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Bacteriological and Immunological Study of Some Sepsis Biomarkers among Bacteremic Patients in Hilla City

A thesis

Submitted to the Council of the College of Science, University of Babylon as a Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيم

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SUMMARY

Summary

Bacteremia is a bacterial infection that has spread from the primary site of infection and reached the bloodstream. Continuously present bacterial agents within the circulation can cause serious body illnesses leading to sepsis, particularly when the laboratory procedures fail to recognize the actual etiological agents. Sepsis is a complex inflammatory response that is strongly associated with multiple organ dysfunction and considered as a major cause of morbidity and mortality worldwide. Bacteremia and sepsis confirmation can achieve using many assays including cultural, immunological, and molecular methods.

The current study aims to evaluate the potential sepsis biomarkers among culturable and unculturable bacteremia among clinically suspected sepsis patients. Blood culture and the PNEUMOBACT IFA test were used to detect the culturable and unculturable causative agents of bloodstream infection. Complete Blood Count (CBC), C-Reactive protein (CRP) and Lactate dehydrogenase (LDH) were used as valuable routine screening test for bacteremia/septicemia while Procalcitonin (PCT), Presepsin (PSN) and Pentraxin-3 (PTX3) were used as three potential biomarkers of sepsis.

A total of 100 blood samples from patients with symptoms of sepsis who resided in the Hilla City hospitals were collected as well as 25 samples were collected from healthy persons as a control group. Each blood sample was divided into three containers: 2 ml in an EDTA tube for the CBC test, 3ml in a centrifuged tube to separate serum for the CRP, LDH, PCT, PSN, PTX3 and IFA test, and 5 ml in brain heart infusion broth (BHI) for blood culture.

The result showed that 65/100 (65%) samples had abnormal (elevated CBC parameters, CRP and LDH) screening tests, whereas only 25/65 (38.4%) of them

gave positive results for blood culture. All control group persons have normal results of CBC, CRP and LDH and they gave negative results for blood culture. The 25 bacterial isolates identified by Vitek 2 compact system were: *Staphylococcus aureus* 6 (24%), *Staphylococcus epidermidis* 4 (16%), *Stenotrophomonas maltophilia* 2 (8%), *Escherichia coli* 2 (8%), *Streptococcus pneumonia* 2(8%), *Salmonella typhi* 1(4%), *Staphylococcus hemolyticus* 1 (4%), *Klebsiella pneumonia* 1(4%), *Morganella morganii* 1(4%), *Pseudomonas aeruginosa1*(4%), *Staphylococcus hominis* 1(4%), *Kocuria kristinae* 1(4%), *Enterobacter aerogenes* 1(4%), and *Pseudomonas stutzeri* 1(4%). Most of these isolates were multidrug resistant when tested with the chosen antimicrobial agents.

Regarding routine screening tests (CRP, CBC, and LDH), statistical analysis shows a highly significant difference (P-value ≤ 0.001) between patients (n=65) and control groups (n=25). Non-significant differences in those parameters were observed between culture-positive and culture-negative patients (P-value > 0.05). on the other hand, these parameters had a significant strong correlation among them. In addition to that, there were no significant differences between males and females regarding CBC, CRP, and LDH for those who gave culture positive (P> 0.05). Males and females who gave culture negative also did not differ significantly (P> 0.05).

The serum levels of PCT, PSN, and PTX3 were determined by the ELISA test during the next part of the study. Statistical analysis (Duncan test) reveals that the control group had a highly significant difference from the patients (CPS and CNS) regarding PSN and PCT concentration (having different small letters in one row), while the difference was non-significant for the PTX3 concentration among patients and control group (having the same small letters in one row). Furthermore,

PSN and PCT concentrations did not significantly differ between the culture positive and culture negative groups of sepsis. The PSN and PCT concentrations have a strong positive correlation between them ($r = 0.500$; $P < 0.001$).

The results of the indirect immunofluorescent assay revealed that, 36/40 (90%) out of 40 culture-negative serum samples had anti-bacterial antibodies identified based on this method, and these antibodies related to: *Legionella pneumophila*, *Chlamydia pneumoniae, Mycoplasma pneumoniae*, *Coxiella burnetii* , *Chlamydia psittaci*. Co-infections were present (each test gave positive reactions to different specific antibodies that belonged to more than one microorganism).

The results of all investigated parameters revealed that, no single parameter is ideal enough for sepsis identification, and even though blood culture is most commonly used as a diagnostic method for detecting bacteremia, this method is insufficiently sensitive alone. Hence, multiple methods must be used in order to identify the actual source of sepsis.

List of Contents

Chapter one introduction and Literature Review

List of Contents

List of Contents

List of Contents

List of Contents

List of Figures

List of Tables

List of Tables

List of Abbreviations

No.	Symbol	Definition
1	Ab_S	Antibodies
2	Ag_S	Antigens
3	BHI	Brain heart infusion
4	CA	Chocolate agar
5	CAP	Community-acquired pneumonia
6	CARS	Compensatory anti-inflammatory response syndrome
7	CBC	Complete blood count
8	CLSI	Clinical and laboratory standard institute
9	CNS	Culture negative sepsis
10	CPS	Culture positive sepsis
11	CRP	C-reactive protein
12	DNA	Deoxyribonucleic acid
13	EDTA	Ethylenediamine tetra acetic acid
14	ELISA	Enzyme linked immunosorbent assay
15	EMB	Eosin methylene blue agar
16	FITC	Fluorescein isothiocyanate
17	GAS	Group A streptococcus
18	GRA	Granulocyte
19	HCT	Hematocrit
20	HGB	Hemoglobin
21	HRP	Horse radish peroxidase
22	ICU	Intensive care unit
23	IFA	Indirect immunofluorescent assay
24	IFN	Interferon
25	IgG	Gama Immunoglobulin.
26	IgM	Macro Immunoglobulin.
27	IL	Interleukin

List of Abbreviations

No.	Symbol	Definition
28	IV	Intravenous
29	LDH	Lactate dehydrogenase
30	LPS	Lipopolysaccharides
31	LRT	Lower respiratory tract
32	LYM	Lymphocyte
33	MAC	MacConkey agar
34	MALT	mucosa-associated lymphoid tissue
35	MCH	Mean Corpuscular Hemoglobin
36	MCHC	Mean Corpuscular Hemoglobin Concentration
37	MCP-1	Monocyte chemoattractant protein 1
38	MCV	Mean Corpuscular Volume
39	MHC	Major-histocompatibility-complex.
40	MR	Methyl red
41	MSA	Mannitol salt agar.
42	NF-κB	nuclear factor-kappa B.
43	NLRs	Nod-like receptors.
44	OD	Optical density
45	PAF	Platelet activating factor
46	PAMP	Pathogen associated molecular pattern
47	PBS	Phosphate buffer saline
48	PSI	Pound per square inch
49	PCR	Polymerase chain reaction
50	PCT	Procalcitonin
51	PGL	Peptidoglycan layer
52	PIA	Pathogenicity island
53	PLT	Platelets
54	PRR	Pattern recognition receptor

List of Abbreviations

No.	Symbol	Definition
55	PSN	Presepsin
56	PTX 3	Pentraxin 3
57	RCU	Respiratory care unit
58	RDW	Red cell distribution width
59	SIRS	Systemic inflammatory response syndrome
60	SPE	Staphylococcal pyrogenic exotoxin
61	SPI	*Salmonella* pathogenicity island
62	TGF	Transforming growth factor
63	TLR	Toll-like receptors.
64	TNF	Tumor necrosis factors
65	TSS	Toxic shock syndrome
66	UPEC	Uropathogenic *Escherichia coli*
67	UTI	Urinary tract infection
68	VP	Voges-Proskauer
69	WBC	White blood cell
70	WHO	World Health Organization
71	XLD	Xylose lysine deoxycholate agar

Chapter one

Introduction
and
Literature Review

1.1. Introduction

Bacteremia is a bloodstream infection. It can evolve into many clinical spectrums, and it is differentiated as septicemia. If left untreated, it can progress to systemic inflammatory response syndrome, sepsis, septic shock, and multiple organ dysfunction syndrome (Corey, 2009; Butler-Laporte *et al*., 2018).

Human usually acquired bacteria either because of normal activities such as toothbrushing, dental procedures or from infection due to use of indwelling genitourinary or IV catheters, after wound, burns or others for example, urinary tract infection. Bacteremia that occurs during ordinary activities does not result in infections because bacteria are present in small numbers and are quickly cleared from the bloodstream by the immune system (Cantor and Kainth, 2019).

However, bacteremia can lead to other infections and occasionally induce a catastrophic body-wide response called sepsis if bacteria are present long enough and in large enough quantities, especially among the elderly or those who have a weakened immune system (Mammen *et al*., 2018; Paul, 2020). Bacteremia can be caused by a wide range of bacteria entering the human body and reaching the bloodstream, such as enteric bacteria, *Staphylococcus aureus*, and coagulase negative *Staphylococcus epidermidis* (Kleinschmidt, *et al*., 2015).

Sepsis represents an uncommon systemic reaction that most likely indicates a pattern of immune system response to injury by pathogens such as fungi, viruses, or bacteria, with mortality risk ranging from moderate to considerable depending on a variety of pathogen and host factors, as well as the promptness with which the disease is recognized and treated. A

dysregulated immune response to infections causes a clinical condition of life-threatening organ failure (Singer. *et al*., 2016).

When a dangerous and sometimes fatal sickness develops, it must be diagnosed as soon as possible so that it can be treated. Even though Blood culture is the most used diagnostic method for detecting bacteremia (Nannan *et al*., 2019). Sometimes blood culture insufficiently sensitive when the patient has already received antibiotics or in the presence of fastidious organisms that cannot grow in normal conditions, and in case of unculturable bacteria (Peters *et al*.,2004; Peker *et al*., 2018; Preiswerk *et al*., 2020).

Numerous research has been proved that several biomarkers may support the recognition of sepsis patients and reduce the mortality rate associated with severe sepsis (Faix, 2013; Hassuna *et al*., 2021; Velissaris *et al*., 2021).

The routine non-specific tests for sepsis include the complete blood count parameters (CBC), cytokines (tumor necrosis factor, interleukin 1β and interleukin 6), C-reactive protein (CRP), and Lactate dehydrogenase (LDH) (Akin and Akgun, 2021). Many studies propose Procalcitonin (PCT), Pentraxin3 (PTX3), and presepsin (PSN) as specific potential biomarkers for bacterial sepsis identification (Behnes *et al*., 2014; Song *et al*., 2019; Conway-Klaassena *et al*., 2020). An indirect immunofluorescent method (IFA) has also been shown to provide evidence for confirming infection by unculturable atypical bacteria.

Aim of the Study

The current study aims to evaluate the potential sepsis biomarkers in culturable and unculturable bacteremia among clinically suspected sepsis patients. Blood culture and PNEUMOBACT IFA test will be used to detect culturable and unculturable causative agents of bloodstream infection.

Complete Blood Count (CBC), C-Reactive protein (CRP) and Lactate dehydrogenase (LDH) will be used as valuable routine screening test for bacteremia/septicemia while Procalcitonin (PCT), Presepsin (PSN) and Pentraxin-3 (PTX3) will be used as three potential biomarkers of sepsis. The study was designed depending on understandable criteria and via the following objectives:

1. Blood sampling, processing, and separation of the serum.
2. Measurement of CBC, CRP, and LDH as routine screening tests for septicemia.
3. Culturing of blood Samples by manual and automated blood culture device.
4. Determination of the antibiotic susceptibility for each bacterial isolates by disk diffusion method.
5. Detection of unculturable bacteria using PNEUMOBACT IFA IgM and IgG (*L. pneumophila*, *M. pneumoniae*, *C. burnetii*, *C. pneumoniae*, and *C. psittaci*).
6. Measurement the serum level of procalcitonin (PCT), pentraxin3 (PTX3), presepsin (PSN) among patients with culturable and unculturable bacteremia as well as healthy control.

1.2. Literature Review

1.2.1. Bacteria: Their Existence and Significance

Bacteria are unicellular, invisible, microscopic germs present everywhere on our planet, including soil, water, and even in or on the human body. A few groups of them are dependent on other living things to survive (Gracelin, 2021). Although there is lack nucleus and organelles, their genetic information is contained in a single nucleic acid. Many bacterial species could acquire an extra genetic material by horizontal genetic transfer, such as plasmids and bacteriophages, which gives the bacteria an advantage over others (Pfeifer *et al*., 2021). The availability of entirely sequenced genomes related to pathogenic and non-pathogenic has substantially advanced our understanding of genome organization in recent years (Hochhut *et al*., 2005).

Whereas many bacteria are benefit or have no effect however, certain potentially harmful bacteria can have serious consequences for people, and they may be the source of a variety of infectious diseases (Lorenz *et al*., 2020). The terms pathogenic and non-pathogenic bacteria were coined based on the Koch's Postulates to distinguish between them. The fundamental difference between these two groups is that the pathogenic bacteria have several virulence factors encoded by additional genes that give them the ability to cause diseases, while nonpathogenic bacteria lack such genes (Naor-Hoffmann et al., 2022). Some virulence factors are encoded within pathogenicity islands for example *staphylococcus* islands which encode superantigens leading to toxic shoch syndrome consequence in infected human (Novick *et al*., 2001; Novick and Ram, 2017). The acquisition of virulent genes that are normally expressed as effector proteins such adhesins, toxins and invasins may make it easier for bacteria to get into the bloodstream, resulting in bacteremia and meningitis.

Classically, human acquired pathogen via different sources either by direct or indirect contact. When a human is accidently exposed to these bacteria, the consequence of that exposure is determined by three essential determinants: (a) agent infectivity and virulence (b) host immunity and susceptibility to infection (c) Environmental factors that render the host more vulnerable to exposure, such as physical and social behavior (Van Seventer, and Hochberg, 2017). During the course of infection, when the living microorganisms reach to the blood stream it can become high risk of mortality and morbidity. In fact, bacterial invasion of the blood circulation is one of the most serious outcomes of infection leading to medically critical disorders and possibly developing into more serious diseases such as endocarditis (Keynan and Rubinstein, 2013).

1.2.2. Bacteremia and Its Consequence

The term bacteremia refers to the occurrence of bacteria in the bloodstream, while septicemia or sepsis refers to the catastrophic outcome of bacteremia (Munford, 2006).

Numerous species of pathogenic bacteria can enter the body of the host through different portal of entry and disseminate from the local site of infection to reach another tissue or organ if the immune system is disabled for self-recovery or absent correct diagnosis and misuse of antibiotics. Bloodstream infections are connected with high morbidity and mortality worldwide (Seifert, 2009). Based on the period of the infection, bacteremia can be categorized as transient, intermittent, or persistent (Hafez *et al*., 2019). Transient bacteremia is characterized by the presence of bacteria in the blood stream for a few hours or minutes before being eliminated by the intrinsic immunity of a healthy individual. It is usually caused by dental procedures, stomach biopsies, or brushing of the teeth. Intermittent bacteremia refers to the discontinuous presence of the same microorganisms that cause infections in the same patient because of clearance and return such

as abscesses. However, the clinical picture of persistent bacteremia is connected to the ability of pathogen to survive in the circulatory system for an extended period of time leading to serious outcome like endocarditis (Horstkotte *et al*., 2004). Persistent bacteremia can also develop during the early phases of systemic illnesses including Malta and typhoid fever. Generally, the long-term existence of bacteria in the blood stream may lead to seriously ill phase of infection known as sepsis (Minasyan, 2017).

1.2.3. The Concept of Sepsis

Our comprehending of the sepsis and its complications has increased over time, as has our capacity to define sepsis. Over the last 30 years, sepsis management has changed dramatically. The term sepsis comes from the Greek word which means "decomposition". It was later employed by Hippocrates, Galen, Fleming, and other researchers in their publications (Funk *et al*., 2009). The "Germ theory" of disease was proposed in the nineteenth century, and there was some acknowledgment that sepsis was caused by serious microbes. In 1914 Hugo made the first effort at a current definition (Gül *et al*., 2017). Several previous studies conducted throughout the twentieth century were able to provide a noticeable history of sepsis while also describing the infectious pathogens and pathogenesis caused by microbial toxins and host mediators. In fact, the risk of sepsis is correlated to the occurrence of immune system dysregulation, which lead to the development of dangerous consequences. Although the infectious pathogen represents the main causative agent, the manifestation of sepsis can also be related to the hyper-inflammatory responses of the immune system, which include profuse production of cytokines, significant alterations in the coagulation cascade, and several other biomarkers that eventually lead to hypotension and septic shock. Because of the complexity of the illness process, identifying, treating, and studying sepsis has been difficult. However, our knowledge of the sepsis concept and its serious complications

has become much more developed especially at the recent years. Cell biology, biochemistry, immunology, and organ function are all more understood now than they were before. This knowledge has resulted in improved sepsis management and a shift in sepsis epidemiology (Gyawali *et al*., 2019). Currently, researchers were used some sepsis biomarkers in their investigations to confirm the relationships between these biomarkers and the development of sepsis symptoms for monitoring and promptly diagnosing the causative agent of illness.

1.2.4. Characteristics of Sepsis

Sepsis and its manifestations are of substantial clinical significance because, they are linked to high rates of morbidity and mortality. Sepsis begins as an infection of sterile blood stream (bacteremia) and gradually develops if proper diagnosis and treatment are delayed. Since infection trigger both innate and adaptive immune system in response to pathogen-associated pattern, sepsis may emerge as a hyper-inflammatory state leading to the immune system down regulation (Henriquez-Camacho and Losa, 2014). Severity of sepsis ranges from mild inflammation with few clinical effects to multiorgan dysfunction in septic shock, which is associated with a high death rate. According to a study conducted in the United States in 2001, total mortality in sepsis is estimated to be around 30% (Angus *et al*., 2001).

Despite advances in medicine, the frequency of sepsis continues to rise. According to several epidemiological research, exogenous Gram-positive bacteria are more complicated in most cases of sepsis than that of Gram-negative bacteria. This episode could be due to improved therapy against Gram-negative pathogens and the spread of antibiotic resistant Gram-positive (Martin *et al*., 2003). Numerous bacteria have evolved to move from the primary to secondary site of infection and disseminate through host tissue, eventually reaching the bloodstream and eliciting a hyper-inflammatory response, resulting in sepsis. *Staphylococcus aureus* and

group-A Streptococcus pyogenes (GAS) are two of the most serious bacteria in this area, as they release exotoxins that cause hyper-inflammatory response that led to toxic shock syndrome (TSS), which is characterized by hypotension and multiorgan dysfunction (Darenberg, 2006).

1.2.5. potential Source of infection

Numerous research documented the presence of bacteria in everywhere and their ability to transfer as well as adapted themselves under any changeable condition (Kaláb *et al*., 2008). The acquisition of bacterial infection occurs through an environmental source that has been classically considered either nosocomial or community-acquired and then reaches the bloodstream in a number of different ways otherwise, the blood stream is normally sterile (Loonen *et al*., 2014). However, there are the specific route of entry for each major group of bacteria. Based on the source of bacterial infection, bacteremia placed in to two groups: (a) primary or healthcare-related bacteremia denotes to the infections result from directly introducing of exogenous pathogen into the previously intact human body by contaminated needle, catheters or any medical device and invasive procedures for example, *Streptococcus epidermidis*. (b) secondary or habitat-related bacteremia refers to the infections caused by the entry of pre-existing bacteria from another site of infection (endogenous) such as through breach the human body through injury, burns, trauma or from gastrointestinal, respiratory, or genitourinary tract for example, *Pseudomonas aeruginosa* (Carroll *et al*., 2019). The main classes of bacteria which causes bacteremia are:

1.2.5.1. Gram Positive Bacteria

The incidence of Gram-positive bacterial infections is gradually rising and represents an important cause of bacteremia due to the normal presence of most of them on the skin. The best and more common example is *Staphylococcus epidermidis*, which can form bifolium on the plastic medical

device through which it enters the circulatory system. Methicillin-resistance Staphylococcus aureus highly correlated with the hospital-acquired Gram-positive bacteria (Cervera *et al*., 2009). Streptococcus and enterococcus species are also implicated in bacteremia infections.

1.2.5.2. Gram Negative Bacteria

Gram-negative bacteria cause half of all community-acquired bacteremia, with the majority being isolated from elderly or immunocompromised patients. Gram negative bacteria usually enter the bloodstream as a result of infections in the respiratory, urinary, or gastrointestinal tract (High and Kevin, 2017). *E. coli* is the most common isolate from UTI patient which represent clinically important etiologic agent of bacteremia. other Gram-negative Enterobacteriaceae family that can cause bacteremia infections include *Salmonella typhi, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis* (Deen. *et al*., 2012).

1.2.5.3. Atypical Bacteria

Atypical bacteria include bacteria that do not color with Gram and ziehl-neelsen stains, as well as bacteria that are unculturable and require special nutrition and growing conditions. *Mycoplasma, Legionella, Chlamydia*, and *Coxiella* are the most prevalent species of these pathogenic bacteria found throughout the world. Gram-positive and Gram-negative bacteria have thick and thin peptidoglycan layers, which allow them to easily obtain their unique color after treatment with the basic and counter stain, depending on the thickness of their PGL layers. peptidoglycan is important for the most common bacterial species due to its critical role in cell division and sustaining cell shape. However, atypical bacteria lack PGL layers such as *Mycoplasma* or contain too thin of this layer preventing visible such as *Chlamydia* (Liechti *et al*., 2014).

Most of these atypical bacteria are implicated in lower respiratory tract

infections, which can cause a particular type of illness known as atypical pneumonia, recent research has revealed the existence of a synergistic interaction between atypical bacteria and common cold virus, coinfection emerged as a result of this interaction (Mina *et al*., 2014). While it is impossible to isolate these bacteria and extract them from clinical samples via cultivation, the standard method for detection depends on the use of polymerase chain reaction (PCR) and/or immunofluorescent assay (IFA). Direct immunofluorescent used primary IgM/IgG antibodies conjugated to a specific fluorophore to label the target antigen in patient sera, whereas indirect immunofluorescent used primary unconjugated and secondary fluorophore-conjugated antibodies directed against antigen- specific antibody. Indirect mode of detection is more sensitive (Im *et al*., 2019).

1.2.6. Route of Entry

The upper respiratory tract is regarded as one of the most common portals of entry for infections transmitted directly from person to person or indirectly through contact with air, food, or waterborne pathogens. Other community sources of infection include blood, sexual, or vector borne as well as environmental situations such as burns and injuries. A medical device could be a source of infection in the case of a nosocomial infection (Doron and Gorbach, 2008).

The various portal of entry for different types of pathogens include:

1- Respiratory tract throughout the mucous membranes of the oral and nasal cavity.
2- Skin in cases of burns, wounds, vectors, or medical devices.
3- Genitourinary tract, either through direct contact or due to the use of a medical device.

1.2.7. Risk Factors

In general, an infection acquired through poor population practices (personal hygiene) or exposure to healthcare facilities (Horcajada et al., 2013). Extreme age and underlying illnesses, as well as treatments and procedures whose primary purpose is to restore health, are all factors that predispose an individual to bloodstream infection (Hernández *et al*., 2015). Although premature newborns and the elderly are the main risk factors due to deficiency of normal defense mechanisms related to immaturity or senescence of the immune system respectively, there are several other factors associated with increased bacteremia which include malignant disease, renal failure, diabetes, cirrhosis, and patients who have lost their skin, which serve as the first line of defense, as a result of burns or injuries. Regarding healthcare procedures, surgery, intravascular and urinary catheters could be a prominent source of infectious bacteremia. Moreover, certain therapeutic agent such as corticosteroids and chemotherapy alter the efficacy of adaptive immune system resulting an increase the risk of infection with intracellular pathogen (Zembower, 2014).

