrobial agents varies in different studies, in different parts of the same country and at different times in the same hospital. Indiscriminate and widespread uses of antibiotics imposes a high selection pressure favoring drug resistance among microorganisms (Masro et al., 1993) particularly in developing countries which may be responsible for the difference in sensitivity in different regions (Khatua et al., 1986).

In our study, out of 233 suspected septicemia, 175 (75.11%) patients were CRP positive (>6mg/l) and 58 (24.89%) patients were CRP negative (<6mg/l). Similarly Makija et al. (2005) in India found CRP positive in 84.3% cases of suspected septicemia patients. The suspected septicemia cases were categorized on the basis of blood culture, CRP and clinical signs. Culture proven septicemia (both blood culture and CRP positive) were 39 (16.74%) cases, probable septicemia (only CRP positive) were 136 (58.37%) and non-septicemic febrile patient (both blood culture and CRP were negative) were 58 (24.89%) cases. Control children were sampled for baseline CRP levels measurement. Similar categorization was done by Bhartiya et al. (2000) in India and Messer et al. (1996) in France. Messer et al. (1996) in France found 15.49% cases of proven septicemia, 49.29% probable septicemia and 35.21% cases were non-septicemic febrile patient. This is consistent with our present study.

Mean serum CRP values of blood culture proven septicemia group, probable septicemia group, non-septicemic febrile group and control group were 70.42 mg/l, 34.05 mg/l, 3.08 mg/l and 0.98 mg/l respectively. Statistically significant difference ($p<0.001$) was found when mean CRP level of proven septicemia group, probable septicemia group and non-septicemic febrile group each compared with control group. None of the control group showed the concentration of CRP above cut-off level. On the other hand, it was found that

culture proven septicemia group and probable septicemia group, both showed CRP level positive (cut-off value 6 mg/lt).

We also attempted to find out the average age (mean ± SD) of the culture proven septicemia, probable septicemia, non-septicemic febrile and control groups (2098.23 ± 1860.09, 1093.75 ± 1484.79, 1011.44 ± 1427.67 and 1631.8 ± 1681.04 days, respectively). There were no significant differences among the study groups with respect to age.

Chapter-7
Summary

SUMMARY

Blood samples from 233 clinically diagnosed cases of septicemia in children were studied. On the basis of blood culture, CRP and clinical signs of septicemia, these patients were categorized as culture proven septicemia 39 (16.74%), probable septicemia 136 (58.37%) and non-septicemic febrile patients 58 (24.90%). 40 control children were sampled for measurement of baseline CRP level study.

Out of these 233 cases studied, the highest number of patients 71 (30.47%) were upto 1 month of age followed by 48 (20.60%) from 1 month to < 1 year age group. Total isolated bacteria were in 39(16.74%) cases. The highest rate of blood culture positivity was found among the patients of 5 years -<10 years age group (25.64%). *Salmonella typhi* was the most frequently isolated bacteria (41.03%) followed by Klebsiella pneumoniae and Acinetobacter spp. (10.26%) each. The rate of blood culture positivity was significantly higher ($p < 0.001$) among patients without antimicrobial therapy (23.74%) than those in patients with antimicrobial therapy (6.38%).

Antibiogram pattern revealed that *S. typhi* were 75% sensitive to Ceftriaxone. Sensitivity to ciprofloxacin, chloramphenicol, ampicillin and cotrimoxazole were 43 -62%. Multi drug resistant (MDR) *S. typhi* isolates were 43.75% in our study. *E. coli* and *Klebsiella pneumoniae* were highly sensitive 100% to imipenem. *Acinetobacter baumannii* were 75% sensitive to ciprofloxacin and imipenem. *Pseudomonas* spp. was 100% resistant to Ceftriaxone, cotrimoxazole, cefixime and imipenem. Majority percent antibiotics used in commonly isolated gram negative bacteria were gentamicin, amoxyclavonic acid.

S. aureus were 100% sensitive to oxacillin, lynezolid and vancomycin but 100% resistant to ampicillin, penicillin, cefepime and doxycycline.
Enterococcus sp. was 100% sensitive to lynezolid and vancomycin but 100% resistant to ampicillin and penicillin.

Out of 233 suspected septicemia cases in children 175 (75.11%) were noted CRP positive and 58 (24.89%) were negative. Both proven septicemia and suspected septicemia cases showed CRP concentration above the cut-off value but statistically significant difference ($p<0.001$) was found between the two study cases.

Chapter-8
Conclusion

CONCLUSION

- Most of the gram negative bacteria isolated from blood culture showed resistance to commonly used antibiotics.
- CRP is increased not only in septicemia but also any in inflammatory reaction and in tissue injury. Only CRP may not be a good marker for diagnostic and prognostic value.
- Measurement of CRP along with interleukin-6 may be a good marker in diagnosing septicemia.
- Antimicrobial susceptibility pattern revealed in this study may not be dependable due to small sample size.
- Multidrug resistant (MDR) *S. typhi* was 43.75%.
- There is no significance of CRP levels among age group of study cases.

Chapter-9
Recommendations

RECOMMENDATIONS

- Blood culture and antimicrobial susceptibility must be done in every cases of suspected septicemia.
- Before starting of antimicrobial therapy, blood culture should be done.
- Large sample size is needed to establish the findings.
- To reduce contamination, blood should be collected by venipuncture of peripheral veins.
- Adequate volume of blood must be drawn for culture.
- Both aerobic and anaerobic blood culture should be done together to determine the complete picture of etiological agents.