1.2.8. Virulence Factors Implicated in the Infection

Certain pathogenic bacteria evolve a wide variety of proteins, enzymes, toxins, or other molecules that assist them to attach, survive, and grow in their hosts. Despite the presence of host defense mechanisms, these molecules promote the ability of bacteria to evade immune response, invade the tissue, and then cause host damage. Thus, virulence factors of bacteria are responsible for determining bacterial pathogenicity (Ribet and Cossart, 2015). Although the human body has a huge number of bacteria, they are restricted to select bodily locations in healthy people, such as the skin, oral and nasal cavities, the vaginal as well as microflora within gastrointestinal

area (Schommer and Gallo, 2013), otherwise the internal tissues and body fluid are normally sterile. The mucosa of the respiratory, digestive, and urinary tracts are all key sites of bacterium interaction. This mucosa act as first line of defense, preventing invasion bacteria. A mucus layer covers the epithelia and interacts with the extracellular ambient, and hence with bacteria, providing protection against any intruders due to its composition of mucins, antimicrobial peptides, and secretory antibodies (McGuckin *et al*., 2011). Most pathogenic bacteria are either resistant to antimicrobial peptides or have flagella that make them capable of moving through this mucus layer to reach epithelial cells (Celli *et al*., 2009), hence a difficult problem arises during host infection. However, renewal epithelial cell plays an essential role in the prevention of bacterial infection. In the gut, there is a delicate balance between the production of new epithelial cells and their removal. Replacement of the infected epithelial cells is used by the host to limit infection by the intestinal pathogens (Kim *et al*., 2010). However, some bacteria can induce cell death in order to gain access to deeper tissue. Other crucial virulence factors are pili, which are hair-like structures protrude from the outer surface of many bacterial species. pili assist bacterial adhesion and colonization of mucosal tissue and initiate the first step of bacterial infections. moreover, sex pili also known as conjugative pili responsible for cell-to-cell interaction to permit the transfer of genetic materials between bacterial cells via conjugation. In addition to that, some pathogenic bacteria, such as *pseudomonas aeruginosa*, characterized by the presence of type IV pili on their surface, act as invasion factors and aid in movement, allowing bacteria to spread in the tissue by a process called twitching motility (Jonson *et al*., 2005). With regard to bacterial toxins, enzymes, and other virulent proteins, each of which has a particular function during infection events. In many cases virulence determinants are encoded by bacterial genome when

pathogens sense and respond to several environmental signals such as, temperature, oxygen, PH, and iron availability (Piattelli *et al*., 2020).

1.2.9. Virulence Expression and Gene Regulation

The availability of entirely sequenced bacterial genomes, including those of pathogenic species, has substantially advanced our understanding of the organization of the bacterial genome in the recent years. It has become obvious that the ideal bacterial genome involves of a conserved genes encoding critical structural properties, as well as flexible, variable genes encoding non-essential traits that are only used in specific growth circumstance. Conserved regions of the genome are relatively stable in their genotypes in closely related species, whereas flexible parts of the genome contain variable and mobile regions of the genome, including transposable elements, plasmids, and bacteriophages that are horizontally transferred between various bacterial species in different transferring processes. In contrast to toxins and other virulence-associated proteins, which are encoded by variable genetic elements, endotoxins, proteins, and other enzymes that are necessary for metabolic pathways, are encoded by conserved genes (Hochhut *et al*., 2005). The conserved areas of the chromosome of closely related species are interspersed by huge regions that have dissimilar features from the original region; these foreign regions may have been acquired through the event of genetic transfer and are termed to as pathogenicity island (PAI). Pathogenicity islands have different G+C content in their sequence and flanked by direct repeats, in addition to that it is possess functional genes encoding for proteins (integrase, transposase) that are essential for genetic transfer. In general, such PAI are well identified in *Escherichia coli* UPEC strains, which encode quantified virulence determinants that assist the bacteria in attaching to epithelial cells and facilitating tissue invasion, resulting in urinary tract infections (Oelschlaeger

et al., 2002), and occasionally disseminating far into the blood stream and causing septicemia in infants.

With the regard of virulence factors regulation, now it can be explained and answered the question: how bacteria evolved themselves to both free and parasitic living? In the simple terms, bacteria have developed complex signaling transduction systems to regulate the genes important in virulence. These systems sense and respond to environmental signals or cues such as (temperature, acidity, oxygen level, growth factors, and specific ion strength). Binding of the sensor (signal) to a specific extracellular receptor on the surface of a microorganism triggers intracellular events that result in the activation of DNA-binding protein (transcription factor) by phosphorylation and subsequent translocation into the nucleus after activation where it binds to a specific-binding site on the virulence gene, resulting in the expression of virulence protein. For instance, *Listeria monocytogenes* motility is at 25 C˚, the environmental temperature that allows them to spread in their niches, while it is non-motile at 37 C˚, the human body temperature (Gründling *et al*., 2004). It is become well known that the function of virulence genes will be crucial in the bacterium ability to cause disease. Virulence or pathogenicity determinants are the results of such genes that aid the bacterium colonization and survival in the host or cause harm to the host (Thomas and Wigneshweraraj, 2014).

1.2.10. Resistance of Bacteria to Antimicrobial Agents

Medical therapies for pathogenic infections have developed dramatically since the discovery of antibiotics, but regrettably, each discovery is followed by the growth of resistance. Hence, it is now very difficult to treat bacterial infections with the existing medicines due to the emergence of multidrug-resistant bacteria. Antibiotic-resistant bacteria have resulted in increasing

morbidity and mortality among hospitalized patients (Reygaert, 2018). The most dangerous bacteria that are resistant to existing medications are listed by the WHO, such as beta-lactamase resistant *Streptococcus pneumoniae* and methicillin resistant *Staphylococcus aureus* (Asokan *et al*., 2019). Gram-positive bacteria are among the most common pathogens that cause serious clinical problems. In fact, there is evidence that the percentage of Gram-positive bacteria resistant to widely used antibiotics is increasing (Lode, 2009). The misuse of antibiotics due to inappropriate diagnosis is one of the leading causes of the emergence of antimicrobial-resistant pathogens. To tackle this issue, antibiotic use must be optimized, and ongoing efforts must be made to develop new techniques. Furthermore, a proper diagnosis is a prerequisite to determining the appropriate antibiotic to target the pathogen.

1.2.11. Sequential Steps of Bacterial Pathogenicity

Pathogenesis, the ability of microorganisms to cause disease and the mechanism that lead to the development of signs and symptoms. A disease is any disorder that disrupt or impaired the normal structure or functions of the body. An infection is the capability of pathogen to colonization of a host and can lead to disease. In most cases, after the pathogen defeats the first line of defense or enters the host throughout mucosal surfaces, the non-antigen specific mechanism of defense provides prompt responses against pathogens through the production of antimicrobial peptides by phagocytes and epithelial cells, as well as activation of complement pathways and acute phase proteins in order to hydrolyze the pathogen and prevent it from binding to the receptors on the cells of the primary site of infection (Moresco *et al*., 2011).

After exposure, in order to cause disease, bacteria need to evade immune defense to adhere to host cells. Non-adherent bacteria are released with mucus fluid. Evasion involves numerous strategies adopted by pathogens to

escape from both the innate and adaptive arms of the immune response. For extracellular bacteria, surface M-protein of certain GAS strains are able prematurely breakdown complement activation. Many bacteria, especially those that cause pneumonia and septicemia, possess distinct capsule. Bacterial capsule also affects the normal activation of complement system (poor activation) due to hide the antigenic determinant on the cell surface. On the other hand, the occurrence of antigenic variation against the production of specific antibody for example, *Streptococcus pneumonia*, exist in a variety of antigenic types, each serotype possesses the distinct structure of its polysaccharide capsule (Janeway *et al*., 2001). For intracellular bacteria, are normally become inaccessible to humoral defense mechanisms and interfere with the adaptive arm of the immune defense through a variety of mechanisms.

Adhesion is the first and most important step in the infectious process, which is followed by the colonization of infectious agents and other subsequent steps in the infection. Each one of the sequential steps is achieved by a specific virulence protein. Adhesion is mediated by the binding of bacterial (ligands) such as adhesin proteins and fimbriae to a specific host cell receptor. Diverse bacterial species as well as varied strains within the same species, have different adherence molecules and adhesion abilities. *E. coli* that causes urinary tract infection (UTI), have type Pap-pili which connect to a component of the P-blood group antigens. Depending on the *E. coli* strain, the type of pili and the exact molecular mechanism of adhesion appear to be distinct (Terlizzi *et al*., 2017). To summarize, adhesion is essential for extracellular lifestyle of bacteria since it allows them to persist in the host. For bacteria that exhibit intracellular lifestyle, adhesion represent a first important step that occurs before their internalization into the host cells. After initial contact between the bacteria and the host cell, colonization (multiplication) to sufficient numbers on the

surface of the host cell occurs, either as extracellular colonization or as internalization of the bacterial pathogen. Regarding to the internalization, invasion of the host cells is used to prescribe the entry of pathogen to professional or non-professional phagocytic cells.

Many bacterial species produce various proteins that cause the host cells to phagocyte bacteria through endocytosis process. Although macrophages, dendritic cells, and other cells of the immune system are responsible for detecting foreign intruders in the mucosal layers and coordinating an appropriate immune response to prevent pathogen colonization, these cells also play a role in pathogen dissemination deeper instead of limitation. This adverse role of phagocytes is related to the effectiveness of some virulent bacteria. Anyone can ask how intracellular bacteria subvert the normal function of phagocytic cells and exploit them for their distribution throughout the body? the answer to this question, illustrated in following examples: A zipper process can cause *Listeria monocytogenes* to internalize into non-professional phagocytes, such as specific types of epithelial cells. After a contact between the surface bacterial protein (Internalin A) and E-cadherin in the epithelial cell, signaling transduction inside the host cells is activated, resulting in cytoskeleton rearrangement and bacterial engulfment. *Salmonella* also causes cytoskeleton reorganization and stimulates its internalization into non-professional phagocytes via a relatively different method known as the trigger mechanism (Cossart and Roy., 2010). After a pathogen is ingested, the epithelial cells sample the pathogen for macrophages and other professional phagocytes. Inside the macrophages, *Salmonella* uses its Salmonella pathogenicity island protein type C (Spi C) to impede phagosome fusion with lysosome or produces Salmonella invasion protein type B (Sip B) that kills the macrophages by apoptosis. In the case of *Legionella pneumophila,* the internal PH of the phagosome is neutralized by these bacteria, and it is also resistant to oxidative burst

activity of macrophages whereas *Listeria monocytogenes* uses its pore-forming toxin to escape from the lysosome and preferentially remain within the cytoplasm. The ability of the phagocytic cells to migrate from the periphery to the blood circulation, is exploited by different pathogens. In conclusion, each pathogen can be internalized in a variety of cell types and create a variety of pathogenic proteins during infection. Each infected cell may play a certain role in disease progression in these cases (Ribet and Cossart., 2015). In general, host damage and clinical signs appear either directly as the infectious process progresses or as a result of hyper-inflammatory responsiveness mediated by the immune system.

1.2.12 Genuine Pathogen and Opportunistic Pathogens

In general, healthy individuals harbor a variety of microorganisms referred to as normal flora or microbiota, which normally reside in the skin and mucous membranes of the nasopharynx, gastrointestinal tract, and genital tract. The members of these microorganism are characterized by their ability to adapt to a non-invasive lifestyle and persist in a restricted environment. In this instance, these microflorae provide many different advantages for the site where they are present (Khan *et al*., 2019). Unless disrupted, microflora represent the first line of defense against pathogens.

Despite the presence of commensals and an intact immune system in healthy people, several community-acquired infections occur when they are exposed to a large number of pathogens. These types of pathogens are termed "genuine" or "primary" pathogens such as food-borne bacterial species (*Salmonella*, *Listeria* and, *E. coli*). In contrast, an “opportunistic” pathogen can only cause disease in unusual circumstances such as elderly, immunocompromised, or hospitalized patients, severe wound and burn patients, as well as those that admitted to cancer therapy. Opportunistic bacteria such as *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*,

and *Proteus* are among the most frequent pathogen that cause hospital-acquired infections (Otto, 2009). Members of the avirulent commensal species are characterized by their ability to form biofilm in catheters and other medical device, and when introduced directly into blood circulation via intravenous catheters, they can cause more severe infections such as endocarditis, bacteremia, and sometimes severe sepsis and multiorgan dysfunction. The concept of opportunistic pathogens refers to those that non-pathogenic bacteria that are transformed into pathogens when they somehow leave their restricted environment or when the host condition changes.

1.2.13 Human Immunity

In simple terms, our immune compartment is a network of biological mechanisms which defends us against disease. It recognizes and responds to a wide range of harmful microorganisms and transformed cells (cancer cells). The immune system is like a small world in our bodies that is occupied by numerous immune cells that define the state of our general health. Each type of immune cell has a distinct function (Iwasaki and Medzhitov, 2015).

These immune cells are generated from the stem cells in the bone marrow and then differentiate into granulocytes, monocytes, and lymphocytes (Janeway *et al*., 2001). Hematopoietic stem cells are immortal, capable of producing progenitors, which will eventually give rise to a variety of immune cells. Myeloid progenitors and lymphoid progenitors are the two major types of progenitors generated.

1.2.14 Innate Versus Adaptive Immunity

According to the composition of the immune system compartment as well as the specific state of protections, immune defense mechanisms can be divided in to two major arms: **(a)** innate immunity also known as a non-specific mechanism of defense, can be achieved through anatomical barriers

such as skin, secretory molecules from the skin and mucous membranes, and cellular components in the blood circulation that work together to prevent the entry and/or provide initial protection against a wide variety of non-specific pathogens. **(b)** adaptive immunity or antigen-specific mechanism of defense provide delayed responses to a particular bacterial antigens through different sets of lymphocytic cells. The adaptive immune response is more complex than the innate (Hoebe *et al*., 2004). Although, there are many variations between innate and adaptive immunity, both kinds of act for the same purpose. Elements of both systems coordinate equally to produce an effective and long-lasting response. However, the elements of both systems encompass three lines of defense: the first line includes skin, mucous membrane, and the secretions of skin and mucous membrane; the second line includes phagocytic WBC, antimicrobial peptides, complement proteins, cytokines, and other mediators; and the third line of defense represents the specific immunity (adaptive immunity), which includes lymphocytic cells and highly specific antibodies (Bonilla and Oettgen, 2010).

1.2.15. Bacterial Infection and Instigate Inflammation

Skin and mucous membrane are excellent barriers against colonization of tissues by germs. Infection may occur, however, if germs penetrate these barriers and reach deeper tissues. Once infectious agent has infiltrated tissues, another natural mechanism of defense comes in to play, known as acute inflammation. The inflammatory response is started by a series of complicated events, including the released of diverse mediators by infected cell, and aids to establish a barrier to prevent the spread of (Stvrtinová *et al*., 1995).

1.2.15.1. Acute Inflammation

The initial response to pathogens is called acute inflammation, which is achieved through the influx of phagocytes, complement components, and other plasma proteins into the infection site.

A vast range of mediators are involved in the early stages of inflammation, some of which act directly on the smooth muscle wall of arterioles to change blood flow. Others produce transitory opening of endothelial junctions, allowing plasma to pass through (Barth *et al*., 2013). Soluble mediators like histamine and thrombin help leukocytes migrate out of the bloodstream by inducing the production of adhesion molecules on the endothelium and leukocyte surfaces, allowing them to connect. Other mediators, including C5a and leukotriene B, act as chemotactic agents, attracting active leukocytes to the site of inflammation. Cytokines such as IL-1β and TNF are also involved in the activation of more phagocytes during the events of acute inflammation (Lotze and Tracey 2005). Following their recruitment to the infection site, phagocytes engulf or use their various killing mechanisms to eradicate the microorganisms. Pattern recognition receptors (PRRs) are cell surface receptors found on innate immune cells that recognize compounds by bacteria but differentiate from human molecule, generally known as PAMPs. These cells become activated when an infection begins and produce inflammatory mediator that cause the clinical signs of inflammation for example fever and pains. Complement pathways, are other major contributors in the acute inflammation process.

Once activated, complement either directs opsonizing pathogens to phagocytes or kills them directly by forming membrane attack complex on their surface (Janeway *et al*., 2005). Depending on the severity of infection, the coagulation cascade may be involved.

Inflammation is originally controlled at both the humoral and cellular levels. The inflammatory response is terminated once the pathogenic agents

have been eradicated to prevent it from getting out of hand. After infections have been cleared, inhibiting cytokine production and immune cell proliferation after clearance of pathogens is crucial for preventing excessive damage to the host. However, the inflammatory components might be persisted due to the inability of the acute inflammatory response to eliminate infections. Numerous virulent bacteria can develop strategies to subvert the regulation of the immune system, resulting in chronic inflammation.

1.2.15.2 Chronic Inflammation

The properties of inflammation are modified in some infectious diseases when the initial immune defense fails to recover the host from a particular antigen. Profuse cytokine production and the site of infection become dominated by macrophages, which may form epithelioid cells. Others aggregate, forming giant cells known as granuloma, which surround the bacterial antigens. Eventually, the infectious agents persist, and the immune system becomes out of control. Chronic inflammation represents the hyper-inflammatory reaction toward a particular pathogen as an attempt to expel it from the body (Gauldie, 2020).

1.2.16 Bacterial Infection and Sepsis Propagation

As we mentioned above, the primary response to any infection involves the release of pro-inflammatory cytokines including TNFα, IL-1β, and mediators such as platelet activating factor (PAF). The functions of these molecules are to induce a proper response to limit infection and tissue repair. When the main task is completed, the pro-inflammatory response is down-regulated through the production of anti-inflammatory cytokines such as IL-10 and TGFβ. In this instance, the immune response remains under normal control and the body is protected from the destructive activities of the excessive production of pro-inflammatory cytokines.

In cases of bacterial sepsis, the regulating of the primary response to infection is diminished, because of the virulent bacterial products that

promote activation of the body's defense mechanisms, resulting in a hyper-inflammatory response. This situation also resulted in the loss of homeostasis in multiple key body pathways (Esmon, 2005). Lipopolysaccharide (LPS) is an outer membrane of gram-negative bacteria that is a potent endotoxin and, in some cases, the main causative factor of sepsis. Furthermore, in gram-positive bacteria, the cell wall peptidoglycan as well as certain secreted exotoxins are also implicated in the development of sepsis symptoms. Superantigens produced by *Staphylococcus aureus* and *Streptococcus pyogenes,* such as the staphylococcal TSST-1 exotoxin and the streptococcal pyrogenic exotoxin (SPE), cause unusual immune system responsiveness and overproduction of cytokines, leading to toxic shock syndrome (Sriskandan and Cohen., 1999).

1.2.17 Culture Method for Detection of Bacterial Infection

Cultural method is used for bacterial identification based on different criteria, one of which is depending on their morphologic characteristics, bacteria are present in cocci, bacilli, spiral, comma, and corkscrew which can be seen in single cells, pairs, chain, or in clusters under the microscope (Mohamad *et al*., 2014). Other well-understanded classification method depending up on oxygen requirement, staining behavior, and biochemical properties. In vivo, there are several protection enzymes excreted by majority pathogenic bacteria to protect them from human immune system. in vitro, these enzymes represent an essential identification marker when optimally cultivating these bacteria which taking from clinical samples (Talaiekhozani *et al*., 2015) Coagulase, catalase, oxidase, urase test, and so others provide evidence about which pathogen are present in laboratory specimens. Several studies use the blood culture methodology as a first step in identifying the causative agent of bacteremia.

Indeed, the cultural analysis is generally remined the common method till now for isolation and identification vast majority number of the bacterial species (Tissari *et al*., 2010). Artificially predetermined culture media are used to cultivate microorganisms under certain laboratory conditions in order to multiply them and determine a comprehensive characterization of physiological and biochemical properties. If the plate or tube contains all the nutrition compound needed for growth (vitamins, growth factors, carbon source, and so others) and environmental condition (O2, CO2, PH and temperature) are ideal the bacteria can reproduce and increase in their mass during the incubation periods (Maier and Pepper, 2015). Primary media was designed to cultivate different groups of bacteria whereas differentiative media contain certain chemical indicator to differentiate between the closely related species on the other hand selective media contain some growth inhibitor materials like antibiotics to inhibit the growth of unwanted bacteria (Paul, 1980; Bonnet *et al*., 2020), certain microorganisms are fastidious and required specific nutrients with additional supplement and PH level, in this case use enrichment media (Kopke, 2005). In order to isolation of diverse groups of bacteria from different samples, specific culture media and optimal growth condition for each bacterium yield the fast growth and give the purpose which designed for it. Under certain circumstance, culture media did not give desirable growth of bacteria due to the presence of contamination resulting in competition for nutrient among mixtures thus the too faster-growing bacteria thrive at the expense of slow-growing species. Furthermore, several clinically important bacteria need specific requirement and disable to grow in currently available conventional culture media, therefore, the absence of essential materials make bacteria to enter into a temporary state of low metabolic activity accompanied by in ability to form colonies (Vartoukian *et al*., 2010), many unmentioned reasons facing cultural analysis in early diagnosis so,

microbiologists rely on additional strategies for confirmation of unculturable causative agent like immunoassay methods.

1.2.18. Other Sources of Sepsis

The multiple sources of sepsis manifestations represent the main cause of the difficulties in the diagnosis and prognosis of sepsis worldwide. Although scientific research now provides a better understanding of sepsis pathogenesis, The growing importance of sepsis in public health necessitates the development of reliable and valid sepsis diagnosis criteria. Previously, it was thought that sepsis symptoms are predominantly caused by a hyperimmune host response to a specific infection. A huge body of research on sepsis, on the other hand, has revealed a considerably more nuanced and intricate interplay between the infectious agent and the host that results in the diverse manifestations of sepsis (Singer *et al*., 2016). Elderly people, autoimmune diseases, malignant disorders, organ transplants, invasive operations, trauma, burns, antibiotic resistance, and hospital-acquired infections are all predisposing variables that might lead to sepsis. Event of sepsis involves early activation of both proinflammatory and anti-inflammatory responses and significant changes in non-immunologic pathways, including metabolic, neural, hormonal, and coagulation, all of which have prognostic importance (Hotchkiss *et al*., 2013; Deutschman and Tracey, 2014). Because most cases of sepsis are delayed in making an appropriate diagnosis of the causative agent, treatment begins later, therefore more sensitive indicators must be identified for early sepsis diagnosis. The goal of numerous studies is to determine whether bacterial or other factors are the causes of sepsis in hospitalized patients who have undistinguishable signs and symptoms such as fever and abnormal heart beating rate. Blood samples from febrile patients who have an abnormal white blood cell count must be subjected to immunological tests in order to prove the role of some

novel biomarkers in sepsis pathogenicity. Generally, early detection of sepsis is one of the most essential aspects since it allows for rapid treatment and reduces mortality and morbidity rates that may be evolved during the time of infection.