Chapter-10
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Chapter-11
Appendices

DATA SHEET (FOR CASE)

Patient's ID no. …… Date:……..…

Patient's name:

Hospital name: United Hospital. Ltd. Ward/Bed:…….…. Age:……. Sex: M / F.

Father/mother/guardian's name:……..

Date and time of the hospital admission:……

Present complaint:

1. Fever: Yes / No. 2. Chills and rigor: Yes / No. 3. Vomiting: Yes / No
4. Poor feeding: Yes / No 5. Convulsion: Yes / No 6. Difficulty in respiration: Yes / No
7. Abdominal pain: Yes / No. 8. Diarrhea/constipation: Yes / no 9. Others:

Past history:

10. Cancer: Yes / No 11. Dialysis: Yes / No 12. Surgery / Cardiac surgery: Yes / No
13. Others if any:

On examination:

14. Temperature:…… 15. Respiratory rate:……..… 16. Heart rate:……
17. Anaemia: Yes / No. 18. Cyanosis: Yes / No. 19. Tongue coated: Yes / No.
20. Abdominal distension: Yes / No. 21. Skin septic spot/Rash: Yes / No. 22. Abscess: Yes / No.
23. Infected wound: Yes / No. 24. Liver:……..…….. 25. Spleen:……..……..
26. Neck rigidity: Yes / No. 27. Indwelling catheter: Yes / No. 28. IV channel: Yes / No.
29. Dehydration: Yes / No 30. Jaundice: Yes / No.

Drug History:

Antibiotics:

	Name	Dose	Duration
1.			
2.			
3.			

Steroids:……

Others:……

Taken antibiotic before hospitalized within 48 hrs: Yes / No

Laboratory results:

C-reactive protein level (mg/l):

Haematology:

TC:..... DC:..... ESR:.....

Culture:

Growth / No growth

If growth name of the organism:

Name of Antibiotics	**Sensitive**	**Intermediate sensitive**	**Resistant**
Ampicillin			
Amikacin			
Amoxyclavonic acid/Amoxycillin			
Ceftriaxone			
Ceftazidime			
Cotrimoxazole			
Ciprofloxacin			
Cefepime			
Cefixime			
Chloramphenicol			
Gentamycin			
Imipenem			
Netilmycin			
Tazobactum			
Aztreonam			
Doxycycline (DO)			
Fusidic acid			
Penicillin			
Linezolid			
Oxacillin			
Vancomycin			

Investigator

CONSENT FORM

Patient's ID no. …… Date:……..…

Patient's name:

Guardian's name:……..

Hospital name: United Hospital. Ltd. Ward/Bed:…… Age:…… Sex: M / F.

Date and time of the hospital admission:……

1. I hereby give consent to Md. Al Jahidi Hasan Chowdhury to collect blood samples of my child for blood culture and CRP estimation.

2. I have been informed about the aim, nature, fate and probable risk of the tests.

3. I have been assured that the result of the investigation will be given to me.

4. I have been informed that these tests do not guarantee the diagnosis.

5. I am ensuring that I have gone through the consent form/have been read before me and I have signed it after understanding all the relevant matters.

Signature of the parent/Guardian's:……

Name of the person (Who signed):……..…..

Address:……

Date:…… Time:……

Appendix-B

The composition and methods of preparation of different media, chemicals and reagents used in this study are given below:

Media

1. Blood agar

Dehydrated blood agar base	:	40 gm
Distilled water	:	1000 ml
pH	:	6.8

Autoclaved at 121°C for 15 minutes under 15 Ibs. pressure. Cooled down at 45°C, 7% sheep blood (defribrinated, aseptically collected) was added, mixed well and poured into sterile petridishes inside the laminar flow.

2. MacConkey agar

Dehydrated MacConkey agar	:	50.0 gm
Distilled water	:	1000 ml

Media dissolved in distilled water, pH adjusted at 7.2 and autoclaved at 121°C for 15 minutes under 15 Ibs. pressure. After cooling to about 55°C, media is then poured into the Petri dishes inside the laminar flow.

3. Muellar – Hinton agar

Dehydrated Muellar – Hinton base	:	38.0 gm
Distilled water	:	1000 ml
pH	:	7.2 ± 0.2

Autoclaved at 121°C for 15 minutes under 15 Ibs. pressure. When cooled down at 45°C, media is then poured into the Petri dishes inside the laminar flow.

4. Triple sugar iron (TSI) agar media

Dehydrated TSI media	:	65.0 gm
Distilled water	:	1000 ml

The ingredients were dissolved by boiling the pH was checked, distributed into test tube in 5 ml. amounts and by autoclaving at 121°C for 15 minutes under 15 Ibs. pressure. The tubes were then allowed to cool and solidify in such a position which gave a slant and a thick butt.

5. Simmons citrate media

Dehydrated Simmons citrate media	:	24.2 gm
Distilled water	:	1000 ml
pH	:	6.8

Ingredients dissolved, dispensed into test tubes, autoclaved at 121°C for 15 minutes and allowed to set as slopes.