1.2.19. Sepsis Biomarkers

As sepsis is an abnormal systemic disorder that displays a pattern of immune system response to infection, biomarkers produced during this condition may allow for early detection, lowering the risk of death. These biomarkers include acute phase proteins such as CRP, neutrophils, monocytes, lymphocytes as well as pro-inflammatory cytokines and chemoattractant factors.

To utilize some sepsis biomarkers as indicators for detection of sepsis episodes, we must first understand what happens during the infection and how certain pathogens might go from pro-inflammatory to instigate hyper-inflammatory immune responses in the early event of sepsis, eventually leading to severe sepsis. The host immune system responds to particular products from different pathogens. The outer membrane LPS of gram-negative bacteria, for example, represents a pathogen-associated molecular pattern (PAMP). Different forms of PAMPs are recognized by innate immune cells which have cell surface receptors for them known as pattern recognition receptors (PRRs) such as TLRs (Kumar *et al*., 2011). After the engagement between bacterial ligands (PAMPs) and their host cell surface receptors (TLRs) occurs, the innate immune cells such as macrophages become activated and generate pro-inflammatory cytokines including TNFα, IL-1β, and IL-6 cytokines. In the case of over production of these cytokines in response to some pathogens, they produce a condition that can be termed " early sepsis ", which takes place if the innate immunity fails to eliminate the pathogen.

For the initial assessment of sepsis, in 1991, an international consensus conference described sepsis as an infection that is combined with the characteristics of systemic inflammatory response syndrome (SIRS) such as changes in normal body temperature and abnormal (WBC) count, whereas severe sepsis is characterized by hypotension (Bone *et al*., 1992). Since the criteria for sepsis description are continually updated over time, in 2001, the experts changed the first description of sepsis to include other signs and symptoms. As a result, this update is therefore based upon the fact that the early definition of sepsis involves signs and symptoms that are insufficient to identify the infection in individuals (Levy *et al*., 2003).

Ten years later, Dr. Roger C. Bone helped to emphasize the relevance of a compensatory anti-inflammatory response syndrome (CARS), which commonly occurs after SIRS, particularly in individuals with severe sepsis (Balk and Roger, 2011). This means that the pathogenesis of sepsis is progressive across the course of infection, and each stage has its own biomarkers. Hence, different biomarkers must be used to detect and monitor the course of sepsis.

The pro-inflammatory cytokines generated by innate immune cells and CRP were considered as a potential indicator for sepsis before the discovery of the relationships between elevated levels of procalcitonin (PCT) and bacterial infection in the 1990s (Karzai *et al*., 1997). Another indicator has been identified, and the increasing levels of CRP and PCT were added to the modified criteria for sepsis in 2003.

It has become much easier to distinguish the early and less severe from the late and more severe sepsis, as shown in [Figure1.1], which summarized the development of sepsis manifestations during the infection. In conclusion, while no single sepsis biomarker is perfect, many are useful in identifying severity ill patients who require monitoring so that the condition can be detected and treated.

Some sepsis biomarkers, such as complete blood count (CBC), lactate dehydrogenase (LDH), C-reactive protein (CRP), procalcitonin (PCT), presepsin (PSN), pentraxin3 (PTX3), and monocyte chemoattractant protein-1 (MCP-1), assist in early identifying the source of sepsis, and some of them have an essential role in the monitoring the development of sepsis events, like the PSN biomarker (Masson *et al*., 2015).

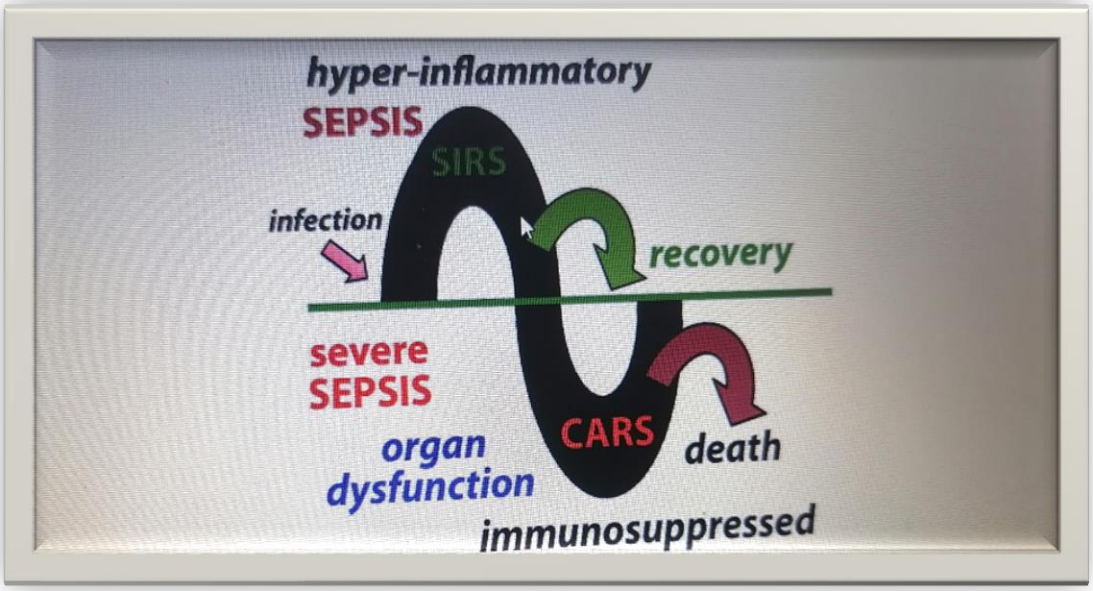


Figure 1.1 Sepsis might be divided into 2 stages. After infection, a hyper-inflammatory response is identified by the systemic inflammatory response syndrome (SIRS). This stage may be resolved, or the patient's condition may develop severe sepsis, leading to events of immunosuppression and multiple organ dysfunction. This stage is known as (CARS) compensatory anti-inflammatory response syndrome (Faix, 2013).

1.2.19.1. Complete Blood Count (CBC)

A complete blood count is a common screening test for certain illnesses. It is performed to detect white blood cells (WBC), granulocytes (GRA), lymphocytes (LYM), hemoglobin (HGB), hematocrit (HCT), red cell distribution width (RDW), and platelets (PLT). Among all the sepsis indicators being studied, CBC values may be the most helpful because they can be easily performed, cheap, and are available in all medical facilities.

This test is important for the early diagnosis of many health issues, which should then be further investigated through laboratory examination (Kumar *et al*., 2021). The presence of acute inflammation caused by unidentified causative agents is indicated by an increase or decrease in normal value of CBC parameters. Since there is no single sepsis parameter that is perfect enough to detect bacterial sepsis, the CBC value with blood culture and other indicative biomarkers can provide essential clinical data to identify and treat sepsis.

1.2.19.2. Lactate Dehydrogenase (LDH)

The LDH enzyme is present in all body tissues and allows the tissues to survive in a low-oxygen environment (hypoxic environment) due to its ability to anaerobically convert pyruvate to lactate during glucose metabolism (Krishnamurthy *et al*., 2021). It is another potent parameter for sepsis detection (Miglietta *et al*., 2015). Septic patients can be detected from the measurement the serum levels of LDH and heart beatings rate (Duman *et al*., 2016). In cases of sepsis, elevated levels of lactate are typically indicative to decrease oxygen supply to the body cells (hypoxia) or reduced tissue perfusion, which results in and anaerobic glycolysis (Rabello., 2016).

1.2.19.3. C-reactive Protein (CRP)

It is a well-known acute phase protein originating from the liver and has a normal serum level ranging from 0.8 mg/L to 3 mg/L in healthy individuals. CRP test is one of the most commonly used diagnostic tests to determine whether patients with sepsis have elevated levels in response to infection and inflammation. Once an infection has occurred, activated macrophages produce IL-6, that in turn stimulates the liver's synthesis of acute phase proteins, such as CRP, and triggers more production of PMNs in the bone marrow (Gabay and Kushner.,1999). Although the role of CRP during acute

inflammation is unclear, it may adhere to the phospholipid components of bacteria, allowing macrophages to more easily remove them. The serum levels of CRP rise considerably during acute inflammation; hence the test is used to detect the presence of infectious disease, particularly in children (Kool *et al*., 2016) and, used as a marker for the inflammation that associated with atherosclerosis and heart disease (Benzaquen *et al*., 2002). Even though it has low specificity as a biomarker of sepsis, it is often used to screen for early onset sepsis because its sensitivity is generally thought to be very high in this situation. C-reactive protein is also commonly used to follow patients after surgery; levels are normally elevated, but they quickly diminish unless a post-operative infection develops (Welsch *et al*., 2008). Finally, despite its relatively non-specific nature, the CRP is considered the first screening test because it provides evidence of specific infection caused by any microorganism such as viruses and bacteria, allowing other confirmatory tests to be performed later. CRP level is increased in acute and chronic inflammatory states (Sankar and Webster, 2013).

1.2.19.4. Procalcitonin (PCT)

Procalcitonin, a protein of 116 amino acids with a molecular weight of 13 kDa, was first discovered 25 years ago as an intracellular precursor of calcitonin. After being cleaved by endopeptidase (proteolytic enzyme), PCT transforms into active calcitonin, which plays a vital function in regulation of calcium concentration. Procalcitonin was recognized for the first time in the 1970s. It is formed in the thyroid gland by parafollicular cells (C-cells), and it is also generated by different cells throughout the body, such as neuroendocrine cells in the lung and intestine (Fan *et al*., 2018). The normal value of the PCT in the blood stream is less than 0.1 ng/mL, whereas it rises within 2 to 4 hours during infection and has a half-life of 22 to 35 hours

(Reinhart *et al*., 2000). However, it is considered a sepsis biomarker because its higher value is associated with the bacterial infection (Schuetz *et al*., 2008; Bréchot *et al*., 2015).

The PCT emerged as a potent biomarker for the diagnosis of bacterial infection (Maruna *et al*., 2000). PCT levels rise during the course of infection and inflammation in response to bacterial toxins and cytokines induced by immune cells in response to specific bacteria, such as IL-1, IL-6, and TNF. If the causative agent is effectively managed by the immune system and appropriate antibiotics, these high levels of PCT will decrease. It is not substantially increased during viral disease. This is because one of the host cell responses to a viral infection is the production of interferon (IFNγ) which impedes the initial creation of PCT (Sandkovsky *et al*., 2015). Because of this preferential cellular mechanism, PCT is a valuable diagnostic marker that is more specific for bacterial infections than other inflammatory markers, and it also helps to distinguish between viral and bacterial infections. The importance of this protein in the diagnosis and monitoring the patients suffers from invasive bacterial infection, was discovered in the 1990s (Assicot *et al*., 1993). Following research, it was discovered that PCT is a component of the systemic response that leads to severe sepsis. PCT, like CRP, may have pro-inflammatory properties. PCT has been recommended as a beneficial test for severely ill patients who develop a new fever (O'Grady *et al*., 2008). However, the usefulness of PCT as a sepsis biomarker in recent years may have lessened the significance of CRP. In fact, several studies have been conducted in recent years to evaluate the diagnostic utility of PCT, usually in comparison to CRP. Recently, PCT value was found to be more efficient and more specific than other inflammatory biomarkers in cases of bacterial infection. Several clinical studies have shown that it may be useful in predicting blood culture findings in patients who are suffering from severe sepsis (Riedel *et al*., 2011).

Furthermore, PCT aids in the early identification of sepsis and differentiates between cases with symptoms of non-infectious SIRS and cases related to severe bacterial infections (Uzzan *et al*., 2006). Moreover, PCT is not just separate test and should not be used in place of other important tests and clinical examination assessments of patients. PCT offers additional information and assists clinicians in making reasonable medical decision in patient cases. The usefulness of one biomarker to accurately predict of infection may be misleading. Despite the facts that it refers to the significant of the PCT levels for primary detection of sepsis, keep in mind that more than one marker must be used to achieve accepted results for such infection.

1.2.19.5. Presepsin (PSN)

Presepsin is a plasma soluble form of CD14 (sCD14), which is cleaved from the main membrane-bound form of CD14 (mCD14). mCD14 is a member of pattern recognition receptors (PRRs), which is upregulated on the surface of the phagocytes as a receptor for various groups of ligands particularly bacterial LPS endotoxin (Ciesielska *et al*., 2021). Presepsin was discovered as a novel and a promised indicator whose value is used in the diagnosis and management of sepsis (Yaegashi *et al*., 2005). This biomarker appears to be more sensitive and specific for sepsis detection. It is also favorable for the evaluation of sepsis severity. (Zou *et al*., 2014).

As was earlier mentioned regarding the existence of non-infectious SIRS in many critical illness cases, the discrimination between SIRS and sepsis at the early stage of disease leads to an accurate diagnosis and accelerates treatment. Hence, in addition to CRP and PCT being recognized in various studies several years ago, presepsin was developed later to complement them in terms of sepsis diagnosis and assessment. Presepsin in the blood circulation indicates that phagocytes have been activated in response to infection (Chenevier *et al*., 2015). The reference value of presepsin ranges

from 55 to 184 pg/ml (Giavarina and Carta., 2015). As with PCT, PSN has a powerful diagnostic accuracy, according to a recently published meta-analysis comparing PSN with other biomarkers. In addition to that, presepsin is also used for early detection of a probable bacterial infection and to predict the risk of death due to its level gradually increasing with the progression of a sepsis events (Wu *et al*., 2017). Despite the fact that it has received a lot of attention from investigators, the clinical relevance of presepsin has remained ambiguous or controversial till now. Several current investigations have approved the importance of PSN level in sepsis diagnosis and suggested that it can be a more reliable biomarker for the diagnosis of bacterial sepsis, as well as comparing its specificity with other sepsis biomarkers such as PCT in order to determine the suitable antibiotic for the correct treatment (Venugopalan *et al*., 2019).

1.2.19.6. Pentraxin 3 (PTX3)

The ambiguity of the sepsis events and their causative agents led that the researchers to exert more effort to find new biomarkers that could help to improve the diagnostic methodologies. They also hypothesized that using multiple biomarkers would increase the diagnostic value compared to using a single biomarker because sepsis is formed up of a range of signaling proteins from diverse cascades.

Pentraxin 3 is another new protein employed as a diagnostic marker for bacterial-originated sepsis. The PTX3 is an acute phase protein that is structurally like CRP but is produced by innate immune cells, specifically macrophages, dendritic cells, neutrophils, fibroblast, and endothelial cells, rather than the liver (Mauri *et al*., 2010).

The PTX3 has a normal value of less than 2 ng/ml in healthy individuals, but it can be promptly increased in cases of inflammation when inflammatory cells generate and release it in response to pro-inflammatory

cytokines including IL-1β and TNFα (Doni *et al*., 2008). The PTX3 has an essential role during the acute phase of inflammation because it binds to C1q protein and activates the classical pathway of the complement component system to facilitate pathogen recognition by phagocytic cells such as macrophages and dendritic cells. Hence, its function as an opsonizing protein, facilitating pathogen identification and engulf it (Garlanda *et al*., 2005). Furthermore, there is evidence referring to the interaction between PTX3 and tissue factors expression, which induces coagulation stimulation during sepsis (Napoleone *et al*., 2004). like other mentioned biomarkers, elevated serum levels of PTX3 also linked to the severity of sepsis. It is, however, high in non-infectious inflammatory conditions such as autoimmune disease, therefore it has no advantage over CRP (Ogawa *et al*., 2010). The study of PTX3 in sepsis could lead to a better understanding of the pathogenesis of this condition and, potentially, the development of more effective treatments. Further research is needed to prove the specificity of PTX3 in the diagnosis bacterial sepsis.

1.2.19.7. Monocyte Chemoattractant Protein-1 (MCP-1)

Chemokines are chemotactic cytokines that constitute a large family of chemoattractant proteins and are grouped into four families according to the spacing and arrangement of the first two cysteine residues in their N-terminus, which are CX3X, CXC, CC, and C. Based on their behaviors, the main roles of these signaling proteins are to recruit leukocytes and other cells to the site of infection, leading to activation of the innate immune system (Deshmane *et al*., 2009). Monocyte chemoattractant protein-1 is a member of the CC family (CCL2), with a normal serum level of 69.5 to 175.2 pg/ml in healthy individuals (Valković *et al*., 2016). MCP-1/CCL2 is encoded by *ccl2* gene located on chromosome 17 and secreted by several cells in response to pro-inflammatory cytokines to regulate the migration and

infiltration of the innate immune cells particularly mononuclear cell during injury or infection (Bozza *et al*.,2007). CCL2 and its receptor, CCR2, have been shown to be induced and involved in a wide range of illnesses. Several studies attempted to use MCP-1 as an indicator for the onset of sepsis, and the majority of them concluded that a high serum level of MCP-1 in adult individuals indicates the existence of sepsis related to specific infections (Zhu *et al*., 2017). However, there has been no single effective prognostic biomarker for sepsis to be used alone in the clinical field. As a result, of that, more than one biomarker is employed in the previous studies, including CRP, PCT, PTX3, presepsin, and the values of MCP-1 in the hope of resolve and success to overcome the difficulties correlated with the diagnosis of bacterial sepsis. Early detection of the sepsis sources has a critical role in term of the minimize antibiotic abuse and misuse while also accelerating the healing from the infection.

1.2.20. Sepsis Management and Proper Treatments

Infections are a common health issue in individuals of all ages and genders. Typically, the immune response given to them is appropriate, so minimal treatment is needed. However, sometimes the response to the infection is inadequate and may lead to sepsis (Rello *et al*.,2017). The life-threatening sepsis that results from a dysregulation of the host response to infection is a major health issue and affects millions of individuals worldwide. Usually, the most cases of sepsis require treatment in the intensive care unit (ICU). Early detection and appropriate therapy in the first hours after the onset of sepsis improves outcomes. Several screening methods are being developed to improve early detection of sepsis; however, their diagnostic accuracy varies greatly, with the majority having low predictive values, despite the fact that their use has been linked to improvements in treatment processes. In order to properly manage of sepsis

in the medical setting, there are numerous essential guidelines to follow. One of the most important guidelines is to determine the infectious agents prior to starting antimicrobials wherever it is possible (Rhodes *et al*., 2017). Early detection of patients who are at risk, prompt therapy, and suitable supportive care are all part of the strategy which in turn can be enhance the management of sepsis and can made considerable outcomes. In spite of the appearance of some current progress in the diagnosis and management of sepsis, however, the search for clinically meaningful biomarkers is still underway, with no promising results yet. The surviving sepsis campaigns (SSC), an international consortium of professional societies engaged in emergency medicine and critical care, provided international rules that have enhanced sepsis management (Levy *et al*., 2018). The use of SSC care bundles is linked to better results. The early control of infection needs conducting a probable diagnosis, performing blood cultures, starting appropriate and prompt empirical antibiotic therapy, as well as eradicating the source of infection. Many efforts must be made to resolve the most important issue, which concerns patients who are likely to develop sepsis manifestations after experiencing some laboratory tests for the optimal diagnosis of bacterial sepsis.

Chapter two

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Equipment and Instrument

All Equipment and Instrument utilized in this study are listed in Table 2.1

Table 2.1 Equipment and instrument with supplier and origin

Equipment	Supplier	Origin
Autoclave	Hirayama	Japan
Biological safety cabinet	Labogene	Denmark
Candle jar	Conda Lab.	Spin
Centrifuge	Hettich	Germany
chemistry analyzer	FUJIFILM	Japan
Distillator	Fine tech	Korea
ELISA test system	Biotech	USA
Eppendorf tube	Eppendorf	Germany
Eppendorf rack	Eppendorf	Germany
Flasks and beakers	Hirschman	Germany
Gas burner	GFL	Germany
Glass slides and cover slides	Bio-zzeik	China
Hematology analyzer	Sysmex	Japan
Immunofluorescent microscope	Olympus	Japan
Incubator	Memmert	Germany
Latex gloves	Broche	Malaysia
Light microscope	Olympus	Japan
Mask	HAD	China

Equipment	Supplier	Origin
Micro centrifuge	Beckman	Germany
Micro centrifuge tube 1.5ml	Bio basic	Canada
Micro pipettes 1-1000 µl	Fisher Bio basic	USA Canada
Micro pipettes tips 10-1000 µl	Promega	USA
Millipore Filter 0.22 µm apparatus		China
Oven	Memmert	Germany
Petri dish	Bio-zzeik	China
Platinum wire loop	Himedia	India
Refrigerator	Concord	Lebanon
Sensitive electron balance	A&D	Japan
Sterilized cotton	AFCO	Jordan
Sterilized needle	AFCO	Jordan
Tube with screw cap	AFCO	Jordan
Vitek 2 system	Biomerieux	France
Vortex	Gemmy	Taiwan
Water bath	GFL	Germany

2.1.2 Chemicals

There is a list of the chemicals utilized in this study in [table 2.2].

Table 2.2 Chemical materials with their company and origin.

Chemicals	Company	Origin
Catalase reagent	HIMEDIA	India
Ethanol (70%, 95%)	Merck	England
Glycerol	Merck	England
Kovac's reagent	Sigma	USA
McFarland 0.5	Biomerieux	France
Methyl red (5 ml vial)	HIMEDIA	India
Mineral oil	Fisher	USA
Oxidase reagent	HIMEDIA	India
Potassium hydroxide VP2	Sigma	USA
α- naphthol VP1	BDH	England

2.1.3 Media:

In [table 2.3] the cultural media utilized in this study are mentioned along with their manufacturer company and origin.

Table 2.3 Media with their supplier and origin.

Media	Supplier	Origin
Bacteriological Peptone water	HIMEDIA	India
Blood agar (BA) base	PRONADISA	Spain
BHI broth (powder)	OXOID	England
BHI broth (vials)	HIMEDIA	India
Eosin methylene blue (EMB) agar	OXOID	England
Kligler iron agar	Himedia	India
MacConkey agar	OXOID	England
Mannitol salt agar	Liofilchem	Italy
MR-VP broth	Himedia	India
Mueller Hinton agar	OXOID	England
Nutrient agar	Liofilchem	Italy
Salmonella-shigella agar (SS agar)	Himedia	India
Simmons citrate agar	Himedia	India
Urea agar base	Himedia	India
Urea supplement 40%	OXOID	England
Xylose-lysine deoxycholate (XLD) agar	Himedia	India

2.1.4 Antibiotics:

All antibiotics utilized in this investigation were listed in Table 2.4.

Table 2.4 Antibiotics with their class, supplier, and origin.