Appendix-C

Catalase test

Few isolated colonies having same morphology from the culture to be tested were picked up from the plate by a sterile toothpick and diped into 1 ml. of 3% hydrogen peroxide (H_2O_2) contained a small test tube. A positive reaction was evidenced by the immediate production of gas bubbles from the surface on the bacterial colonies diped with the wooden stick. *Staphylococcus aureus* ATCC 25923 was used as positive control and *Streptococcus* spp. as negative control.

Coagulase test

- **Slide Coagulase test**

  One drop of normal saline (0.85% Nacl) was placed on a clean glass slide and with a minimum of spreading; one or two colonies of test organism were taken and emulsified in the drop of saline to form a smooth milky suspension. One drop of undiluted human plasma was added to the bacterial suspension with the help of straight inoculating wire. Appearances of coarse clumping visible to the naked eye within 10 seconds indicate positive reaction. *Staphylococcus epidermidis* was used as negative control (Colle et al., 1996).

- **Tube Coagulase test**

Plasma was diluted 1 in 6 normal saline. 1 ml. of diluted plasma was taken in a clean test tube and a colony of strain under test was emulsified. The tube was then incubated at 37oC and tested to see clot formation by tilting the tube through 90oC after 1, 3 and 6 hours. The tubes showing negative reaction were kept overnight at room temperature and re-examined. Positive and negative controls were run side by side.

Oxidase test

A piece of filter paper was placed in a clean petridish and 2 to 3 drops of 1% freshly prepared oxidase reagent (tetramethyl-p-phenylene diamine dihydrochloride) was added. With a sterile glass rod 1 or two colonies of the test organism were taken and rubbed on the filter paper. Development of blue-purple within 10 seconds indicates positive test. *Pseudomonas aeruginosa* was used as positive control and *Escherichia coli* as negative control (Colle et al., 1996).

Appendix-D

All the organisms were identified by their colony morphology, staining characters, pigment production, motility and other relevant biochemical tests as per standard methods (Collee et al., 1996; Cheesbrough, 2002; Sonnenwirth and Jerret 1980)

S. typhi

Morphology and staining characters: Gram negative bacilli (2-4x0.6μm) in size.

Colony morphology: In MacConkey agar, pale colonies (non lactose fermenter) 1-3 ml in diameter (Old, 1996).

Motility Test: Motile

Biochemical test: Oxidase test- Negative, Nitrate reduction test – Positive, Indole – Negative, Voges-Proskauer reaction – Negative, Citrate utilization – Negative, Urease test – Negative, H_2S –Produced, Sugar fermentation tests: Glucose – positive with production of no gas, Lactose – negative, Mannitol – Positive, Maltose – Positive, Sucrose – Negative.
Final identification of *S. typhi* was done by slide agglutination test using specific antisera.

Klebsiella spp.

Morphology and staining characters: Straight and thick Gram-negative rods (1-2x 8 μm) in size.

Colony morphology: In MacConkey agar, large, mucoid, pink colonies (lactose fermenter)

Motility Test: Non motile

Biochemical test: Oxidase test- Negative, Nitrate reduction test – Positive, Indole – Negative, Voges-Proskauer reaction – Positive, Citrate utilization – Positive, Urease test – Positive. Gas – Produced, H_2S –Not produced. Sugar fermentation tests: Glucose – positive with reduction gas, Lactose – Positive, Mannitol – Positive, Inositol – Positive, Sucrose – Positive.

E. coli

Morphology and staining characters: Gram-negative straight rod (1.1-1.5x2-6μm).

Colony morphology: In MacConkey agar, circular, 1-3 mm in diameter low convex, smooth surface, pink (lactose fermenting) colonies (Crichton, 1996).

Motility Test: Motile.

Biochemical test: Oxidase test- Negative, Nitrate reduction test – Positive, Indole – Positive, Voges-Proskauer reaction – Negative, Citrate utilization – Negative, Urease test – Negative, Methyl red reaction – Positive. H_2S –Not produced, Sugar fermentation tests: Glucose – positive with reduction of gas, Lactose – Positive, Mannitol – Positive, Inositol – Negative, Maltose – Positive.

Pseudomonas spp.

Morphology and staining characters: Gram-negative rod 0.5-1μm).

Colony morphology: In MacConkey agar plate, non lactose fermenting colonies. In Nutrient agar, blue green pigment production. Culture produces characteristic grapelike smell of amino-acetophenon (Govan, 1996).

Motility Test: Motile.

Biochemical test: Oxidase test- Positive, Citrate utilization – Positive, H_2S –Not produced, Arginine dihydrolase test – Positive, Lysine decarboxylase test – Negative, Ornithin decarboxylase test – Negative.

Acinetobacter spp.

Morphology and staining characters: Gram-negative coccobacillary to coccal in appearance, 1-1.5x1.5-2.5 μm, frequently arranged in pairs (Allen et al., 2000).

Colony morphology: In MacConkey agar plate, colonies are non pigmented, domed and mucoid with smooth to pitted surface.

Motility Test: Non motile.

Biochemical test: Oxidase test- Negative, Nitrate reduction test – Negative, Indole – Negative, Catalase test – Positive, H_2S –Not produced.

Serratia spp.

Morphology and staining characters: Gram-negative rods 0.6-6.0x0.3-1.0μm.