Class	Antibiotics	Supplier	Origin
Aminoglycosides	Amikacin (AK) 30 mg	Liofilchem	Italy
	Tobramycin (TOB) 10 mg	Liofilchem	Italy
Ansamycin	Rifamycin (RA) 5 mg	Liofilchem	Italy
	Rifampicin (RF) 5 mg	Liofilchem	Italy
Carbapenems	Imipenem (IMI) 10 mg	Liofilchem	Italy
	Meropenem (MRP) 10 mg	Liofilchem	Italy
Cephalosporin	Cefotaxime (CTX) 30 mg	Liofilchem	Italy
	Ceftazidime (CAZ) 30 mg	Liofilchem	Italy
	Ceftriaxone (CTR) 30 mg	Liofilchem	Italy
Folate	Trimethoprim (TM) 5 mg	HIMEDIA	India
Glycopeptide	Vancomycin (VA) 30 mg	Liofilchem	Italy
Lincosamides	Clindamycin (CD) 2 mg	Liofilchem	Italy
Macrolides	Azithromycin (AZM) 15 mg	Liofilchem	Italy
	Clarithromycin (CLR) 15 mg	Liofilchem	Italy
Penicillin and beta-lactam combination agent	Augmentin (AUG) 30 mg	Liofilchem	Italy
	Methicillin (MET) 5 mg	Liofilchem	Italy
	Piperacillin (PRL) 100 mg	Liofilchem	Italy
Phenicol	Chloramphenicol (C) 30 mg	Liofilchem	Italy
Quinolones	Ciprofloxacin (CIP) 5 mg	Liofilchem	Italy
	Levofloxacin (LEV) 5 mg	Liofilchem	Italy
Tetracycline	Doxycycline (DXT) 30 mg	Liofilchem	Italy

2.1.5 Commercial Kits

The commercialized kits utilized in this research were listed with their manufacturer company and origin in Table 2.5

Table 2.5 Commercial kits with their components and origin

Kits	Company	Origin
PNEUMOBACT IFA kit for in vitro diagnostic use.	Vircell	Spain
C-reactive protein kit: CRP latex reagent, control positive, and control negative.	Spinreact	Spain
Gram stain kit: Crystal violet dye, Iodine, ethanol 70%, and counter stain (safranin).	Merck	Germany
Hematology work solution: hemolytic agents, diluents, cleaning solutions, and concentrated cleaning solutions.	Sysmex	Japan
Human Pentraxin3 ELISA	BT- LAB	China
Human Presepsin ELISA	BT- LAB	China
Human Procalcitonin ELISA	BT- LAB	China
Lactate dehydrogenase	Sigma	USA
Vitek 2 kit: reagent cards (cassette), which have 64 wells for automated biochemical tests.	Biomerieux	France

2.2 Methods

2.2.1 patients

The study's population consists of hospitalized patients who display the signs and symptoms of sepsis. Most of them are in the Intensive Care Units (ICU). Prior to sampling, the medical histories and demographic information of the participants such as, patient's name, identification number, sex, age, date and time of sample collection, clinical symptoms, and medical history, were recorded on a questionnaire form.

2.2.2 Ethical Approval

The first is informed consent, in which patients who agree to participate in a study understand what they are agreeing to and authorize the researcher to collect information on their history without coercion. to avoid causing harm to others or exposing the patient to undue risks.

2.2.3 Clinical Sample

The collection of samples is a significant part of the study. Through the process, standard measures are followed, and strict aseptic conditions are maintained. Ten milliliters of blood were drawn from the patients using a sterile syringe after disinfecting the injection sites on their hands with alcohol 70% and iodine.

During the period of sample collection at (Murjan Medical City, AL-Sadiq hospital, AL-Hilla educational hospital, AL-Nour hospital for children, and Al-shomaly hospital) in province of Babylon, a total **100** blood samples from both male and female patients between the age of (13-85 years) were collected according to inclusion and exclusion criteria. In addition to that, there are **25** sample from healthy individuals were taken to use as a control in this study through the period from January to June 2022.

2.2.4 Sample Processing

Each blood sample was dispensed into three parts: 2ml placed into an EDTA tube for the screening test, 3ml into a centrifuged gel tube for using the serum in the immunological procedures, and 5 ml into BHI vial for blood culture. Blood samples were taken from patients as soon as possible before they were admitted to antibiotic treatment. All the samples were accurately labeled with the known information before being transported to the laboratory. The brain heart infusion broth bottles containing blood sample were incubated at **37°C** in the incubator for 24-48 hours, to at least two weeks before being disposed. During these periods, one or two drops from the sample were transferred to Blood agar, and MacConkey agar to re-incubating at **37°C** to check for bacterial growth if present.

2.2.5 Inclusion Criteria

All the samples were taken from hospitalized patients, the majority of whom were in the ICU (intensive care units), RCU (respiratory care units), and burn unit. All the participants in the study have a history of some clinical signs such as fever, abnormal heart beating, and other symptoms of sepsis.

2.2.6 Exclusion Criteria

As a crucial step toward achieving a more homogeneous study population, patients with a history of the following confounding variables are excluded: Patients having a history of antibiotic usage, and history of autoimmune disease were excluded from the trial, as were other immunological disorders, and patient with HIV, COVID-19 positive.

2.2.7 Study Design:

The case control study was designed to include 125 blood samples, divided into 100 from septic patients and 25 from healthy controls.

[Figure 2.1] summarizes the sequential steps of our study.

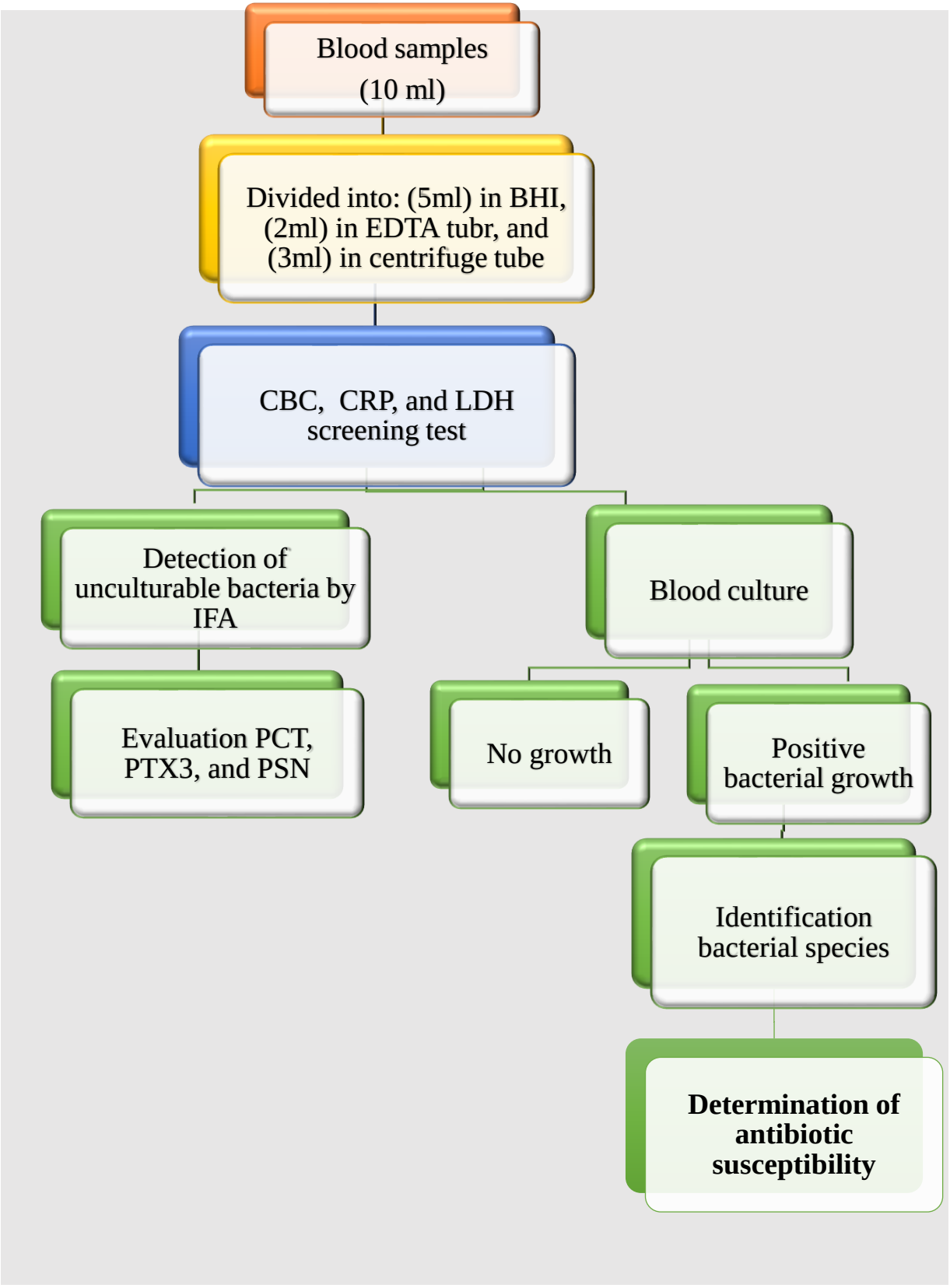


Figure 2.1 Study design

2.2.8 Screening Test Method

Complete blood count and CRP measurements were chosen as the main criteria that reveal whether an infection and inflammation is present in the patient of interest.

2.2.8.1 Complete Blood Count (CBC)

The CBC is a common screening test for most diseases. It is detected by a full automated hematology analyzer (Sysmex, Jaban). This apparatus was used to measure of white blood cells, granulocytes, lymphocyte counts, hemoglobin, hematocrit, platelets, and red distribution width in the blood containing EDTA tubes. They normally have a value of:

Table 2.6 The normal value of the complete blood count parameters.

No.	CBC parameters	Normal value
1	WBC	4.0-11.0*10^g/L
2	Granulocytes	40.0-70.0 %
3	Lymphocytes	20.0-50.0 %
4	HCT	35-50%
5	HGB	11-16 g/dL
6	PLT	150-450 10^g/L
7	RDW	10-15%

2.2.8.2 C-reactive Protein

By using serial dilutions of serum and saline, the semiquantitative slide agglutination method has been used to detect CRP. This procedure based on clumping between latex particle coated with anti-CRP and serum containing CRP. The CRP sensitivity in the serum can be detected by the kit when its concentration above 6 mg/L. In accordance with manufactured guidelines (Spinreact, Spain), the procedure was performed as follows:

1- The serum samples and latex were brought to room temperature.

2- With a micro pipette, 50µL of saline were placed into each circle of the slide.
3- Serial dilutions (two-fold) of the sample with saline were prepared on each circle of the slide.
4- The CRP-latex was mixed well before adding 50 µL to each circle on the same test slide containing the sample-saline solution.
5- With the wooden stick, the preparation was mixed and diffused on the entire surface of each circle.
6- The slide was tilted back and forth sluggishly for two minutes.
7- With the naked eye, the presence or absence of agglutination was seen. The occurrence of agglutination indicates a CRP concentration greater than 6 mg/L and the CRP-titer is the highest dilution display a positive result.
8- The formula used to determine the CRP concentration was 6× dilution factor.
9- Positive and negative control were performed.

2.2.8.3 Lactate Dehydrogenase Assessment

The LDH has been measured by using FUJI DR-CHEM NX500 automated clinical chemistry analyzer (FUJIFILM, Jaban).

2.2.9 Culture Method for Isolation of Bacterial Species

2.2.9.1 Preparation of Culture Media and Reagents

The culture media and solution utilized in this investigation, together with the protocol used to prepare them, are mentioned below.

2.2.9.1.1 Brain Heart Infusion Broth (BHI)

The calibrated flask was used to dissolve 3.7 grams of BHI medium in 100 ml of distilled water according to the manufacturer's instructions (HiMedia, India). The mixture was boiled in order to completely solve the

media, then the solution was sterilized in autoclave apparatus at 15 psi, 121C° for 15 minutes. The medium was poured in to sterile 10 ml test tubes with screw cap.

About 10-15% glycerol was added to the part of the broth that would be used later for the freezing (maintenance) of bacterial isolates at -20 C° (Tedeschi, and Paoli., 2011).

2.2.9.1.2 Blood Agar (BA) Preparation

A weight of 40 grams of blood agar base was melted in one liter of distilled water in accordance with manufactured guidelines (Pronadisa, Spain). The medium was completely dissolved by boiling the mixture. The mixture was sanitized by autoclaving for 15 minutes at (121 C° and 15 psi pressure) to sanitize it. After the medium has been autoclaved, let it cool to 50-45 °C before adding the blood 5 % v/v (Buxton, 2005). Blood agar is an enrichments medium used for the growth of both Gram-positive and Gram-negative bacteria as well as to differentiate between hemolytic and non-hemolytic Gram-positive bacteria (Suvarna, and Mahon, 2022).

2.2.9.1.3 Chocolate Agar (CA) Preparation

The procedure for preparing of CA is the same as that for preparing BA, with the exception of the step where red blood cells are lysed to liberate intracellular nutrients. Blood agar base was prepared according to manufactured procedures. 5 % of defibrinated sheep blood was aseptically added to the solution after it had been autoclaved, and it was then heated in a 75°C water bath. Pour it into sterile petri dishes under aseptic circumstances after allowing it to cool to 50 °C. chocolate agar is an enrichment medium used for cultivating the fastidious bacteria (Atlas and Snyder ,2013).

2.2.9.1.4 MacConkey Agar (MAC) preparation

A weight of 4.953 gm of the MacConkey powder was dissolved in 100 milliliters of purified (distilled) water in accordance with manufactured guidelines (HiMedia, India). The mixture was boiled for 1 minute, then the completely dissolved powder was sanitized by autoclaving for: 15 minutes at 121 C° and 15 Ibs pressure. The medium was cooled to 45-50°C and mixed well before pouring into Petri dishes. MacConkey agar is a selective medium, it contains bile salts and crystal violet that inhibit the growth of Gram-positive bacteria. This medium is also considered a differentiative medium due to it is ability to differentiate between lactose and non-lactose fermenter Gram-negative Enterobacteriaceae (Suvarna, and Mahon, 2022).

2.2.9.1.5 Mannitol Salt Agar (MSA) Preparation

A weight of 11.1 gm of the powder was solved in 100 ml of purified water in accordance with manufactured guidelines (liofilchem, Italy). The solution was mixed well and boiled for 1 minute until entirely dissolved. The mixture was sanitized in autoclave at (121°C for 15 minutes). After the medium has been autoclaved, let it cool to 50 °C before pouring into sterile Petri plates. The MSA is a selective and differentiative medium used in the identification of *Staphylococcus spp*. It contains NaCl and thus selects for those bacteria that can tolerate high salt concentrations. The MSA also differentiates among *staphylococci* based on their ability to ferment the sugar mannitol (Shields, and Tsang, 2006).

2.2.9.1.6 Eosin methylene blue (EMB) Agar Preparation

A weight of 3.596 gm from the EMB powder was solved in 100 ml of distilled water in accordance with manufactured guidelines (OXOID, England). The solution was mixed well and heated to dissolve the medium entirely. After that the mixture was Sanitized in autoclave at (121°C for 15

min.). After the autoclaving is completed, let it cool to between 45 to 50 °C, mix it, and then pour it onto sterile Petri dishes.

The EMB agar is selective for Gram-negative bacilli. It is also suitable in differentiating between lactose and non-lactose fermenter Gram-negative enteric bacteria. The bacteria that ferment lactose form-colored colonies, whereas those that do not ferment lactose seem colorless (Lal, and Cheeptham, 2007).

2.2.9.1.7 Xylose lysine deoxycholate (XLD) Agar Preparation

A weight of 5.668 gram of the powder was dissolved in 100 milliliters of distilled water in accordance with manufactured guidelines (Himedia, Iindia). The solution was mixed well and heated until it boiled to dissolve the medium entirely. This medium is not Sterilized in an autoclave. let it cool to between 45 and 50 °C and mix it gently before pouring it onto Petri dishes. The XLD agar is a selective medium for the growth of enteric Gram-negative bacteria, and it is also favorable for differentiating between Enterobacteriaceae based on xylose fermentation and the production of hydrogen sulfide (H2S) from the sodium thiosulphate (Wehr and Frank, 2004).

2.2.9.1.8 Mueller Hinton Agar Preparation

A weight of 38 gm of powder was solved in 1000 ml of purified water in accordance with manufactured guidelines (OXOID, England). The solution was mixed well and heated until it boiled for melt the powder entirely. The mixture was Sanitized in autoclave at (121°C for 15 minutes). The mixture has been cooled, and then poured into sterile Petri dishes.

Mueller Hinton agar is used for antibiotic susceptibility testing for the disc diffusion method (Fernández *et al*., 2009).

2.2.9.1.9 Salmonella Shigella (SS) Agar preparation

A weight of 6.302 gm of powder was dissolved in 100 milliliters of distilled water in accordance with manufactured guidelines (Himedia, Iindia). The solution was mixed well and heated until it boiled to dissolve the medium entirely. This medium is not Sterilized in an autoclave. The medium has been cooled, and then poured into sterile Petri dishes.

The SS agar is a selective medium for the growth of *Salmonella* and *Shigella spp*. It is also suitable for differentiating between them. Sodium thiosulfate and ferric citrate allow detection of H2S produced by *Salmonella typhi* (Bayu *et al*., 2013).

2.2.9.1.10 Urea Agar Base Preparation

In accordance to manufactured procedures (Himedia, India), a weight of 2.451 grams of urea base agar was dissolved in 95 ml of distilled water. The solution was mixed to completely dissolve the powder. The mixture was sanitized in autoclave at (15 psi, 121C°, for 15 minutes). After cooling in between 40-45 C, 5 ml sterile 40% Urea Supplement was added to the solution. The mixture was distributed into sterile tubes each with 3ml.

This preparation was used for urease test to identify bacteria that are capable of hydrolyzing urea into ammonia and carbon dioxide (Brink, 2010).

2.2.9.1.11 Simmons Citrate Agar Preparation

A weight of 2.428 gm of Simmons citrate powder was solved in 100 ml of distilled water in accordance to manufactured guidelines (Himedia, India). The solution was heated to dissolve the mixture entirely. Then it was sanitized by autoclaving for 15 minutes at (15 psi pressure, 121C°), and then poured into test tube after cooling at 50 C°.

The Simmons citrate agar was used to test if bacterial isolates were able to utilize citrate as a carbon source (MacWilliams, 2009).

2.2.9.1.12 Kligler Iron Agar Preparation

A weight of 5.752 gm of Kligler Iron agar was solved in 100 ml of distilled water in accordance to manufactured guidelines (Himedia, India). Heat was applied to the mixture to completely melt the powder. The mixture was distributed into test tubes, and then sanitized by autoclaving at (15 psi pressure at 121C° for 15 minutes). After the sterilization was completed, the tubes were cooled in a slanted position to form slopes.

Kligler iron agar is test for the ability of bacteria to ferment glucose and lactose, gas, and H2S production (Church, 2010).

2.2.9.1.13 Peptone Water Preparation

In this study, Peptone water was used for the detection of indole by certain microorganisms. The indole test screens if bacteria can break down the amino acid tryptophan and create indole ring (MacWilliams, 2012). In accordance to manufactured procedures (Himedia, India), a weight of 1.5 grams of Peptone was solved in 100 milliliters of distilled water. The mixture was dissolved completely and poured onto tubes with scraw cap. Then the tubes were sanitized in autoclave at 121C°, and 15 psi for 15 minutes.

2.2.9.1.14 MR-VP Broth

It is readily available and supplied by (Himedia, India) for the performance of MR and VP tests after the incubation period has been completed. The MR-VP broth bottles contain 5 ml of medium and are store at 2-8 °C.

2.2.9.1.15 Barrett's Reagent

It is composed of two solutions: VP1(α-naphthol) solution 5% w/v was prepared by dissolving a volume of 6 grams of α-naphthol powder in 100 milliliters of 95% ethanol, while the VP2 (KOH) solution was made by dissolving 16 grams of KOH powder in 100 milliliters of distilled water (McDevitt, 2009).

2.2.9.1.16 Oxidase Reagent

The oxidase reagent is N, N-Dimethyl p-phenylenediamine hydrochloride. It was available for direct use from Himedia, India.

2.2.9.1.17 Catalase Reagent

Hydrogen peroxide 3% (H2O2) provided by (Himedia, India) was utilized to determine whether bacteria could produce catalase.

2.2.9.1.18 Gram Stain Kit

It was readily available from (Merck, Germany) and consisted of**:** Crystal violet dye, iodine, 70% ethanol acts as a decolorizer, safranin acts as a counter stain.

2.2.9.2 Cultivation and Biochemical Identification

The collection of samples is an important part of the blood culture procedure. Throughout the procedure, standard measures are performed, and stringent aseptic conditions are maintained. Blood cultures were gotten in according to guidelines of (Towns *et al*., 2010), to increase the quality and clinical relevance of blood culture investigations while also lowering the risk of sample contamination.

2.2.9.2.1 Cultivation with Brain Heart Infusion Broth

The blood samples (5ml) were cultivated in BHI vials (50ml) and incubated at 37°C for 24 hours to at least two weeks before being disposed. During these periods, positive blood culture (turbid vial) was directly transferred and streaked onto several selective and differential solid media and re-incubated at **37°C** to check for bacterial growth if present.

2.2.9.2.2 Cultivation with Solid Media

Aseptically, one or two drops from positive broth (turbid vials) were transferred to solid media (Blood, and MacConkey). Re-incubations were performed at the same standard conditions. This method was used to

differentiate Gram-positive from Gram-negative bacteria. Presence of anti-bacterial materials in the MacConkey agar components that inhibit the growth of Gram-positive bacteria and only allow Gram-negative growth. After Gram staining were achieved, sub-cultures were performed by using other selective and differential media to differentiate among pathogenic bacteria. Mannitol salt agar was used to differentiate between gram-positive cocci (coagulase- positive and coagulase-negative). However, Eosin methylene blue and xylose lysine deoxycholate were used to differentiate between lactose fermenter bacteria and hydrogen sulfide production, respectively. Other media were included in this study such as salmonella shigella agar to provide more information about biochemical and morphological features of bacteria.

2.2.9.2.3 Gram Staining

A single bacterial colony was spread on a glass slide and heated to fix it. The crystal violet (primary stain) was applied and allowed to act for 1 min. The slide was washed gently by water for 2 second before flooded with iodine for 1minute. The slide was washed again before flooded with the decolorizing agent (70%ethanol) for 15 second. Counterstain (safranin) was applied for one minute. Then the slide was washed with water and dried by the hot air. Under an oil immersion microscope, the Gram-positive bacterium appear blue or purple and the Gram-negative bacterium appear pink or red (Smith and Hussey, 2005).

2.2.9.2.4 Motility Test

Motility was assessed through the hanging drop method. Aseptically, a single colony from 24-48 hours of bacterial growth has been placed on the glass slide. The colony was diluted by adding one drop of normal saline, mixing it, and covering the diluent specimen with a cover slip. The slide containing the specimen was transferred for examination under a light

microscope at 40x and 100x magnifications, respectively. The test is positive, if it shows any type of bacterial movement (Aygan and Arikan, 2007).

2.2.9.2.5 Coagulase Test

Slide method was adopted for achieved this test in order to differentiate among Gram-positive cocci. A sterile loop was used to transfer some colonies from overnight bacterial culture, and they were placed on the glass slide. A sterile dropper was used to cover bacterial colonies on that slide with one or two drops of plasma. This preparation was mixed well by wooden stick. The test is positive, if it shows clumping (coagulation) with in 10 second. Additional slides were used for control positive and control negative by utilizing coagulase-positive species (*Staphylococcus aureus*) and coagulase-negative (*Staphylococcus epidermidis*) respectively (Katz, 2010).

2.2.9.2.6 Catalase Test

After 24-48 hours of bacterial growth, some colonies were transferred by sterile disposable loop onto the glass slide. On the same glass slide, two drops of H2O2 were placed on these colonies. Wooden stick was used for mixing the colonies with the reagent. Bubbles indicate the existence of catalase enzyme; it is a positive finding. Additional slides were used for both control positive and negative (Reiner, 2010).

2.2.9.2.7 Oxidase Test

Two drops of the reagent were placed on the filter paper. By platinum loop, some colonies were transferred from 24-48 hours of bacterial growth and placed on the prepared filter paper for biochemical testing. The appearance of purple color within 10 seconds reveals the positive result (Shields and Cathcart, 2010).

2.2.9.2.8 Urease Test

A part of freshly isolated colony was streaked on the surface of urea agar or 1-2 drops from broth culture were inoculated by stabbing method. Urea agar was incubated at standard conditions (24-48 hours at 37˚C). If the growing microorganisms on urea agar containing an indicator at 6.8-7 PH produce urease enzyme, the enzyme will split urea into ammonia and carbon dioxide, resulting in an alteration of the origin color of the agar from yellow to pink due to the raise in the PH of the medium, this is positive result (Hemraj *et al*.,2013).

2.2.9.2.9 Kilgler Iron test (sugar fermentation)

This medium was employed to identification of bacteria based on sugar fermentation (lactose and glucose) and H2S production. Phenol red acts as an indicator based on the change in its color from red to yellow due to the change in the medium from alkaline to acid if bacteria are able to utilize the sugar component of the medium. The procedure was performed as follows:

1- With an inoculating sterile needle, some colonies from well-chosen solid media such as MacConkey were inoculated into KIA by using the stabbing method. The slant surface of the KIA was streaked after withdrawing the needle.
2- The test tubes containing the media was loosely closed before incubating for 24 to 48 hours at 37˚C.
3- After the incubation period was completed, the tubes were seen for acid production (slant/butt), gas, and H2S.
4- The results were recorded (Lehman, 2005).

2.2.9.2.10 Indole Test

Indole test evaluates the capacity of microorganisms to break down the amino acid tryptophan and generate indole. It is used to differentiate among enteric bacteria, particularly those that ferment lactose sugar. The procedure was performed as follows:

1- With a sterile loop, some colonies from solid media were inoculated into peptone water-containing tubes. The tubes were incubated for 24 hr. at 37˚C.
2- After incubation period is completed, one to two drops of Kovac's reagent were added to each tube.
3- If the growing microorganism degrades tryptophan producing, a red-colored ring from indole, this is positive result (Hemraj *et al*.,2013).

2.2.9.2.11 Methyl red and Voges-Proskauer tests

The MR/VP test is intended to determine the fermentation pathway that uses glucose in order to differentiate among enteric bacteria.

Methyl red test evaluates the ability of bacteria to produce mixed acid from glucose fermentation. In addition to the glucose, MR/VP broth contains peptone and phosphate buffer. Enteric bacteria were divided into two groups: those that perform mixed-acid fermentation and then produce an abundance of acid to overcome the broth's buffering potency (PH below 4.4), and those that perform other types of fermentation and cannot overcome the broth's buffering potency (PH above 6).

Voges–Proskauer test evaluate the ability of bacteria to produce acetoin from glucose fermentation. The PH indicator α- naphthol (VP1 reagent) and KOH (VP2 reagent) were used in this method. If the enteric bacteria degrade glucose into acetylmethylcarbinol, the VP1 and VP2 reagents will

reacts and give positive result (red color). The procedure was performed as follows.

1- Some colonies from fresh media were inoculated into previously prepared 5 ml MR/VP-broth-containing tubes and incubated for 48 hours at 37˚C.
2- In the case of the MR test, 5 drops of methyl red were added to these tubes as a PH indicator after the incubation period was completed. If the color of the broth turns red, this is a positive-result, whereas a yellow color is considered a negative-result.
3- In the case of the VP test, 12 drops of VP1 reagent and 4 drops from VP2 reagent were added to these tubes and shake it's for 30 second. The test tubes were allowed to stand for 30 minutes before the result were recorded. If the color of the broth turns red, this is a positive result, whereas a yellow color is considered as a negative result.
4- Control positive and negative were included to correctly interprets the results (McDevitt, 2009).

2.2.9.2.12 Citrate test

The test was carried out to see if the bacteria could use citrate as a carbon source. The PH indicator bromothymol blue was used in this method. If the microorganisms utilize the citrate compound, resulting in an alkaline product, the color of the media will change from original green to blue (Claus, 1989). The procedure was performed as follows:

1-Some colonies from fresh media were inoculated into previously prepared Simmons citrate agar. Agar containing plates were incubated for 48 hr. at 37˚C.
2-The color change was observed after the incubation period was completed. Blue color reveals that the test is positive.

2.2.9.3 Automated Method for Bacterial identification

For accurate species identification and to compare the efficiency of the VITEK 2 system with conventional manual procedures for identifying pathogenic bacteria in patients with a serious illness like sepsis, all the manually identified bacterial isolates were confirmed by the VITEK®MS device (bioMérieux). Vitek has an expanded identification database for all common identification tests, which increases the effectiveness of microbiological diagnosis and decreases the need for additional tests, hence raising safety for both the test and the users (Ali, 2017). This method is based on a fully automated option for performing about 64 biochemical tests through the presence of reagent cards, which have 64 wells to do this. In accordance with manufacture instructions, the procedure was performed as follows:

1- With sterile loop, a single colony was transferred from fresh (24 hour) solid media and inoculated into a tube containing 3 ml of normal saline
2- To standardize the colony to McFarland's standard solution (1.5×10^8 cell/ml), the tube was placed into the dens check machine.
3- The standardized inoculums were loaded into the cassette, and a barcode was used to inter a sample identification number into the computer.
4- After being placed in the filler module, the cassette was moved to the reader incubator module once the card had been fully filled.
5- The Vitek device controls the incubation temperature, reads the card, and sends test results to the computer for analysis.

2.2.9.4 Antibiotic Susceptibility

The testing for antibiotic sensitivity was done using commercial antibiotic disks and the conventional disk diffusion method on Mueller

Hinton agar in accordance with the guidelines of the CLSI (Clinical and Laboratory Standards Institute) Edition 2022 (Schuetz *et al*., 2022). The original Kirby-Bauer procedures is being updated and modified by the CLSI in accordance with a process of international consensus.

- **Purpose:**

The disk diffusion susceptibility method (Kirby-Bauer) measures the resistance or sensitive of pathogenic bacteria to numerous antimicrobial drugs to assist a physician in selecting the most effective course of therapy for his patients. The pathogen was cultivated on Mueller-Hinton agar with several antimicrobial disks. The presence and/or lack of growth around the antibiotic disk is an indicator of the antibiotic's ability to suppress the pathogen.

- **Test Requirement:**

Two milliliters of normal saline in a test tube; fresh colonies (24 hr.) from non-selective media; 0.5 McFarland act as standard; Wickerham card; Mueller-Hinton agar in a petri dish; sterile inoculating loop; sterile swab; antibiotic disks; ruler and forceps.

- **Inoculum Preparation:**

1- With a sterile loop, some colonies from fresh (24 hr.) non-selective media were transferred into test tube containing 2 ml of normal saline.
2- Smooth suspension was created by using a vortex.
3- Suspension turbidity was setup to be corresponding to the standard McFarland by placing the McFarland standard and the inoculum tubes in front of a Wickerham card. If the suspension is lighter than standard, more organisms must be added, or the suspension must be diluted with normal saline if it is heavier than standard.

- **Inoculation of the Mueller Hinton Petri dishes:**

1- sterile swab was dipped into the suspension tube. The swab was pressed and rotate on the internal surface of the tube above the fluid to remove extra fluid.
2- Streaking method was used to inoculate Mueller Hinton agar with this swab. The plate was rotated, and the streaking was repeated three times until the inoculum covered the entire surface of the agar.
3- The swab was disposed of in the proper container.

- **Placing the Disks of the Antibiotic:**

1- An appropriate number of antibiotic disks were placed on the surface of the Mueller Hinton agar and dispensed one at a time by using forceps.
2- To avoid contamination, the cover of the plate must be partially removed and returned at each time the disk is placed.
3- After placing all the desired disks, the plate's cover was replaced, the plate was inverted, and it was placed in an incubator at (35-37 °C for 24 hours).
4- After the incubation period was ended, the inhibition zone was measured with a ruler, and the result was recorded.

2.2.10 Immunological Method

The immunological approach for this investigation was divided into two parts: the first part used an indirect immunofluorescent assay (IFA), which relies on the interaction between sample's antibodies and the antigen adsorbed on the surface of the slide to detect IgG or IgM antibodies against unculturable bacterial agents that are present in patients' serum. The second

part evaluated the levels of procalcitonin, presepsin, and pentraxin3 in serum from patients (culture positive & culture negative) and serum from a healthy population as a control group by using an ELISA test.

2.2.10.1 IFA Method for Abs Detection in CNS

Depending upon the indirect immunofluorescent assay (IFA), the patient's serum of culture-negative sepsis (CNS) was tested for IgG and IgM antibodies against the main bacterial agents that cause atypical pneumonia in the respiratory tract: *Legionella pneumophila*, *Coxiella burnetii*, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Chlamydophila psittaci* using the PNEUMOBACT IFA kit supplied by (Vircell, Spain).

The indirect immunofluorescent procedure is relied on the response of the immunoglobulins in the samples (serum or plasma) when tested with an antigen that has been adsorbed on the surface of the slide. The specific antibodies (Ab_s) in the samples interact with the antigens (Ag_s), whereas any non-specific immunoglobulins that are not bound to the antigen were removed during the washing step. The antigen-antibody complexes then interact with the fluorescein-labeled anti-human globulin in the next step and are examined with an immunofluorescence microscope.

- **PNEUMOBACT IFA kit components**: all the contents of the kit were described in detail as shown in the [Table 2.7]
- **Storage**: store at 2-8°C.
- **Materials required:** micropipettes, incubator, coverslips, fluorescence microscope, humid chamber, and distilled water.

Table 2.7 PNEUMOBACT kit components

Components	Quantity
PNEUMOBACT slide	10 slides with the following Ags, each with 2 rows of 5 wells: *L. pneumophila*, *M. pneumoniae*, *C. burnetii*, *C. pneumoniae*, *C. psittaci*.
PBS powder (pH 7.2)	1 vial
PNEUMOBACT IgG positive control	300 µl of sodium azide-containing, IgG positive control.
PNEUMOBACT IgM control positive	300 µl of sodium azide-containing, IgM positive control.
PNEUMOBACT control negative	600 µl of sodium azide-containing, negative control.
Anti-human IgG (FITC conjugate)	2 vials containing 1.1 milliliters of the anti-human IgG that has been labeled with fluorescein in phosphate buffer with (Evan's blue, sodium azide, and a protein stabilizer).
Anti-human IgM (FITC conjugate).	2 vials containing 1.1 milliliters of the anti-human IgM fluorescein conjugate that has been labeled with fluorescein in phosphate buffer.
Mounting medium	Buffered glycerol with sodium azide in 3 ml
Anti-human IgG globulin (sorbent)	one vial contains 1.5 milliliter

- **Procedure:** Only the PBS was prepared beforehand by adding the PBS powder in the vial to one liter of purified water and shaking the preparation till it completely dissolved, then, keep it at 2 to 8ºC.

➢ **IgG determinations:**

1- Before use, all the solutions and slides were brought to the room temperature.

2- An 1/128 dilution of samples was prepared by addition ten microliters of samples to 1270 microliters of the PBS. The control reagents (positive and negative control) not to be diluted.

3- A volume of 20 µl of the dilutions were applied into the wells of the slide. Similarly, 20 µl of positive control and 20 µl of negative control were added to all wells 1 to 5 of the upper rows and 1 to 5 of the lower rows, respectively, on that slide.

4- Then the slide was incubated in a moist chamber (for 30 minutes at 37ºC).

5- Briefly rinsed the slide with the PBS (avoid directing PBS at wells) and immersed in the buffer (PBS) while shaking for ten minutes on a shaker. The slide was submerged in distilled water.

6- the slide was dried with air.

7- A volume of 20 microliters of anti-human IgG (FITC conjugate) was added to all wells.

8- The steps number four, five, and six were repeated sequentially.

9- Before covered with a coverslip, a small drop of mounting medium was addended to all wells.

10- The slide was observed in a fluorescence microscope at 400x magnification.

- **IgM determination**:

1- Before use, all the reagents and the slides were brought to the room temperature.

2- An 1/2 dilution of the samples was prepared through addition 25 microliters of the sample to 25 microliters of the PBS buffer. The control reagents not to be diluted.

3- The samples were treated with antihuman IgG sorbent by addition 15 microliters of diluted samples to 75 microliters of sorbent and mixed thoroughly. The control sera cannot be sorbent treated.

4- A volume of 15 µl of sorbent-treated samples were applied into the wells of the slide. Similarly, 15 µl of positive control and 15 µl of negative control were added to the 1-5 wells on the upper row and 1-5 wells on the lower row, respectively, on that slide.
5- Then the slide was incubated in a moist chamber at (37ºC for 30 minutes).
6- Briefly Rinsed the slide with a PBS (avoid directing the PBS at wells) and immersed in PBS while shaking for ten minutes on a shaker. The slide was submerged in the distilled water.
7- Then the slide was dried.
8- A volume of 20 microliters of anti-human IgM (FITC conjugate) was added to all wells.
9- The steps number 5, 6, 7 were repeated sequentially.
10- Before covered with coverslip, a small drop of mounting medium was added to all wells.
11- Then the slide was observed in a fluorescence microscope at 400x magnification.

2.2.10.2 ELISA tests the Levels of PCT, PTX 3, and PSN

Human PCT, PTX3, and Presepsin biomarkers were accurately and quantitatively detected in serum or plasma using the sandwich kits supplied by (BT LAB, China). Each plate in these kits was precoated with human-PCT, PTX3, or Presepsin antibodies. when serum samples are added to these plates, the PCT, PTX3, or Presepsin bind to the Abs fixed on the wells, then secondary Abs was added (biotinylated human PCT, PTX3, or Presepsin Abs) and bound to one of these biomarkers in serum sample. After that. Streptavidin HRP was applied. Streptavidin will bind to the biotinylated PCT, PTX3, or Presepsin Abs. Following the incubation and during the washing phase, unbound Streptavidin HRP is removed. After

addition of the substrate solution, color is developing in proportion to the amount of human PCT, PTX3, or Presepsin. Finally, the process is stopped by adding of a stop solution, and absorbance is measured at 450 nm.

2.2.10.2.1 ELISA Kit for the Procalcitonin Test

Human PCT concentration was detected using the sandwich kit produced by BT LAB/China according to the manufactured procedure:

- **Storage**: 2 to 8°C.
- **Standard Curve Range**: 5pg/ml - 2000pg/ml.
- **Sensitivity**: 2.49pg/ml.
- **Kit components:** [Table 2.8].

Table 2.8 Components of the PCT ELISA kit.

Kit components	Quantity
Standard Solution (2400pg/ml)	0.5 milliliter
ELISA Plate	12 × 8 well strips
Standard Diluent	3 milliliters
Streptavidin HRP	6 milliliters
Stopping Solutions	6 milliliters
Substrate (A)	6 milliliters
Substrate (B)	6 milliliters
Washing Buffer (25x)	20 milliliters
Biotinylated human-PCT Ab	1 milliliter

- **Preparation of reagents**:

Prior to use, all reagents were carried to room temperature. In order to create a 1200 pg/milliliter standard solution, the 120 microliter (2400 pg./ml) of the standard was reconstituted with 120 microliters of diluent. The standard was allowed to sit for fifteen minutes before producing a dilution. The standard stock solution (1200 pg/ml) was serially diluted (1:2) with the standard diluent to create solutions at 600 pg/ml, 300 pg/ml, 150 pg/ml and 75 pg/ml. The zero standard (zero pg/ml) is the standard diluent.

- **Wash Buffer:**

To create 500 ml of 1x wash buffer, Concentrated wash buffer (20 ml) was diluted with deionized or distilled water.

- **Procedure**:

1- All the reagents were prepared in accordance with manufacturer instructions and all serum samples were brought to room temperature.

2- In order to be utilized, the strips were put into the plate.

3- A volume of 50µl standard solution was added to the standard wells. The standard already contains biotinylated antibody, thus there is no need to add antibody into the standard well.

4- A volume of 40µl of serum samples was addended to the sample wells, followed by the addition of ten microliters of anti-PCT antibody to the sample wells, and 50 microliters of streptavidin HRP was addended to the samples well and standards well. Mixed The preparation, and the plate was covered before being incubated for (60 minutes at 37°C).

5- The cover was removed, the wells was washed five times with washing buffer, each time for 30 seconds. The plate was dried with absorbent substance like towels.

6- A volume of 50 microliters substrate A and 50 microliters substrate B was added to all wells, and then the plate was covered and incubated for (10 minutes at 37°C) in the dark.

7- The blue color turned to yellow immediately, after a volume of 50 microliters of Stopping Solution was added to all wells

8- following ten minutes of addition the stop solution, the OD (optical density) of all wells was measured at 450 nm using a microwell reader.

- **Calculation:**

  The concentration for standards was plotted on the Y (vertical) axis while the OD on the X (horizontal) axis, and a fit curve was drawn over the points on the chart to create a standard curve. The calculations were performed with computer-based curve fitting software and the best fit line was determined by regression analysis.

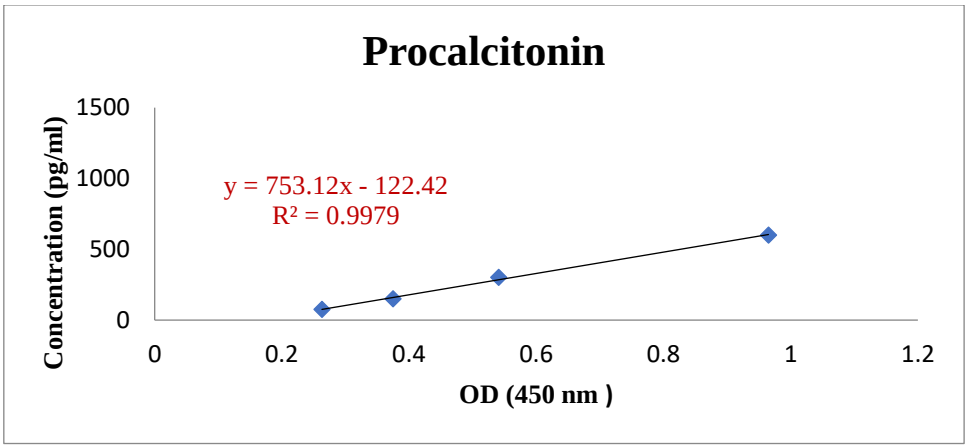


Figure 2.2: Standard curve for human procalcitonin in ELISA.

2.2.10.2.2 ELISA Kit for the Pentraxin 3 Test

Human PTX3 concentration was detected using the sandwich kit produced by BT LAB/China according to the manufactured procedure:

- **Storage**: 2 to 8°C.
- **Standard Curve Range**: 0.1 – 30 ng/ml.
- **Sensitivity**: 0.05 ng/ml.
- **Kit components:** [Table 2.9].

Table 2.9 Components of the PTX3 ELISA kit.

Kit components	Quantity
Standard Solution (32ng / ml)	0.5 milliliter
ELISA Plate	12 × 8 well strips
Diluent	3 milliliters
Streptavidin HRP	6 milliliters
Stopping Solution	6 milliliters
Substrate (A)	6 milliliters
Substrate (B)	6 milliliters
Washing Buffer (25x)	20 milliliters
Biotinylated Pentraxin3 Antibody	1 milliliter

- **Reagent Preparation**: Prior to use, all reagents were carried to the room temperature. To create a 16 ng/microliters standard solution, the 120 microliters of the (32 ng/ml) standard was reconstituted with 120 microliters of diluent. The standard was allowed to sit for 15 minutes before producing a dilution. The stock solution (16 ng/ml) was serially diluted (1:2) with the standard diluent to create solutions at 8 ng/ml, 4 ng/ml, 2 ng/ml and 1 ng/ml. The zero standard (zero ng/ml) is the standard diluent.
- **Wash Buffer:** To create 500 ml of 1x wash buffer, Concentrated wash buffer (20 ml) was diluted with deionized or distilled water.

- **Procedure:**

1- All the reagents were prepared in accordance with manufacturer instructions and the samples were brought to room temperature.

2- The strips were inserted into the plates for use.

3- A volume of 50µl standard solution was added to standard well. The standard already contains biotinylated antibody thus, there is no need to add antibody to the standard well.

4- A volume of 40µl of serum samples was addended to sample wells, followed by the addition of ten microliters of anti-PTX3 antibody to the sample wells, and 50 microliters of streptavidin HRP was added to the sample wells and the standard wells. Mixed the preparation, and the wells was covered before being incubated for (60 minutes at 37°C).

5- The cover was removed, the wells was washed five times with washing buffer, each time for 30 seconds. The plate was dried with absorbent substance like towels.

6- A volume of 50 microliters substrate (A) and 50 microliters substrate (B) was added to all wells, and then the plate was covered and incubated for (10 minutes at 37°C in the dark).

7- The blue color turned to yellow immediately, after a volume of 50 microliters of Stopping Solution was added to all wells

8- Following ten minutes of addition the stopping solution, the OD (optical density) of all wells was measured at 450 nm using a microwell reader.

- **Calculation:**

As with PCT protocols, the concentration for all standard was plotted on the Y (vertical) axis and the OD on the X (horizontal) axis. The best curve was drawn over the points on the chart to generate a standard

curve. These calculations were made by computer and the fit line was determined by regression analysis.

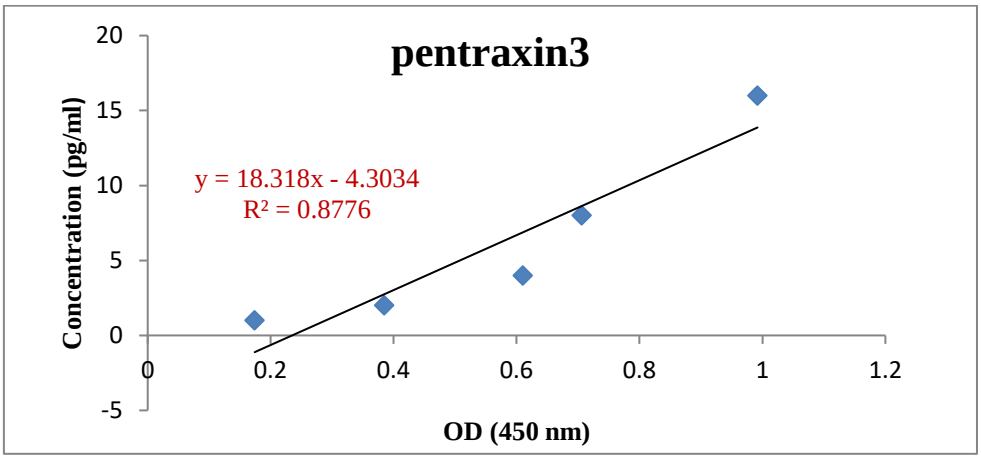


Figure 2.3: Standard curve for human pentraxin 3 in ELISA.

2.2.10.2.3 ELISA Kit for the PSN Test

Human Presepsin concentration was detected using the sandwich kit produced by BT LAB/China according to the manufactured procedure:

- **Storage**: 2 to 8°C.
- **Standard Curve Range**: 5ng/L – 1000 ng/L.
- **Sensitivity**: 2.39 ng/L.
- **Kit components**: [Table 2.10].
- **Reagent Preparation**: As with PCT and PTX3, to create 640 ng/L standard stock solution, the 120 microliter of the standard (1280 ng/L) was reconstituted with 120 microliters of standard diluent. Before producing a dilution, the standard was allowed to sit for 15 minutes with gentle agitation. The standard stock solution (640 ng/L) was serially diluted (1:2) with the standard diluent to create solutions at 320 ng/L, 160 ng/L, 280 ng/L and 40 ng/L. The zero standard (zero ng/L) is the standard diluent.
- **Wash Buffer**: As with PCT and PTX3 procedures the concentrated wash buffer (20ml) was diluted with deionized or distilled water to create 500 ml of 1x wash buffer.