Colony morphology: In MacConkey agar, colonies are convex, red pigmented with relatively opaque center.

Motility Test: Motile.

Biochemical test: Oxidase test- Negative, Citrate utilization – Positive, Voges-Proskauer reaction – Positive, Indole – Positive, Urease test – Negative, Gelatin liquefaction test – Positive, H_2S –Not produced.

S. aureus

Morphology and staining characters: Gram positive cocci of uniform size approximately 1µm in diameter occurring in groups or clusters.

Colony morphology:

In blood agar, colonies are smooth, low convex, densely opaque 1-3mm in diameter. In blood agar in narrow zone of haemolysis surrounds colonies. Pigmentation of colonies ranges from cream through buff to golden yellow (Baird, 1996).

Biochemical test:

Catalase test – Positive, Coagulase (both slide and tube) test – Positive, Oxidase test- Negative.

Enterococcus spp.

Morphology and staining characters: Gram positive cocci in pair or short chains approximately 0.5-1 mm in diameter.

Colony morphology: In blood agar, colonies are circular, low convex and semi transparent.

Biochemical test:

Catalase test – Negative, Coagulase (both slide and tube) test – Negative, Oxidase test- Negative, Bile solubility-positive.

Appendix-E

Turbidity standard equivalent to McFarland 0.5

This is a barium sulphate standard against which the turbidity of the test and control inocula can be compared. When matched with the standard, the inocula should give confluent or almost confluent growth. Standard was shaken immediately before use.

Composition and preparation

1% (v/v) solution of chemically pure (0.36 N) sulphuric acid and 1.75% (w/v) solution of chemically pure (0.048 M) barium chloride was prepared in nutrient broth in two separate sterile flasks. Then 9.9 ml. of sulphuric acid and 0.1 ml. barium chloride were added to the clean screw capped test tube and sealed. The barium chloride suspension corresponds approximately to MacFarland standard tube no. 1 with corresponding cell density of $3x10^8$ organisms/ml.

To make the turbidity standard of cell density to one half of the MacFarland standard tube no. 1 which corresponds to cell density of 1.5×10^8 organisms/ml. for determination of antibiotic sensitivity by Kirby-Bauer technique, 0.5 ml of 1.75% (w/v) barium chloride was added to 99.5 ml. of 1% (v/v) sulphuric acid (0.36N) mixed well and 5-10 ml. was distributed in sterile screw capped test tubes and sealed.

Appendix-F

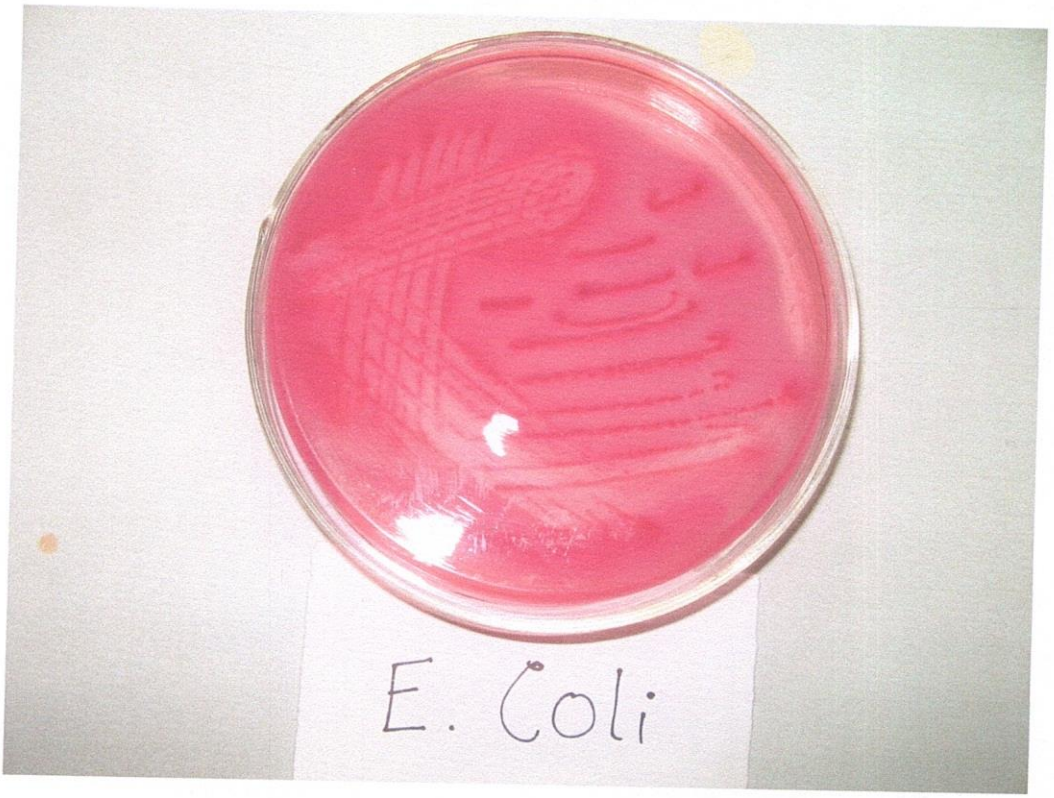


Fig.-6: Growth of *E. coli* on MacConkey agar media.