Table 2.10 Components of the Presepsin ELISA kit.

Kit components	Quantity
Standard (1280ng/L)	0.5 milliliter
ELISA Plate	12 × 8 well strips
Diluent	3 milliliters
Streptavidin HRP	6 milliliters
Stopping Solution	6 milliliters
Substrate (A)	6 milliliters
Substrate (B)	6 milliliters
Washing Buffer (25x)	20 milliliters
Biotinylated Presepsin (PSN) Antibody	1milliliter

- **Procedures**:

1- All the reagents were prepared in accordance with manufacturer instructions and the samples were brought to room temperature.

2- In order to be utilized, the strips were put into the plate.

3- A volume of 50µl standard solution was added to standard well. The standard already contains biotinylated antibody thus, there is no need to add antibody to the standard well.

4- A volume of 40µl of serum samples was addended to sample wells, followed by the addition of ten microliters of anti-Presepsin antibody to sample wells, and 50 microliters of streptavidin HRP was added to

samples well and standards well. Mixed the preparation and covered the plate before being incubated for (60 minutes at 37°C).

5- The cover was removed, and the wells was washed five times with washing buffer, each time for 30 seconds. The wells were dried with absorbent substance like towels.

6- A volume of 50 microliters of substrate (A) and 50 microliters of substrate (B) was added to all wells, and then the plate was covered and incubated for (10 minutes at 37°C in the dark).

7- The blue color turned to yellow immediately, after a volume of 50 microliters of Stopping Solution was added to all wells.

8- Following ten minutes of addition the stopping solution, the OD (optical density) of all wells was measured at 450 nm using a microwell reader.

- **Calculation:**

As with PTX3 protocols. The concentration for standards was plotted on the Y (vertical) axis while the OD on the X (horizontal) axis, and a fit curve was drawn over the points on the chart to create a standard curve.

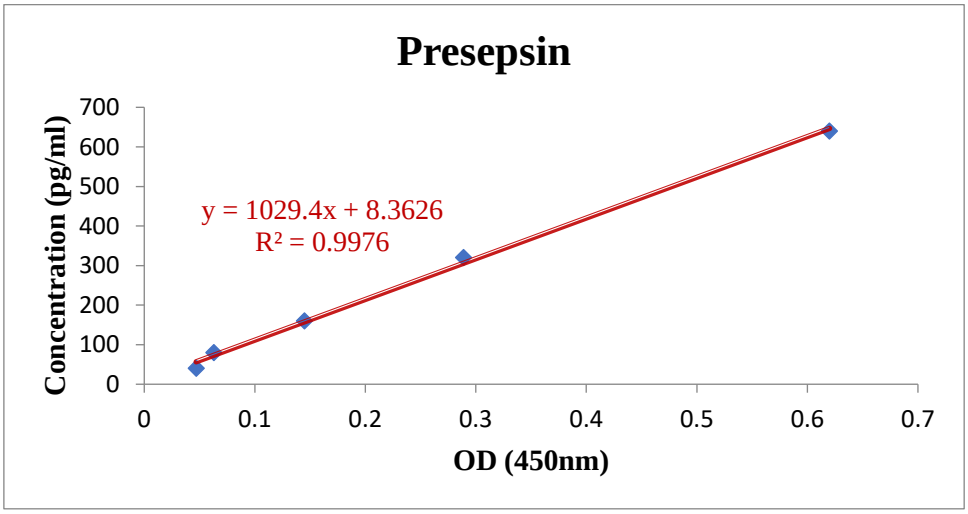


Figure 2.4: Standard curve for human presepsin in ELISA.

2.2.11 Statistical analysis

Statical package for the social science (SPSS), version 23 (SPSS Inc., Chicago, IL, USA) was used for data input and statistical analysis. An independent T-test was used to compare the patients and control groups, patients who gave culture-positive and culture-negative, as well as male and female in both culture-positive and culture-negative groups of patients, regarding the count of WBC, lymphocytes, and granulocytes as well as CRP and LDH. The outcomes were presented as mean± SD. In addition to that, one-way ANOVA (Duncan test) was used for multiple comparison groups, while Pearson's correlation test was used to explain the correlation between pro-inflammatory parameters and the correlation between sepsis biomarkers concentrations in the serum. A p-value < 0.05 was considered to denote statistical significance.

Chapter Three

Results and Discussion

3. Results and Discussion

A total of 125 blood samples were collected and analyzed during the study period. A number of 25 samples from healthy individuals served as the control group, while the remaining 100 samples served as the patient group for the detection of the presence of bacterial sepsis in the bloodstream of hospitalized patients, 18 of them died after a few days of collection. The findings of this investigation encompass those from screening test, cultural test, and immunological test.

3.1 Demographic Distribution of Patients and Control

The 100 patients (52 males and 48 females) who participated in the study had a mean age of 59.60 ± 18.314 years. In addition to that, the 25 healthy controls (12 males and 13 females) had a mean of 56.08 ± 19.925. Statistical analysis reveals that there are no significant differences between the ages of patients and controls (P value = 0.40).

Table 3.1 Comparison between patients and control in accordance with age.

Variables	Groups	NO.	Mean ±SD	P- value
Age (years)	Patients	100	59.60 ± 18.314	0.04
	Control	25	56.08 ± 19.925	

3.2 Screening Test (First Part) of the Study

The screening test revealed that 65 (65%) of the 100 samples had abnormal complete blood count parameters, and LDH values. In addition, 58 (89.2%) of the 65 samples had positive CRP levels (>10 mg/L), while the remaining 7 (10.8%) samples had negative CRP levels (≤10 mg/L). However, 35 (35%) of the 100 samples had unvaluable results of all screening tests.

According to this result, the participants who gave unvaluable result to the screening test (35 participants) may suffer from non-infectious causes of complications. The distinction between sepsis and non-infectious causes of inflammation needs further investigations (Bloos and Reinhart, 2014). The comparison of the patients' group (n=65) who gave abnormal screening tests with the healthy group (n=25) showed significant differences regarding all investigated parameters (WBC, GRA, LYM, HGB, HCT, RDW, PLT, CRP, and LDH) at P- value < 0.001 [Table 3.2].

Table 3.2 Parameters comparison between patients and control

Parameters	Control (n=25) Mean ± SD	Patients (n=65) Mean ± SD	P value
WBC	7.740 ± 1.6335	16.511 ± 4.6542	0.001
LYM	26.108 ± 3.6466	8.546 ± 3.2089	0.001
GRA	62.656 ± 4.8527	87.128 ± 8.8623	0.001
HGB	13.876 ± 1.1110	10.575 ± 1.5122	0.001
HCT	41.888 ± 3.2055	32.549 ± 4.8431	0.001
RDW	12.472 ± 0.6228	14.700 ± 3.6521	0.001
PLT	280.76±40.196	186.82±101.715	0.001
CRP	4.32 ± 1.520	13.45 ± 4.748	0.001
LDH	158.36 ± 73.802	550.77 ± 291.543	0.001

To avoid the serious consequences and to lower the mortality, the quick identification of sepsis is essential. Therefore, the useful of rapid screening test offers a preliminary indication of the presence of a condition that may cause sepsis and is regarded as one of the key indicators for the early detection of sepsis (Mohamed and Youness, 2021).

As with other sepsis parameters under investigation, complete blood count values (CBC) might be commonly useful since they are simple to carry out,

accessible in all healthcare facilities, and the first-line laboratory test that is most frequently ordered in all clinical settings (Agnello *et al*., 2021).

The correct interpretation of this test is essential for the early identification of a variety of clinical problems, which should then be further explored through laboratory and clinical analysis. The WBC, RBC, and PLT are the three CBC parameters. Lymphocytes, monocytes, and granulocytes are types of WBCs. It can be reported either an absolute value or as a percentage. The abnormal WBC value indicates the presence of acute inflammation caused by unidentified causative agents while, the relative value is useful for determining which WBC population is primarily involved in the inflammatory process, providing an etiological diagnosis.

Lymphocytes and granulocytes respond to microbial infection. This responsiveness is characterized by the increase of granulocytes and decrease lymphocytes count. Lymphocytes are an important element of the adaptive immune response. They account for 20-50% of all WBCs. The co-existence of pro-inflammatory and immunosuppressive changes is a defining feature of sepsis (Jarczak *et al*., 2021). These latter ones are distinguished by an early, extensive lymphocyte apoptosis. Overall, apoptosis brought on by sepsis results in lymphocytopenia. According to a lot of literature, lymphocytopenia may be a sign of a higher mortality risk in patients with sepsis. Hohlstein et al. suggested that lymphocytopenia at an intensive care unit (ICU) admission is associated with increased mortality (Hohlstein *et al*., 2019).

Granulocytes are another type of WBC that includes neutrophils, basophils, and eosinophils and their normal value is about 40–70% of the total WBC. They are a crucial element of the innate immune system and serve as sentinels to destroy invasive infections. The granulocyte count significantly increases during infection, and it is typically correlated with the severity of the infection.

However, the ability to diagnose sepsis from granulocyte percentage alone is limited. The fact that the granulocyte-to-lymphocyte ratio has become a reliable sepsis biomarker.

Regarding RBC, Effenberger & Hartmann observed that sepsis is characterized by a decreased RBC count, which may be brought on by a number of mechanisms connected to the altered generation or survival of RBC (Effenberger and Hartmann., 2018) However, a lower RBC count has no diagnostic value for sepsis. Critically ill patients may also have anemia due to lower hemoglobin (HGB<10g/dL) levels. Most patients develop anemia by day 8 throughout their hospitalization in an intensive care unit. (Docherty *et al*., 2018). Hematocrit (HCT) is the percentage of RBC in a whole blood sample. A decrease in HCT percentage is a hallmark of sepsis (van Beest *et al*., 2008; Angus *et al*., 2014). The values of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) do not have a role in patients with sepsis. Furthermore, because RBC survival was reduced and maturation was suppressed during sepsis, the red cell distribution width (RDW) increased (Krishna *et al*.,2021). However, several researchers were unable to find a relationship between sepsis consequence and RDW (Fontana *et al*., 2017).

Platelets (PLT) are one of the blood components. A comprehensive intravascular immune defense response is orchestrated by PLT to prevent the spread of bacteria by acting as sentinels for the quick detection of microbial invasion (McDonald and Dunbar, 2019). During sepsis, multiple processes, including the activation of the coagulation system, cause the activation of platelets. The surface of activated platelets expresses many receptors that either directly bind to and sequester external pathogens or stimulate the aggregation of neighboring platelets and leukocytes (Guo and Rondina, 2019). A typical sign

in septic patients is thrombocytopenia, or a decrease in platelet count. In fact, numerous writers have demonstrated that platelet count is an effective sepsis diagnostic and prognostic indicator (Levi, 2016).

For further progressive in our investigations, CRP was estimated as another diagnostic parameter. Positive CRP responses indicate the presence of infection and inflammation (Escadafal *et al* 2020). Although it is used to screen for early sepsis, CRP has low specificity in the case of bacterial sepsis detection.

The LDH is another potent parameter for sepsis detection. In individuals with sepsis, lactate levels are thought to increase because of reduced tissue perfusion, which results in hypoxia and anaerobic glycolysis (Kang and Park., 2016). Although LDH is unreliable for determining the etiological agent of infection, it has an important role in identifying the severity of sepsis and monitoring the state of patients who reside in intensive care units.

3.3 Cultural Part

The results of the cultural method exhibit differences when the 100 blood samples were inoculated in the BHI vials and then only the turbid vials were transferred to several selective and differential media to be re-incubated under standard bacterial growth conditions as illustrated below:

3.3.1 Findings of the Brain Heart Infusion broth

Only, 25 (25%) of 100 samples had positive blood culture (turbid vials) when monitoring daily during the BHI incubation period. As an existing result, the BHI turbidity may be related to the growth of gram-positive, gram-negative, or other types of bacteria because these artificial media are ideal for their growth and their proliferation. Afterward, the bacterial growth in broth media were transferred into the several types of solid media to differentiate among them. Standard measures are performed, and stringent aseptic conditions are

maintained to improve the quality and clinical relevance of blood culture studies. The first indication of the presence of bacteria in the target blood samples can be obtained using the blood culture method.

3.3.2 Growth on the Selective and Differentiative Solid Media

In this investigation, all 25 (25%) positive BHI vials were incubated immediately on Blood agar, Chocolate, MacConkey, Mannitol salt, Xylose lysine deoxycholate, and Eosin methylene blue agar. After a 24-hour of incubation at 37 °C is completed, all 25 positive BHI vials gave positive results and demonstrated the morphological form and biochemical properties of different bacterial species.

The result of blood culture is illustrated in [Figure 3.1].

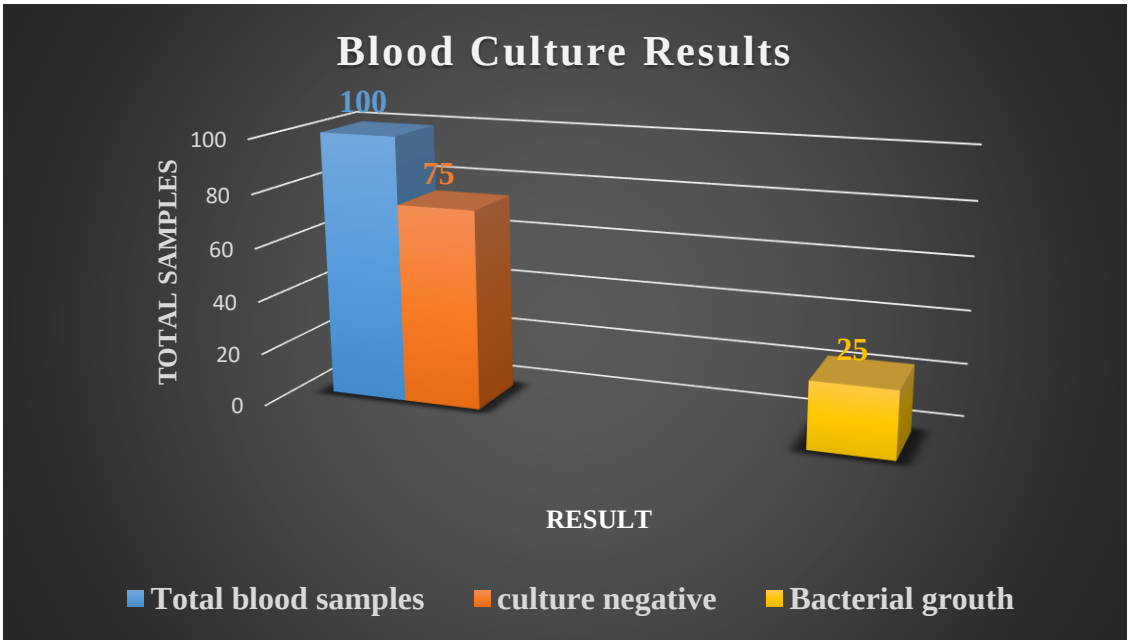


Figure 3.1 Results of the blood culture. First column for all blood samples, second column for culture negative, and third column for positive blood culture.

3.3.3 Manual and Automated Methods for Species Identification

The Gram staining and other major biochemical tests were performed for each culture positive sample (n=25) with a manual methodology for species identification.

Various species of bacteria observed, and these isolates were confirmed later by Vitek 2 system. These isolates are characterized by the predominance of Gram-positive 15 (60%) over the Gram-negative bacteria 10 (40%). According to several investigations, Gram-positive bacteria are now outnumbering Gram-negative bacteria in the epidemiology of sepsis (Al-Sa'ady and Naher, 2016). The results of the current study are compatible with the results of Arora and Devi study in 2007, when the positive blood culture represented 509 (20%) of 2542 cases, Gram positive *Staphylococcus aureus* was the predominant isolate followed by coagulase negative *Staphylococci* and Gram-negative bacteria (Arora and Devi, 2007).

In fact, this change may be caused by greater usage of invasive devices or an increased in patients who are susceptible to sepsis, such as the elderly. Additionally, Gram-positive pathogens are responsible for major healthcare infections and have become more resistant to antibiotics. (Opal, 2003 ; Jubeh *et al*., 2020).

From 25 CPS, 6 (24%), 4 (16%), 2 (8%), 1 (4%), 1 (4%) and 1 (4%) were gave the morphological and biochemical characteristics of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa Staphylococcus haemolyticus*, and *Staphylococcus hominis* respectively when were identified by manual and automated methods. These six bacterial isolates might be actual infectious agents or, correlate with the secondary infection of hospitalized patients acquired through their admission to a health-care facility (hospital-acquired infection). This interpretation is related to the location and time of sample collection, as the sample was collected from Intensive care unit and Respiratory care unit within 2 weeks of hospitalization (Yardena *et al*., 2002). Because a certain number of samples were collected from hospitalized patients with a history of urinary tract

infections and patients with a history of respiratory disease, other isolates such as *Escherichia coli* 2 (8%), *Enterobacter aerogenes* 1 (4%), *Morganella morganii* 1 (4%), *Salmonella typhi* 1 (4%), *Streptococcus pneumonia* 2 (8%), and *Klebsiella pneumonia* 1 (4%) may reflect the origin of the primary site of infection. *Kocuria kristinae*, a rare pathogenic bacterium, was present in 1 (4%) of the 25 CPS. It is a part of the normal skin flora. However, it can cause serious illness in a small number of patients, including catheter-related bacteremia, particularly in those with immune system defects or in elderly and debilitated patients (Dunn *et al*., 2011). Finally, one (4%) of the 25 CPS was *Pseudomonas stutzeri*, a distinct, nonfluorescent bacterium that is common in the environment and has been isolated from patients as an opportunistic disease. Several publications described the isolation of these bacteria from clinical samples, particularly those that were collected from bacteremic patients (Lalucat *et al*., 2006).

A precise diagnosis of the bacteria causing bloodstream infections gives essential clinical data needed to identify and treat sepsis (Towns *et al*., 2010 ; Zhang *et al*., 2022). However, a blood culture alone is not sensitive enough to detect bacterial sepsis especially, when the patient has already taken antibiotics or there are fastidious organisms present that are unable to grow under normal conditions (culture give false negative). Furthermore, the most challenging interpretation is determining if the organism recovered from a blood culture is an actual pathogen that causes bloodstream infection or a contaminant (false positive). If it is a contaminant, the patient can end up getting antibiotics when they are not necessary, which would put the patient at more risk (Richter *et al*., 2002).

The sex distribution of bacterial isolates and the findings of the biochemical tests for each isolate are depicted in [Table3.3].

Table 3.3 Sex distribution of isolates and their biochemical features.

Number of isolates		Gram Staining	Motility	Coagulase	Catalase	Oxidase	Urase	H2S	Indole	MR	VP	Citrate	Species
Male	Female	Biochemical tests											
3	3	+	-	+	+	-	-	-	-	+	+	+	*S. aureus*
-	4	+	-	-	+	-	+	+	-	-	+	-	*S. epidermidis*
2	-	-	+	-	+	+	-	-	-	-	-	+	*S. maltophilia*
-	2	-	+	-	+		-	-	+	+	-	-	*E. coli*
1	1	+	-	-	-	-	-	-	-	+	-		*S. pneumonia*
1	-	-	+	-	+	-	-	+	-	+	-	-	*S. typhi*
-	1	+	-	-	+	-	-	-	-				*S. haemolyticus*
1	-	-	-	-	+	-	+	-	-	-	+	+	*K. pneumonia*
-	1	-	+	-	+	-	+	+/-	+	+	-	-	*M. morganii*
1	-	-	+	-	+	+	-	-	-	-	-	+	*P. aeruginosa*
-	1	+	-	-	+	-	+	+	-	-	-	-	*S. hominis*
1	-	+	-	-	+	-	-	-			+		*K. kristinae*
-	1	-	+	-	+	-	-	-	-	-	+	+	*E. aerogenes*
-	1	-	+	-	+	+	-	-	-	-	-	+	*P. stutzeri*
Total = 25		culture positive of 100 blood samples											

3.3.4 Antibiotic Susceptibility

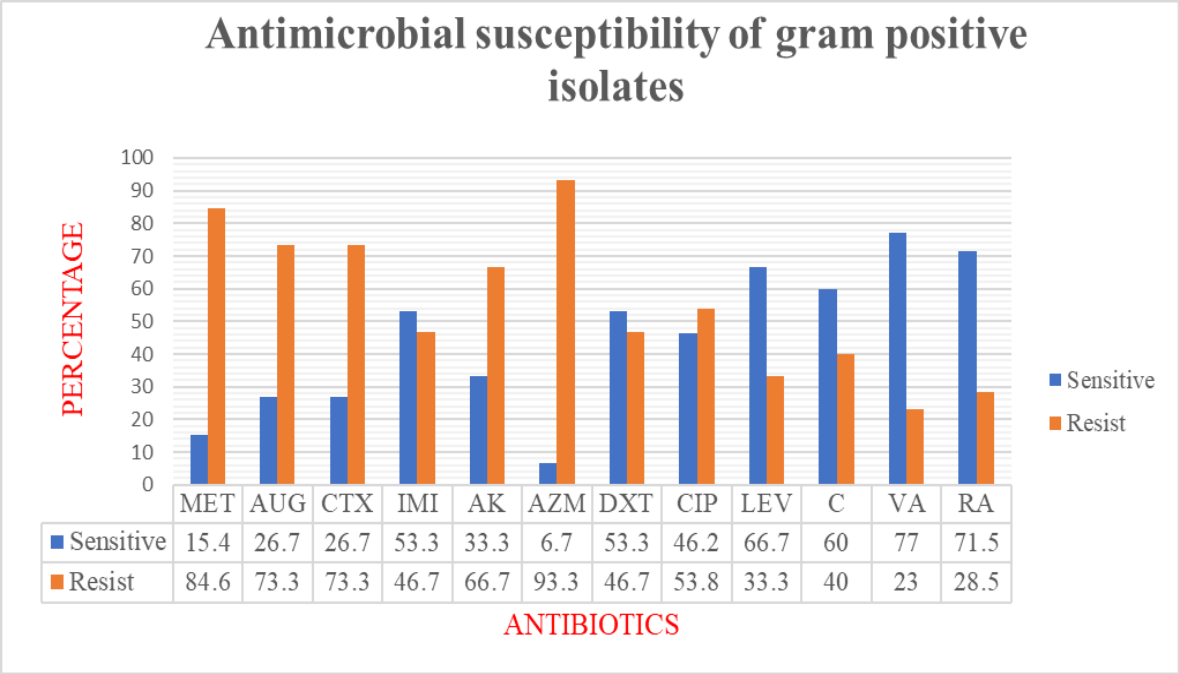
Alongside the discovery of antibiotics, resistance was always acknowledged and continuously developed (Jubeh *et al*., 2020). The administration of early and appropriate antibiotic therapy is a critical in determining the final fate of

patients with sepsis (Lodise *et al*., 2007). When a patient exhibits clinical signs of septicemia or bacteremia, it is normal practice to begin early empirical therapy with broad spectrum antibiotics. Such empirical therapy may be justifiable given the severity of the sepsis, but the specialized therapy will certainly enhance the therapeutic result of patients (Owens, 2008). During this investigation, different antibacterial disks from different classes were used to detect the antimicrobial susceptibility of Gram positive and Gram-negative staining isolates.