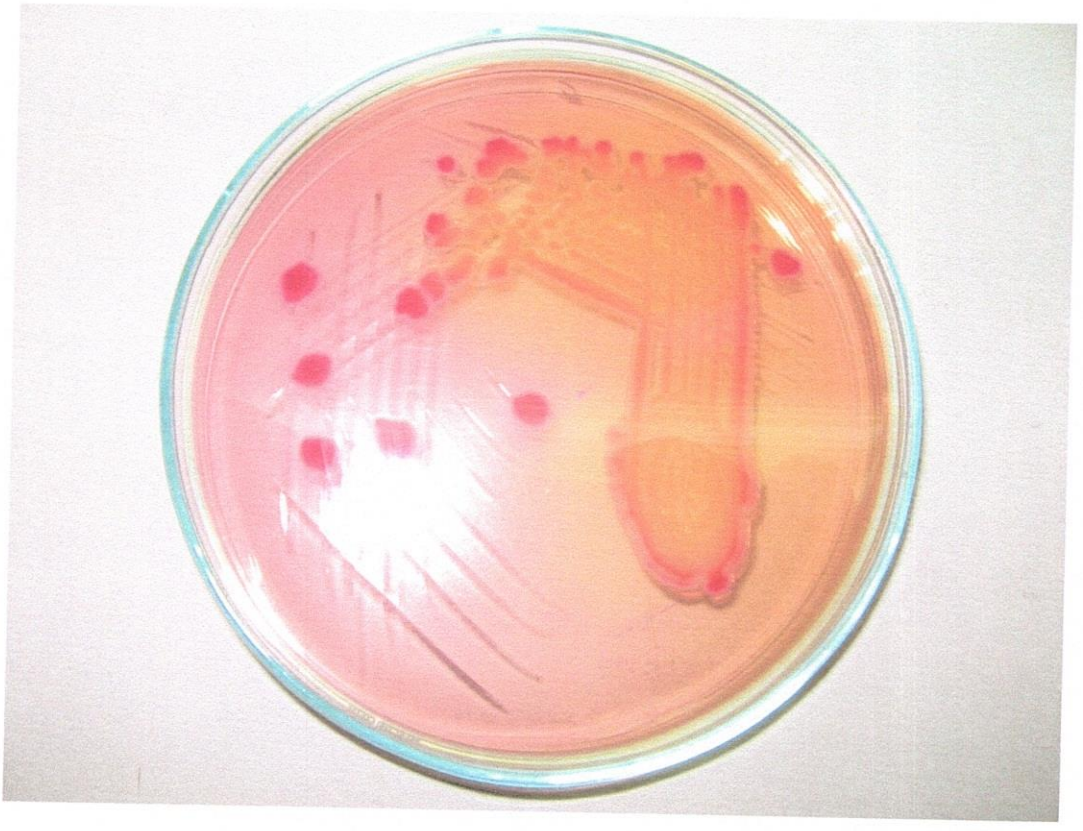


Fig.-7: Growth of *K. pneumoniae* on MacConkey agar media.

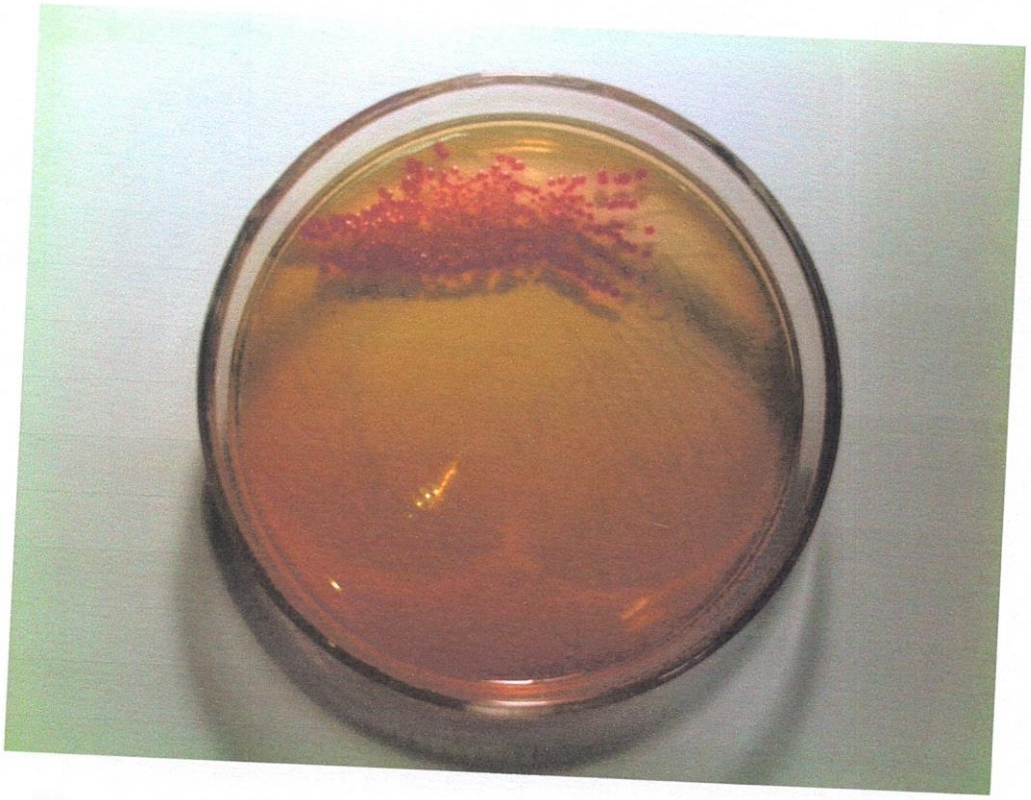


Fig.-8: Growth of *S. marcescens* on MacConkey agar media.

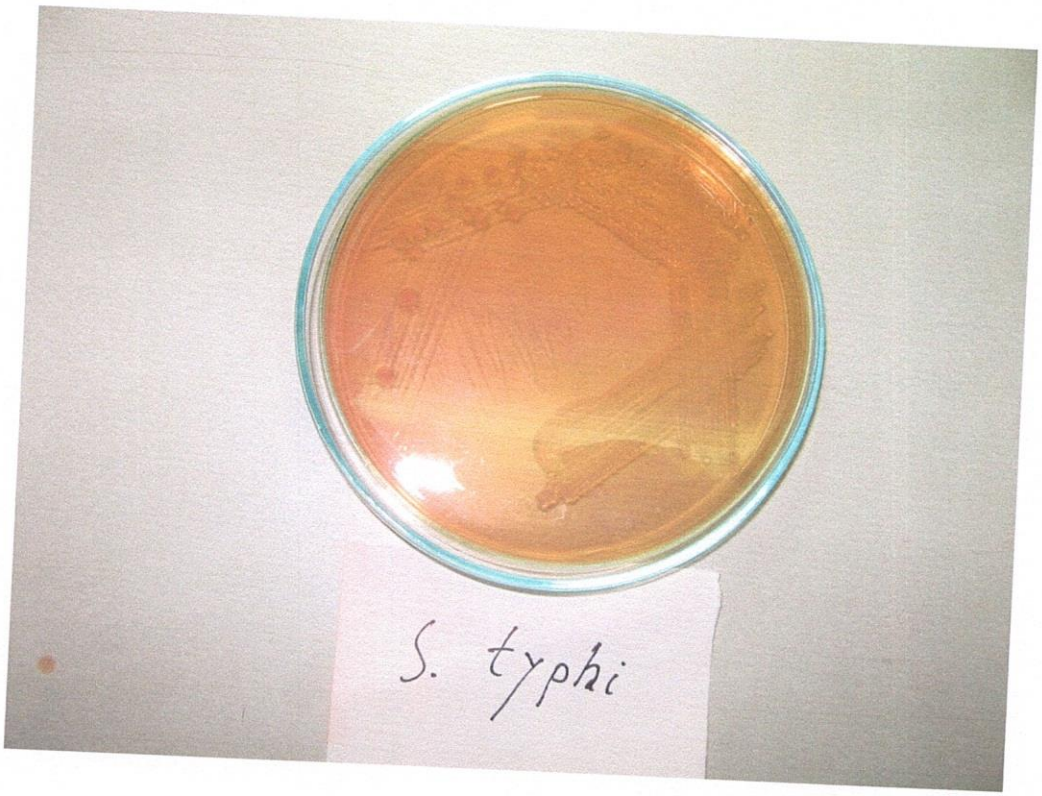


Fig.-9: Growth of *S. typhi* on MacConkey agar media.

Fig.-10: Growth of *Pseudomonas* spp. on MacConkey agar media.