The disk diffusion (Kirby-Bauer) method on the Mueller Hinton agar was done in accordance the guidelines of the CLSI.

3.3.4.1 Susceptibility Results of the Gram-Positive Bacteria

According to the findings of this study, the most common gram-positive pathogens in bloodstream infections were *Staphylococcus* species 12 (48%) of the 25 isolates, followed by *Streptococcus pneumoniae* 2 (8%) and *Kocuria kristinae* 1 (4%). Most of them were antibiotic resistant. Azithromycin, Methicillin, Augmentin, Cefotaxime, and Amikacin have the highest resistance percentages (93.3%, 84.6%, 73.3%, and 66.7%, respectively), while the least resistant percentages were to Chloramphenicol, Levofloxacin, Rifampin, and Vancomycin (40.0%, 33.3%, 28.5%, and 23%, respectively). Both Imipenem and Doxycycline have a moderate and equal effect on these bacteria. The percentages of sensitivity and resistance of tested antibiotics to all Gram-positive isolates are illustrated in the [Figure 3.2].



	MET	AUG	CTX	IMI	AK	AZM	DXT	CIP	LEV	C	VA	RA
Sensitive	15.4	26.7	26.7	53.3	33.3	6.7	53.3	46.2	66.7	60	77	71.5
Resist	84.6	73.3	73.3	46.7	66.7	93.3	46.7	53.8	33.3	40	23	28.5

Figure 3.2 Antibiotic susceptibility percentage of Gram-positive isolates.

S: Sensitive; **R**: Resist; **MET**: Methicillin; **AUG**: Augmentin; **CTX**: Cefotaxime; **IMI**: Imipenem; **AK**: Amikacin; **AZM**: Azithromycin; **DXT**: Doxycycline; **CIP**: Ciprofloxacin; **LEV**: Levofloxacin; **C**: Chloramphenicol; **VA**: Vancomycin; **RA**: Rifampin.

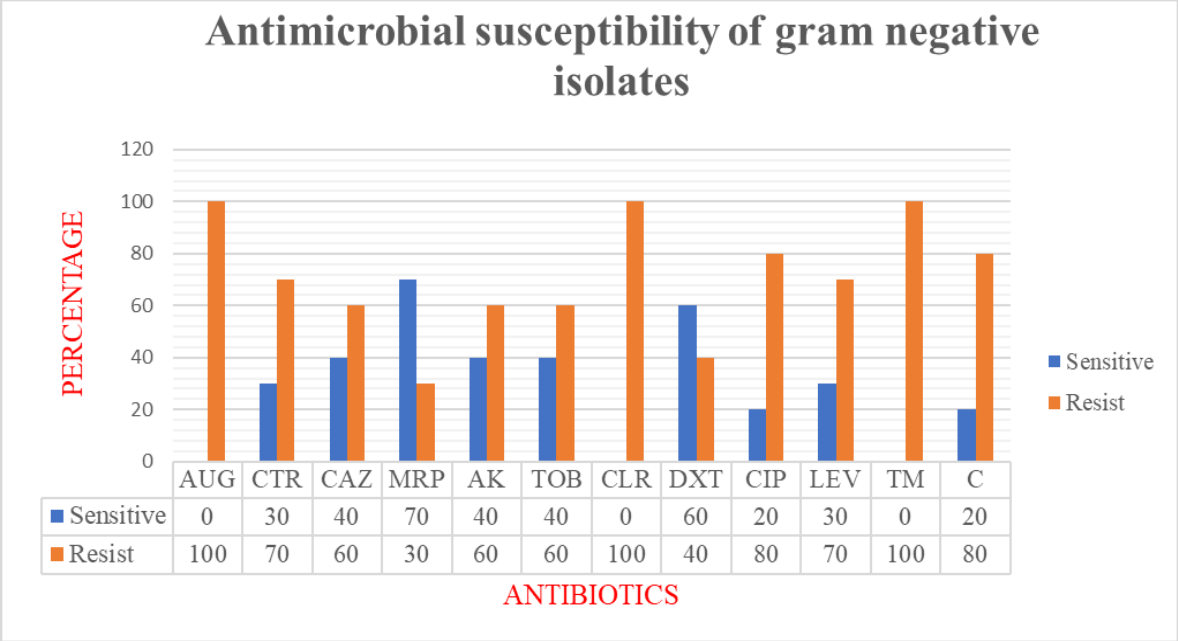
Regarding the problem of antibiotic resistance dissemination, bacteria of the Staphylococcus species are part of the human microflora and at the same time represent the most important opportunistic pathogens. The major pathogen in this group is *Staphylococcus aureus*, which causes a wide range of human infections and is leading cause of bacteremia (Tong *et al*., 2015). Nevertheless, coagulase negative staphylococci, particularly *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, have emerged as a frequent cause of hospital-acquired infections (Becker *et al*., 2014). Increasing the resistance to different antimicrobial classes among staphylococcus species isolated from different hosts, such as *Staphylococcus haemolyticus*, may be related to the acquisition of the antibiotic resistance gene through the exchange of genetic material among these species (Kohler *et al*., 2018). Current evidence reports that the less pathogenic Staphylococcus species act as gene-reservoirs for more pathogenic

species, like *Staphylococcus aureus* (Rossi *et al*., 2017). Other Staphylococcus species, like *S. epidermidis*, have a biofilm forming feature that help them cooperate and hinders their exposure to antibiotics (Flemming *et al*., 2016). The Present findings indicates that all Gram-positive isolates were resistant to most tested antibiotics, especially methicillin and other beta-lactam antibiotics, and the percentage of this resistant is consistent with several previous studies that reported about the decreasing of antibiotic effects among most gram-positive strains such as methicillin resistant *S. aureus*. (French, 2006 ; Boucher *et al*., 2009 ; Kante *et al*., 2014). Another study found that antibiotic susceptibility testing of isolated Gram-positive microorganisms showed good sensitivity to amikacin, meropenem, and vancomycin. However, *S. epidermidis* and *S. haemolyticus* were resistant to ampicillin (85.9%) and penicillin (88.1%). In addition, *S. aureus* displayed resistance to ampicillin (89.9%) and penicillin (86.0%). Gram positives were also found to be resistant to Augmentin (Acheampong *et al*., 2022). A multidrug resistant pathogen may be increasing the risk of death, prolong hospital stays, and necessitate the administration of more expensive drugs. Even though antibiotic resistance restricts therapeutic options for treating septic patients, the current isolates are least resistant to Vancomycin and Rifampin, respectively, and these antibiotics can be used as specific and alternative therapies against Gram-positive bacteria, which cause lower respiratory tract infection and bacteremia.

3.3.4.2 Susceptibility Results of the Gram-Negative Bacteria

Out of 25 isolates, *Escherichia coli* were 2 (8%) and *Stenotrophomonas maltophilia* were 2 (8%), followed by other reported Gram-negative bacteria. All these bacterial isolates demonstrate a high resistance pattern to most tested antibiotics. Augmentin, Clarithromycin, and Trimethoprim are completely

resistant (100%), followed by Chloramphenicol and Ciprofloxacin (80%). Other studies also have found that most of the gram-negative bacteria in their works as multidrug resistant (Gill and Sharma, 2016), this might be due to inappropriate empirical use of antibiotic as urgent treatment. The least proportion of resistance were to Meropenem and Doxycycline (30% and 40%, respectively), while 60% were to Amikacin, Tobramycin, and Ceftazidime. The percentages of sensitivity and resistance of tested antibiotics to all gram-negative isolates are illustrated in [Figure 3.3].



	AUG	CTR	CAZ	MRP	AK	TOB	CLR	DXT	CIP	LEV	TM	C
Sensitive	0	30	40	70	40	40	0	60	20	30	0	20
Resist	100	70	60	30	60	60	100	40	80	70	100	80

Figure 3.3 Antibiotic susceptibility percentage of Gram-negative isolates.

S: Sensitive; **R**: Resist; **AUG**: Augmentin; **CTR**: Ceftriaxone; **CAZ**: Ceftazidime; **MRP**: Meropenem; **AK**: Amikacin; **TOB**: Tobramycin; **CLR**: Clarithromycin; **DXT**: Doxycycline; **CIP**: Ciprofloxacin; **LEV**: Levofloxacin; **TM**: Trimethoprim; **C**: Chloramphenicol.

Acheampong *et al*. performed a study in 2022 that partially agreed with the results of this study regarding the antimicrobial susceptibility pattern among isolated Gram-negative microorganisms, *Klebsiella spp*. demonstrated sensitivity to the meropenem (97.0%) and relatively high resistance to ampicillin (89.2%) and cefotaxime (82.0%). *P. aeruginosa* showed relatively

high resistance to cefotaxime (71.0%). *E. coli* demonstrated moderate sensitivity to amikacin (71.0%) and relatively high sensitivity to meropenem 94.7% (Acheampong *et al*., 2022).

Antimicrobial resistance is a major worldwide health concern and one of the most significant risks to human survival today. Gram-negative bacteria are thought to be more resistant than Gram-positive bacteria due to their unique structure, as well as they cause major morbidity and mortality worldwide. The outer membrane of Gram-negative bacteria (LPS) is the main cause of resistance to a wide range of antibiotics, such as β-lactams, quinolones, and others (Breijyeh *et al*., 2020).

In this investigation, the antibiotic resistance pattern of Gram-positive and Gram-negative isolates showed a high prevalence of resistance, especially to beta-lactams, macrolides, and cephalosporin families. However, some species are sensitive to Glycopeptides (vancomycin), carbapenems, and quinolones, which is agreed with the other studies (Soriano *et al*., 2008 ; Wasihun *et al*., 2015). The rate of the antimicrobial resistance is still increasing due to the use of empirical antibiotics in the treatment of most hospitalized cases, especially those that have more severe infections with unidentified causative agents.

In general, the bacteria that responsible for septicemia are different over time and even from region to region. The pattern of antimicrobial susceptibility differs from country-to-country based on the epidemiology of sepsis. These differences are the main cause of the varying antibiotic susceptibility reported by different researchers (Parajuli *et al*., 2017).

3.4 Analysis the Results of Screening Test and Blood Culture

Since no single test is perfect, there are numerous sepsis parameters that can at least help identify severely ill patients so that the condition can be identified and treated. For this reason, further analysis was done to confirm the clinical importance of general screening tests and blood cultures in the early detection of septic patients. A comparison between culture positive and culture negative septic patients was done. This comparison included all the screening test parameters. Males and females were also subjected to this comparison.

3.4.1 Comparison between CPS and CNS

At this point, the patients were divided into those with culture-positive sepsis (CPS) 25 (25%) of 100, and those with culture-negative sepsis (CNS) 75 (75%). This comparison included 25 CPS, and only 40 samples from 75 CNS that had abnormal screening test.

Statistically analysis of the results reveals that there are no significant differences between two groups of hospitalized patients (CPS and CNS) regarding to CBC, CRP, and LDH results ($P > 0.05$) [Table 3.4]. This finding adds additional evidence for the vital role of CBC, CRP, LDH, blood culture, and other diagnostic parameters to detect bacterial sepsis (Fan *et al*., 2016). According to the present findings, the culture-negative samples might be related to septic patients suffering from fastidious and unculturable bacterial infections. parameters, such as white blood cells count, C-reactive protein, and lactate, have been commonly used to predict the occurrence of life-threatening bacteremia and provide prognostic information (Lien *et al*., 2022). However,

further analysis using other laboratory tests must be done to confirm this prediction.

Table 3.4 Comparison between culture positive and culture negative patients.

Characteristics	CPS (n=25) Mean ± SD	CNS (n=40) Mean ±SD	P- value
WBC	16.476 ± 6.2218	16.533 ± 3.4240	0.96
LYM	6.556 ± 3.5385	7.350 ± 3.223	0.37
GRA	89.084 ± 5.1489	85.905 ± 10.4204	0.16
HGB	10.308 ± 1.7088	10.743 ± 1.3717	0.26
HCT	32.364 ± 6.0613	33.155 ± 4.2601	0.54
RDW	15.004 ± 4.8043	14.510 ± 2.7546	0.64
PLT	216.92±127.977	168.00±77.168	0.09
CRP	12.22 ± 2.21	13.56 ± 3.56	0.08
LDH	594.12 ± 377.099	523.68 ± 223.642	0.34

CPS: culture positive sepsis**; CNS**: culture negative sepsis; **WBC**: white blood cell; **LYM**: lymphocytes; **GRAN**: granulocytes; **Hb**: hemoglobin; **HCT**: hematocrit; **RDW**: red cell distribution width; **PLT**: platelets **CRP**: C-reactive protein; **LDH**: lactate dehydrogenase.

3.4.2 Distribution According to Sex

From 25 culture positive sepsis (CPS), 10 (40%) were males and 15 (60%) females. In addition to that, from 40 culture negative sepsis (CNS) patients chosen, 23 (57.5%) were males and 17 (42.5%) females.

Statistical analysis of the results reveals that there are no significant differences between CPS and CNS males [Table 3.5], as well as no significant difference between CPS and CNS females regarding the investigated parameters (P> 0.05) [Table 3.6].

Table 3.5 Comparison between CPS and CNS males.

Characteristics	CPS male (n=10) Mean ±SD	CNS male (n=23) Mean ±SD	P- value
WBC	13.780 ± 6.7141	17.387 ± 3.2930	0.11
LYM%	6.290 ± 2.0223	7.743 ± 2.8550	0.227
GRA%	89.590 ± 2.2698	84.948 ±12.1732	0.244
HGB	10.280 ± 1.8570	11.035 ± 1.5035	0.226
HCT%	30.178 ± 6.6257	33.965 ± 4.8663	0.146
RDW%	15.220 ± 6.5974	14.348 ± 2.4711	0.58
PLT	219.90 ± 132.687	168.78 ± 61.325	0.136
CRP	12.6544±3.4784	13.6784±3.4784	0.172
LDH	589.00 ± 271.711	578.043 ±240.6821	0.909

Table 3.6 Comparison between CPS and CNS females.

Characteristics	CPS female (n=15) Mean ±SD	CNS female (n=17) Mean ±SD	P- value
WBC	18.273 ± 5.2345	15.376 ± 3.3462	0.128
LYM%	6.733 ± 4.3302	7.052 ± 3.086	0.814
GRA%	88.747 ± 6.4677	87.200 ± 7.6039	0.982
HGB	10.327 ± 1.6697	10.347 ± 1.0904	0.967
HCT%	32.600 ± 5.1926	32.059 ± 3.0749	0.721
RDW%	14.860 ± 3.3958	14.729 ± 3.1638	0.911
PLT	214.93 ± 129.415	166.94 ± 96.654	0.240
CRP	12.9659±2.782	12.9659 ± 2.782	0.999
LDH	597.53 ± 443.042	464.142 ±185.3590	0.259

CPS: culture positive sepsis**; CNS**: culture negative sepsis; **WBC**: white blood cell; **LYM**: lymphocytes; **GRAN**: granulocytes; **Hb**: hemoglobin; **HCT**: hematocrit; **RDW**: red cell distribution width; **PLT**: platelets **CRP**: C-reactive protein; **LDH**: lactate dehydrogenase.

The sex comparison showed no significant difference between CPS males and CPS females [Table 3.7]. There were no significant differences between CNS males and CNS females (P> 0.05) [Table 3.8].

Table 3.7 Comparison between males and females in culture positive patients.

Characteristics Culture Positive	CPS male (n=10) Mean ±SD	CPS female (n=15) Mean ±SD	P- value
WBC	13.780 ± 6.7141	18.273 ± 5.2345	0.07
LYM%	6.290 ± 2.0223	6.733 ± 3.661	0.76
GRA%	89.590 ± 2.2698	88.747 ± 6.4677	0.69
HGB	10.280 ± 1.8570	10.327 ± 1.6697	0.94
HCT%	30.178 ± 6.6257	32.600 ± 5.1926	0.33
RDW%	15.220 ± 6.5974	14.860 ± 3.3958	0.85
PLT	219.90 ± 132.687	214.93 ± 129.415	0.927
CRP	12.6544±3.4784	12.9659±2.782	0.80
LDH	589.00 ± 271.711	597.53 ± 443.042	0.95

Table 3.8 Comparison between males and females in culture negative patients.

Characteristics Culture Negative	CNS male (n=23) Mean ±SD	CNS female (n=17) Mean ±SD	P- value
WBC	17.387 ± 3.2930	15.376 ±3.3462	0.06
LYM%	7.395 ± 2.838	6.935 ± 2.806	0.613
GRA%	84.948 ±12.1732	87.200 ± 7.6039	0.50
HGB	11.035 ± 1.5035	10.347 ± 1.0904	0.11
HCT%	33.965 ± 4.8663	32.059 ± 3.0749	0.16
RDW%	14.348 ± 2.4711	14.729 ± 3.1638	0.67
PLT	168.78 ± 61.325	166.94 ± 96.654	0.942
CRP	13.6784±3.4784	12.9659±2.782	0.53
LDH	578.043 ±240.6821	464.142 ±185.3590	0.13

CPS: culture positive sepsis**; CNS**: culture negative sepsis; **WBC**: white blood cell; **LYM**: lymphocytes; **GRAN**: granulocytes; **Hb**: hemoglobin; **HCT**: hematocrit; **RDW**: red cell distribution width; **PLT**: platelets **CRP**: C-reactive protein; **LDH**: lactate dehydrogenase.

Although the results of the present study are similar for both sexes in terms of abnormal WBC, CRP, and LDH levels, the progression of sepsis events varied between them, and the mortality rate among the patients of the study (100 patients) was higher in the male (11%) than the female (7%). In fact, there are

many studies suggest sex differences in infectious illnesses and sepsis in terms of their immunological system responsiveness especially throughout the severe sepsis events (Kante *et al*., 2014). Sepsis is characterized by a complex interaction of immune cells, endothelial cells, acute phase proteins, and cytokines. The disruptions in the microcirculation result in organ dysfunction or even failure, which increases mortality in those patients. A review published in 2014 discussed the relationships between the sex hormones and their impacts on inflammatory parameters and the degree of immune system responsiveness. This review reveals that the male androgen hormone appears to have immunosuppressive activity, in contrast to the protective effects of the female estrogen hormone (Angele *et al*., 2014). Other previous research has shown sex differences in the emergence of sepsis complications. In 1999, Offner *et al*. identified males as an independent risk factor for the development of severe infection and there appeared to be a higher incidence of bacteremia in males compared with females (Offner *et al*., 1999). The current study's higher mortality rate in the male gender reflects the previously mentioned in these earlier literatures.

3.4.3 Correlation between Diagnostic Parameters

Statistical analysis reveals there was a significant strong positive correlation between WBC, RDW, and LDH (r = 0.373 and 0.332, respectively). That means when WBC is increased, RDW and LDH are also significantly increased. A significant strong negative correlation between LYM and GRA was detected (r= -0.983). That is mean when LYM is increased, GRA is decreased, and vice versa. The correlation between LYM and RDW is significantly positive (r = 0.302), while the correlation between GRA and RDW is significantly negative (r = -0.309). There is a significant, strong positive correlation between HGB

and HCT (r = 0.945), whereas the correlation between HGB and RDW is significantly negative (r = -0.258). A significant, strong negative correlation between HCT and RDW was detected (r = -0.347). Finally, there is a significant positive correlation between RDW and PLT (r = 0.296). Whereas no significant differences have been observed between other parameters [Table 3.9].

Table 3.9 Correlation between diagnostic parameters of sepsis

Parameters		WBC	LYM	GRA	HGB	HCT	RDW	PLT	CRP	LDH
GRA	Correlation Coefficient		-0.983**	1						
	Sig. (2-tailed)		0.001							
HCT	Correlation Coefficient				0.945**	1				
	Sig. (2-tailed)				0.001					
RDW	Correlation Coefficient	0.373**	0.302*	-0.309*	-0.258*	-0.347**	1			
	Sig. (2-tailed)	0.002	0.014	0.012	0.038	0.005				
PLT	Correlation Coefficient						0.296*	1		
	Sig. (2-tailed)						0.016			
LDH	Correlation Coefficient	0.332**							0.311*	1
	Sig. (2-tailed)	0.007							0.012	

****. Correlation is significant at the 0.01 level (2-tailed).**

***. Correlation is significant at the 0.05 level (2-tailed).**

3.5 Immunological Part

The immunological part of the study includes two methods: (a) An Indirect immunofluorescent assay (IFA), which is based upon the reaction of antibodies in the sample with the antigen adsorbed on the slide surface to detect IgG or IgM antibodies against unculturable bacterial agents that are present in patients' serum. (b) the sandwich ELISA method was used to assess some sepsis biomarkers, comprising Pentraxin 3 (PTX3), Presepsin (PSN), and procalcitonin (PCT) in both hospitalized patients (CPS & CNS) and healthy controls.

3.5.1 An Indirect Immunofluorescent Assay (IFA)

According to the manufacturer's guidelines, the pneumobact kit was used for the detection of IgM and IgG antibodies in the serum of 40 culture negative sepsis (CNS) against the main bacterial agents that cause atypical pneumonia in the respiratory tract: *Legionella pneumophila, Coxiella burnetii, Mycoplasma pneumoniae, Chlamydophila pneumoniae, and Chlamydophila psittaci* (Calderaro *et al*., 2022).

These bacteria are unique infectious agents that can infect the lower respiratory tract (LRT) and are known as atypical bacteria. *Chlamydia psittaci* and *Coxiella burnetii* are zoonotic microorganisms that spread from animals, while *Legionella pneumophila*, *Mycoplasma pneumoniae, and Chlamydophila pneumoniae* are three non-zoonotic microorganisms (Al-Dahmoshi, 2016). Atypical pathogens are distinguished clinically from classical bacteria that cause community acquired pneumonia (CAP). Each type of atypical bacteria has the tendency to extrapulmonary involvement and is more probable to result in systemic disease. However, it is difficult to cultivate and diagnose these

microorganisms. For these reasons, a prompt clinical diagnosis is critical for raising the index of suspicion and commencing appropriate therapy (Al-Abbad *et al*., 2022). Most atypical pathogens are usually diagnosed based on an indirect immunofluorescent assay (Cunha, 2006).

In the current study, the results of the IFA assay demonstrate differences among all tested cases regarding the spectrum of disease. Some cases give only positive results for IgM antibodies; these indicate the presence of recent infection or acute phase of the disease (Murdoch *et al*., 2019). However, other cases give positive results for IgG; these indicate past exposure or reinfection (Cunha, 2006). The remaining infected patients give positive results for both IgM and IgG; these indicate the progression of illness episodes and may become severe.