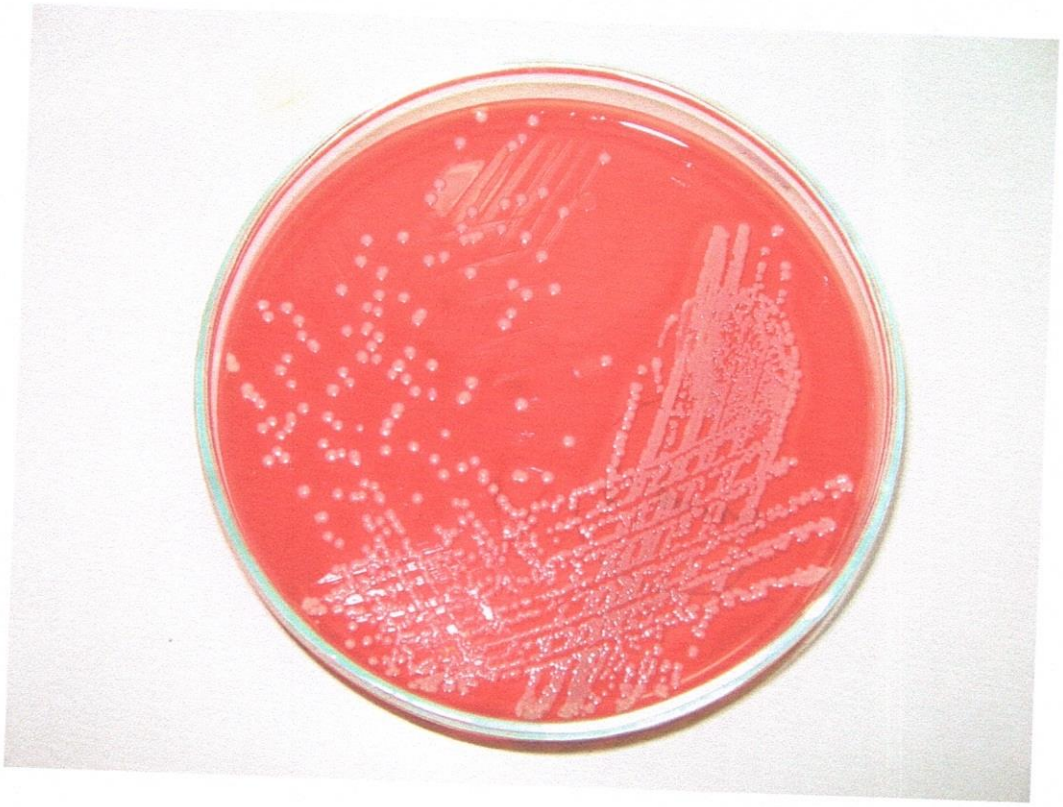


Fig.-11: Growth of *S. aureus* on Blood agar media.

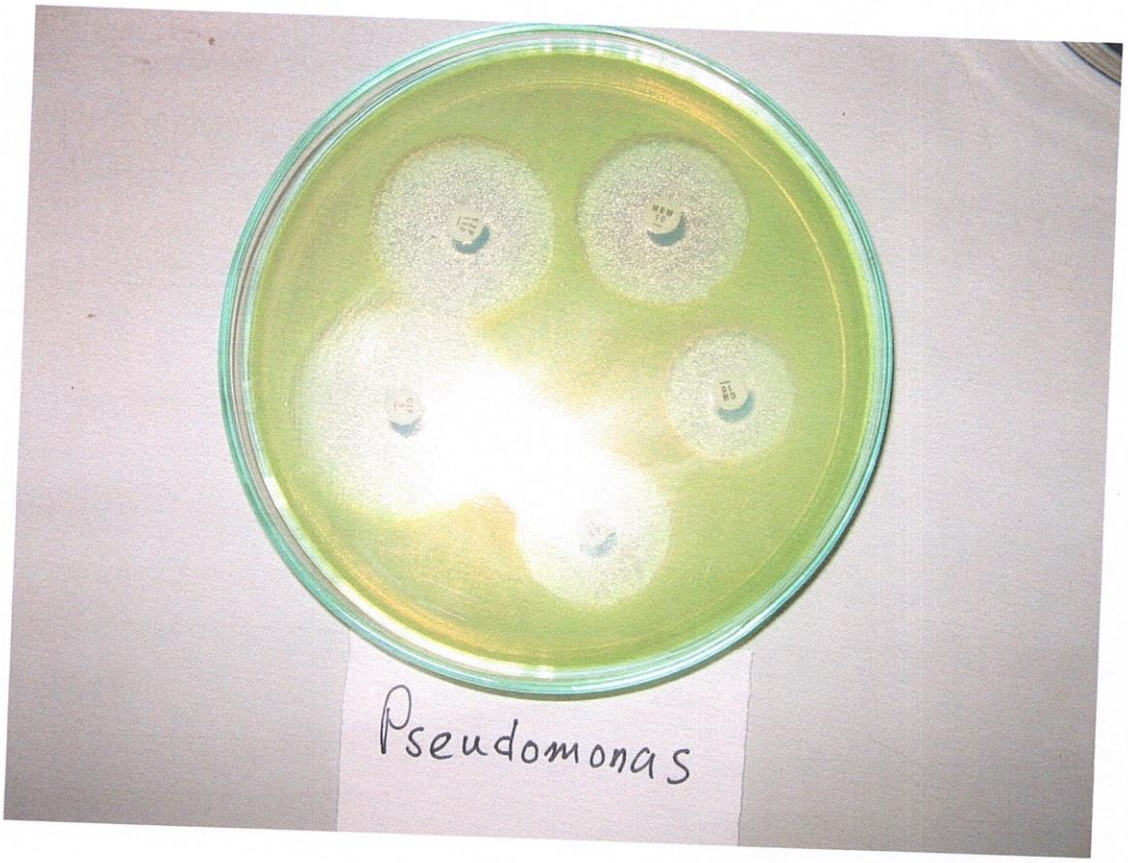
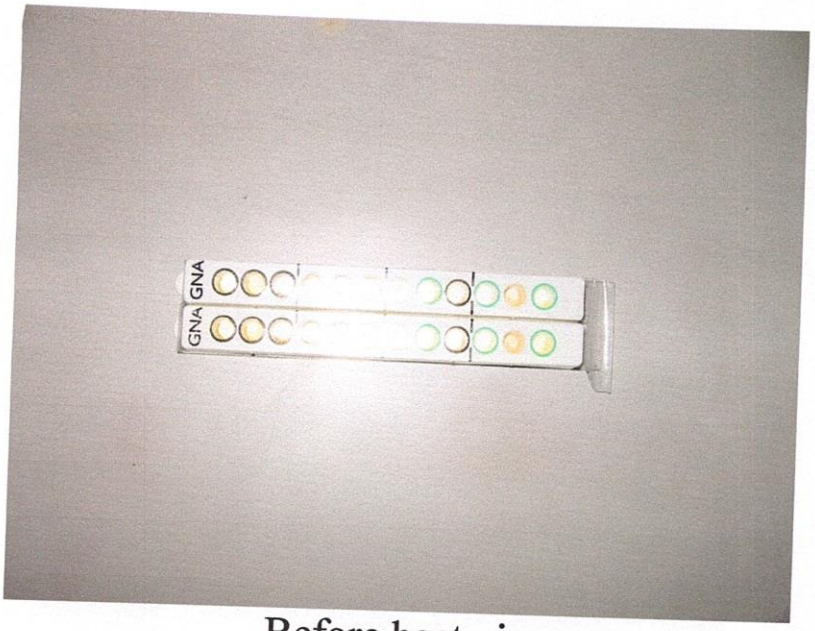
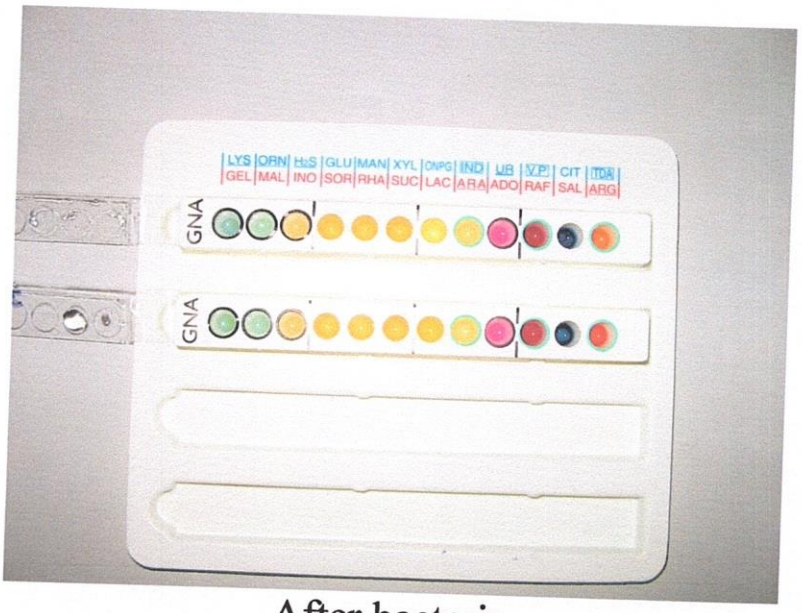


Fig.-12: Antibiogram of *Pseudomonas* spp. in Muellar- Hinton agar media.



Before bacteria



After bacteria

Fig.-13: Microgen strip for biochemical test.

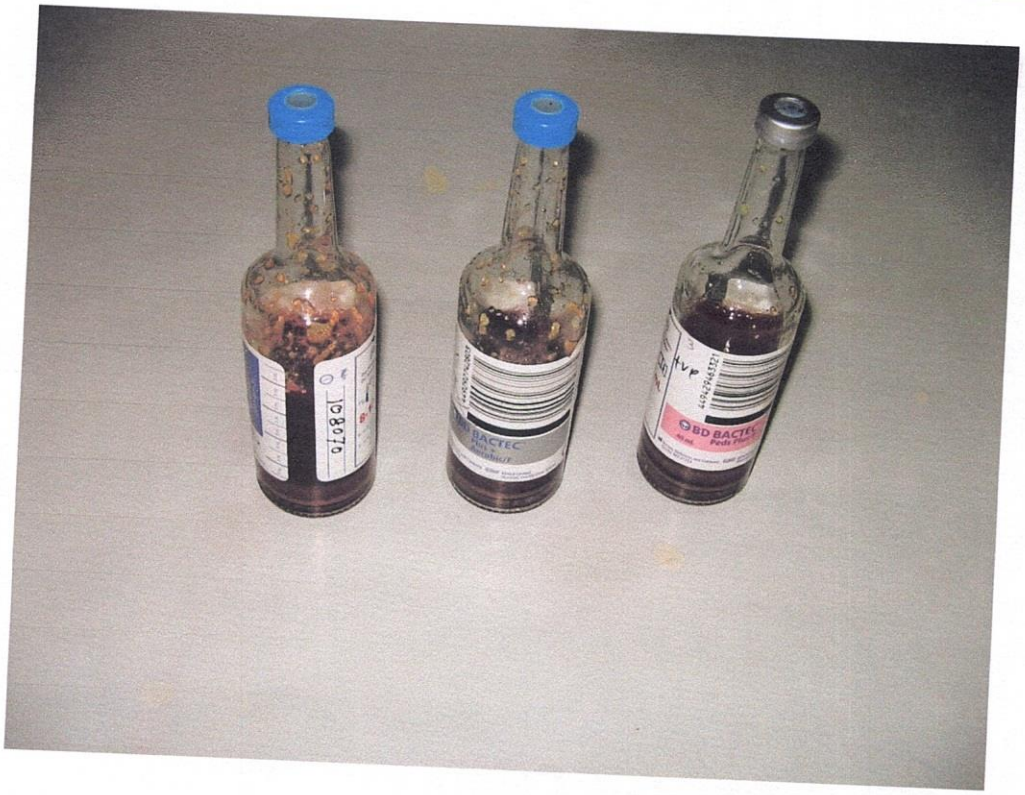


Fig.-14: Blood culture vial.

Fig.-15: 6.5% Sodium chloride & Bile aesculin