The findings of this study revealed that *Legionella pneumophila* was present in 25 (62.5%) of 40 CNS samples. It represents the largest percentage among other detected atypical bacteria. *Legionella pneumophila* is a small gram-negative bacillus normally present ubiquitously, especially in water sources such as showerhead and taps (Iliadi *et al*., 2022), transmitted via inhalation of contaminated aerosols and causes atypical pneumonia (Legionnaires) that can be community-acquired or hospital-acquired. Legionella is the most significant atypical bacteria that can lead to severe community-acquired pneumonia in hospitalized patients (Mandell *et al*., 2005). Prior research has shown that *L. pneumophila* may breach the pulmonary blood vessels as a potential initiating event in bacteremia during the systemic spread of the bacteria through the bloodstream (Chiaraviglio *et al*., 2008). In 2002, an international survey performed by Yu et al. recognized community-acquired legionellosis in 508 patients. 91.5% of the isolates belonged to *Legionella pneumophila* and

serogroup 1 was the predominant 84.2% (Yu *et al*., 2002). The IFA is advised as the reference approach for the diagnosis of *Legionella pneumophila*-associated respiratory tract infection, with a sensitivity of between 75% and 80% and a specificity of greater than 99% when the *Legionella pneumophila* serogroup 1 antigen is used (Skevaki *et al*., 2012).

Of the 40 CNS samples, *Chlamydia pneumoniae* was present in 25 (62.5%), the second-highest percentage among other detected atypical bacteria. *Chlamydia pneumoniae* is an obligate intracellular, Gram-negative bacterium present in two developmental forms: elementary and reticulate bodies (Cosentini *et al*., 2001). As with *L. pneumophila*, transmission occurs through inhalation of contaminated droplets, and its pathogenicity involves extrapulmonary organs. Depending on several previous studies, *C. pneumoniae* recognized as the most common non-viral intracellular human respiratory pathogen. It is accounts for 6-20% of community-acquired pneumonia (CAP). The spectrum of its infectivity varies from mild to severe especially in elderly and immunocompromised patients (Dumke *et al*., 2015).

Mycoplasma pneumoniae is another bacterial pathogen that lacks a cell wall and belongs to the prevalent nonzoonotic atypical infection. It was detected by the IFA approach in 10 (25%) of the 40 CNS patients. According to previous study, *M. pneumoniae* causes approximately 10 to 30 percent of all cases of CAP, particularly among immunocompromised patients (Morozumi *et al*., 2010). It is noteworthy that this investigation shows multiple infections were present when the serum samples were subjected to the IFA test (each test gave positive reactions to different specific antibodies that belonged to more than one microorganism). Coinfection was detected in 24 (60%) of the 40 CNS patients; 11 (27.5%) of them had *L. pneumophila* and *C. pneumoniae*; 6 (15%) had *L.*

pneumophila, *C.* pneumoniae and *M. pneumoniae*; 3 (7.5%) had *C. pneumoniae* and *M. pneumoniae*; 2 (5%) had *C. pneumoniae* and *C. psittaci*; 1(2.5%) had *L. pneumophila* and *C. burnetiid*; 1(2.5%) had *L. pneumophila* and *C. psittaci* [Table 3.10]. The incidence of coinfection is higher in debilitated patients, particularly those that reside in the intensive care unit or respiratory care unit. This is true because more than three quarters of patients' samples were collected from hospitalized patients who reside in those units. In 2021 a retrospective study conducted by De Francesco et al. targeted 721 hospitalized patients; the demographic distribution of their ages was like that of the participants in the current study (median ages ~65 years). This study showed out of 443 patients suffering from SARS-CoV-2, 242 had an antibody against *Mycoplasma* and *Chlamydia* (De Francesco *et al*., 2021). Moreover, Goodarzi et al. were detected coinfection in 7 patients; 6 of them infected by *M. pneumoniae* and *L. pneumophila*, and only one was infected by *L. pneumophila* and *C. pneumoniae* (Goodarzi *et al*., 2020).

Regarding zoonotic atypical pathogens, When the serum samples from the current study were examined, *Coxiella burnetii* and *Chlamydia psittaci* were found in 2 (5%) and 4 (10%) of the 40 CNS cases, respectively. Zoonotic atypical pathogens are less common than nonzoonotic due to their connection to environmental risk factors and the exposure to specific vectors (Dueck *et al*., 2021). The numbers and percentages of each detected antibody against the atypical pathogens mentioned above are documented in [Table 3.11]. The positive and negative IFA results illustrated in [Figure 3.4].

Table 3.10 Number of CNS samples (36/40) that gave positive results when examined by IFA. The first part of the table for samples that gave positive results for a single pathogen. Second part of the table for samples that gave positive results for multiple pathogens.

Single Pathogens	**NO.**
L. pneumophila	6
M. pneumoniae	1
C. burnetii	1
C. pneumoniae	3
C. psittaci	1
Multiple pathogens	**NO.**
L. pneumophila, C. pneumoniae	11
L. pneumophila, M. pneumoniae, C. pneumoniae	6
L. pneumophila, C. burnetii	1
C. pneumoniae, C. psittaci	2
L. pneumophila, C. psittaci	1
M. pneumoniae, C. pneumoniae	3
Total number of positive	**36/40**

Table 3.11: Number and percentage of each human IgM and IgG antibody detected out of 40 culture-negative sepsis serum samples examined by the IFA method.

Bacterial Antigen	**Positive results %**			**Total Positive%**
	Human IgM	Human IgG	Both IgM and IgG	
L. pneumophila	8 (20%)	3 (7.5%)	14 (35%)	25 (62.5%)
C. pneumoniae	6 (15%)	7 (17.5%)	12 (30%)	25 (62.5%)
M. pneumoniae	1 (2.5%)	2 (5%)	7 (17.5%)	10 (25%)
C. psittaci	1 (2.5%)	2 (5%)	1 (2.5%)	4 (10%)
C. burnetii	0 (0%)	0 (0%)	2 (5%)	2 (5%)
Total number of positive =36 of 40 CNS serum samples				

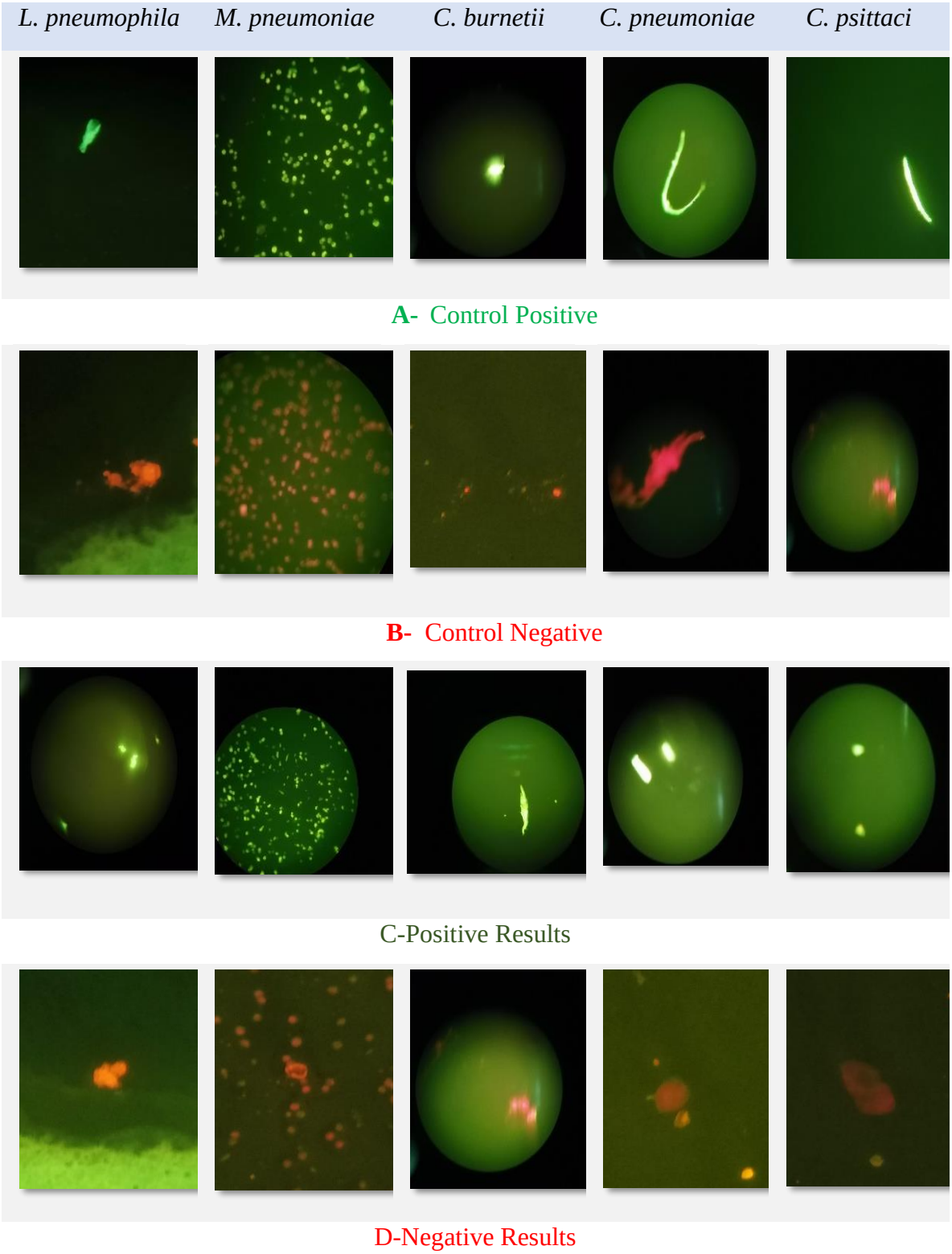


Figure 3.4 The IFA images of determining antibacterial immunoglobulin (IgM and IgG), the green apple color refers to positive results while red indicates to negative results.

3.5.2 ELISA Test of PTX3, PSN, and PCT

During the study period, serum samples from 90 participants (25 control, 25 culture positive, and 40 culture negative sepsis) were subjected to ELISA analysis (sandwich method) for the three selected sepsis biomarkers Pentraxin 3 (PTX3), Presepsin (PSN), and Procalcitonin (PCT).

Statistical analysis (Duncan test) reveals there were no significant differences among the three groups (CPS, CNS, and healthy control) regarding PTX3 concentration (having the same small letters in one row). furthermore, there were no significant differences between culture positive and culture negative groups regarding PSN and PCT concentrations (a, a). However, statistical analysis reveals that the control group had a significant difference from both the culture positive and negative groups regarding PSN and PCT concentration (have different small letter b) [Table 3.12].

Table 3.12: Comparison of the PTX3, PSN, and PCT results among the HC, CPS, and CNS groups (Duncan test)

Parameters	HC (n=25) Mean ± SD	CPS (n=25) Mean ± SD	CNS (n=40) Mean ± SD	P-value
Pentraxin 3	5.93 ± 2.27	5.98 ± 2.56	6.81 ± 3.97	0.56
	a	a	a	
Presepsin	199.26 ± 41.57	274.09 ± 150.7	310.84 ± 254.18	<0.001
	b	a	a	
Procalcitonin	102.07 ± 24.21	134.97 ± 32.36	155.27 ± 97.3	<0.001
	b	a	a	

HC: healthy control; **CPS**: culture positive sepsis; **CNS**: culture negative sepsis.

The current study showed no significant differences between sex and each age, pentraxin 3, presepsin, and procalcitonin. The P value for these parameters were 0.43, 0.31, 0.70, and 0.33 respectively. [Table 3.13].

Table 3.13: Distribution of variables between sexes.

Variables	Gender	NO.	Mean	S. D	P value
Ages (years)	Female	32	60.06	20.26	0.43
	Male	33	56.27	18.30	
Pentraxin 3	Female	32	7.21	3.52	0.31
	Male	33	5.79	2.91	
presepsin	Female	32	307.28	273.59	0.70
	Male	33	286.44	153.90	
procalcitonin	Female	32	166.44	107.82	0.33
	Male	33	129.05	41.87	

The results of the correlation test reveal there was a significant strong positive correlation between PSN and PCT (r = 0.500**) (P <0.001) That means when PSN is increased, the PCT is also increased significantly. However, there is a significant positive correlation among three biomarkers (PTX3, PSN, and PCT) [Table 3.14].

Table 3.14: Correlation between Pentraxin 3, Presepsin, and Procalcitonin

Parameters		Pentraxin	Presepsin	Procalcitonin
Pentraxin	Correlation Coefficient		0.246*	.265*
	Sig. (2-tailed)		0.03	0.012
Presepsin	Correlation Coefficient	0.246*		.500**
	Sig. (2-tailed)	0.03		0.001
Procalcitonin	Correlation Coefficient	.265*	.500**	
	Sig. (2-tailed)	0.012	0.001	

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

The pathogenesis of sepsis is progressive across the course of infection, and each stage has its own biomarkers. Hence, the ability to diagnose sepsis from CBC, CRP and blood culture is limited. For further progressive in our investigations, different biomarkers must be used to detect and monitor the course of sepsis (Balk and Roger., 2011). Procalcitonin (PCT) was considered a sepsis biomarker and was identified to be more effective and more specific than CRP because its higher value is associated with bacterial infection (Ugajin *et al*., 2011). Several studies have shown that it may be helpful in predicting the risk of bacteremic infection indicated by a positive blood culture. In septic patients, a high PCT concentration is a reliable indicator of bacterial infection. (Krüger *et al*., 2009). In this study, PCT levels increased significantly in both CPS and CNS patients (mean=134.97 and 155.27 pg./ml, respectively), in contrast to the low levels in the control group (mean=102.07 pg./ml).

Despite the facts that it refers to the significant of the PCT levels for primary detection of sepsis, keep in mind that more than one marker must be used to achieve accepted results for such infection.

Recently, presepsin (PSN) was discovered as a novel indicator whose value was used in the diagnosis of bacterial sepsis (Memar and Baghi, 2019). In a recent meta-analysis, Kondo *et al*. discovered that PSN had even higher diagnostic accuracies than procalcitonin for identifying mixed-pathogen sepsis in critically sick adult patients. As with PCT, PSN is mainly used for early detection of a possible bacterial infection and to predict the risk of death due to its level gradually increasing with the progression of a sepsis episode (Kondo *et al*., 2019). In the current study, the mean levels of the PSN in both the CPS and CNS groups (274.09 pg./ml and 310.84 pg./ml, respectively) were considerably higher than in the control group (199.26 pg./ml).

The ambiguity of the sepsis events and their causative agents led the researchers to exert more effort to find new biomarkers that could improve the diagnostic methodologies.

Pentraxin 3 (PTX3) is a new protein employed as a diagnostic marker for bacterial-originated sepsis. It is an acute phase protein that is structurally like CRP but is produced by innate immune cells rather than the liver (Mantovani and Garlanda, 2023). The statistical analysis of this study was showed no significant differences among three groups (CPS, CNS, and control) in term of PTX3 concentrations (5.93, 5.98, and 6.81 pg./ml, respectively). In this instance, PTX3 in diagnosing sepsis does not appear to be superior to the other mentioned biomarkers. While the current study is unable to predict the potential role of PTX3 in septic patients' diagnosis, further research is needed to prove the specificity of PTX3 in the diagnosis of bacterial sepsis. Generally, early detection of sepsis is one of the most essential aspects since it allows for rapid treatment and reduces mortality and morbidity rates that may be evolved during the time of infection.

Conclusions and Recommendations

Conclusions and Recommendations

Conclusions

1-Even though blood culture is the gold standard for detecting bacteremia, but it is not valuable for bacteremia caused by unculturable bacteria.

2-The CBC, CRP, and LDH are helpful as routine screening tests but not confirmatory tests for septicemia.

3-The PNEUMOBACT IFA assay is very important for septicemic patients who give negative blood culture or those with a positive blood culture, but not improved successfully after antibiotic treatments.

4-In addition to Procalcitonin, presepsin can be used as a diagnostic biomarker for septicemia.

Recommendations

1-Further studies are needed for the evaluation of presepsin as a biomarker for septicemia and studying the potential SNPs for sepsis patients with a normal or low level.

2-Applying more advanced molecular studies for enhanced early management of septic patients.

3-Studying the serum level of monocyte chemoattractant protein-1 (MCP-1) as a probable biomarker for bacterial sepsis.

4-Studying the (1→3)-β-D-Glucan in serum as potential biomarker for fungal sepsis.

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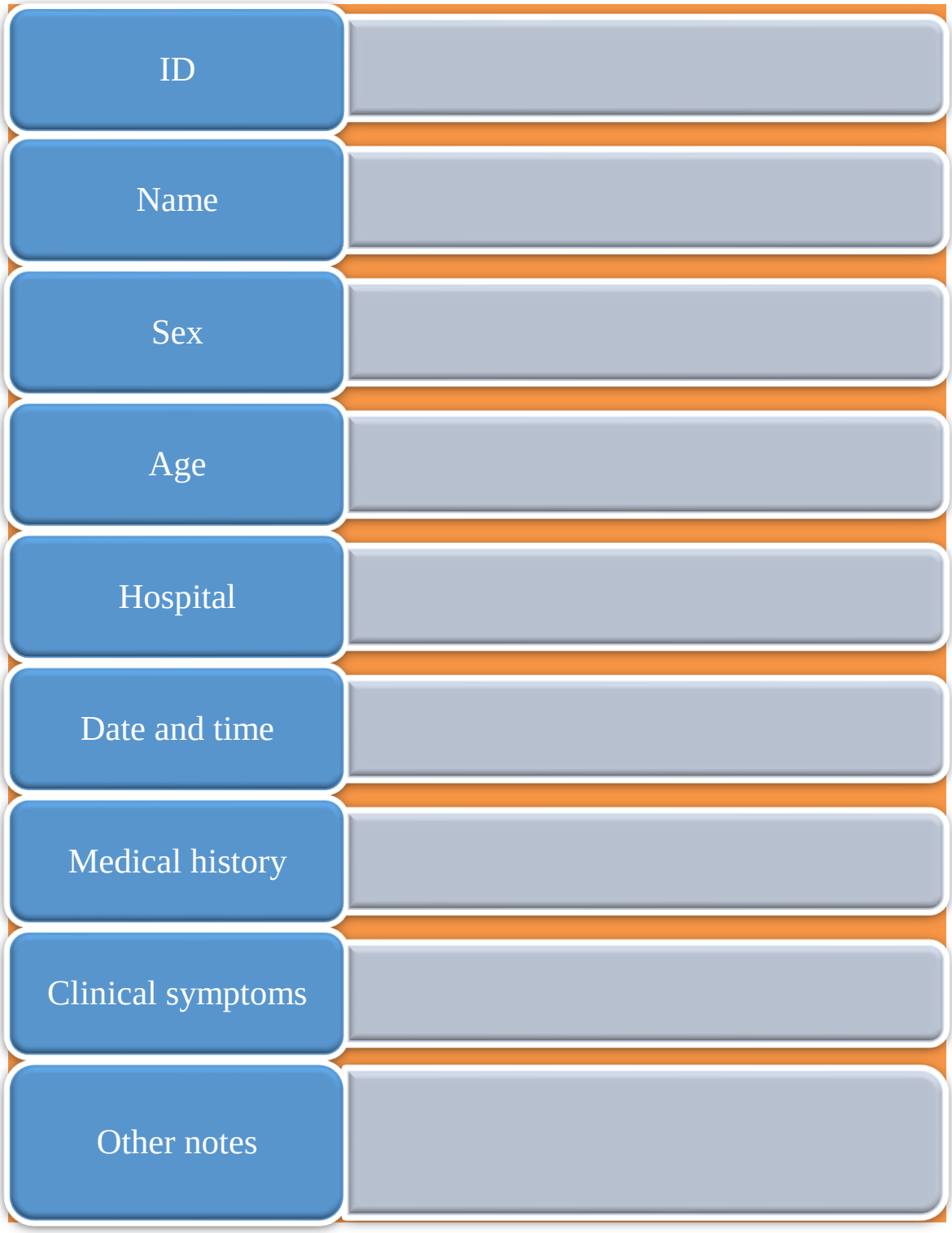
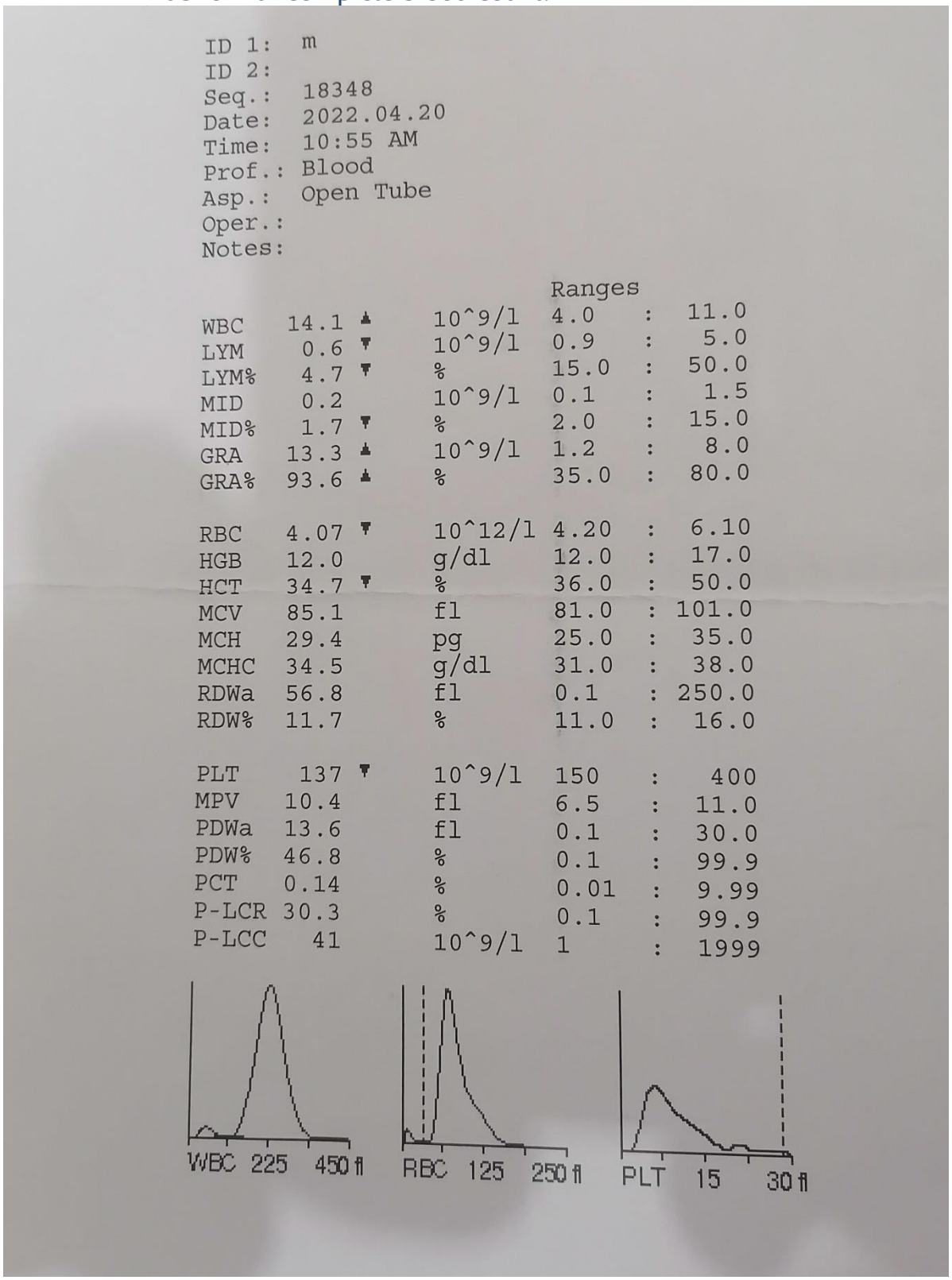
Appendices

Appendix 1: The questionnaire form for each patient who participated in this Study.



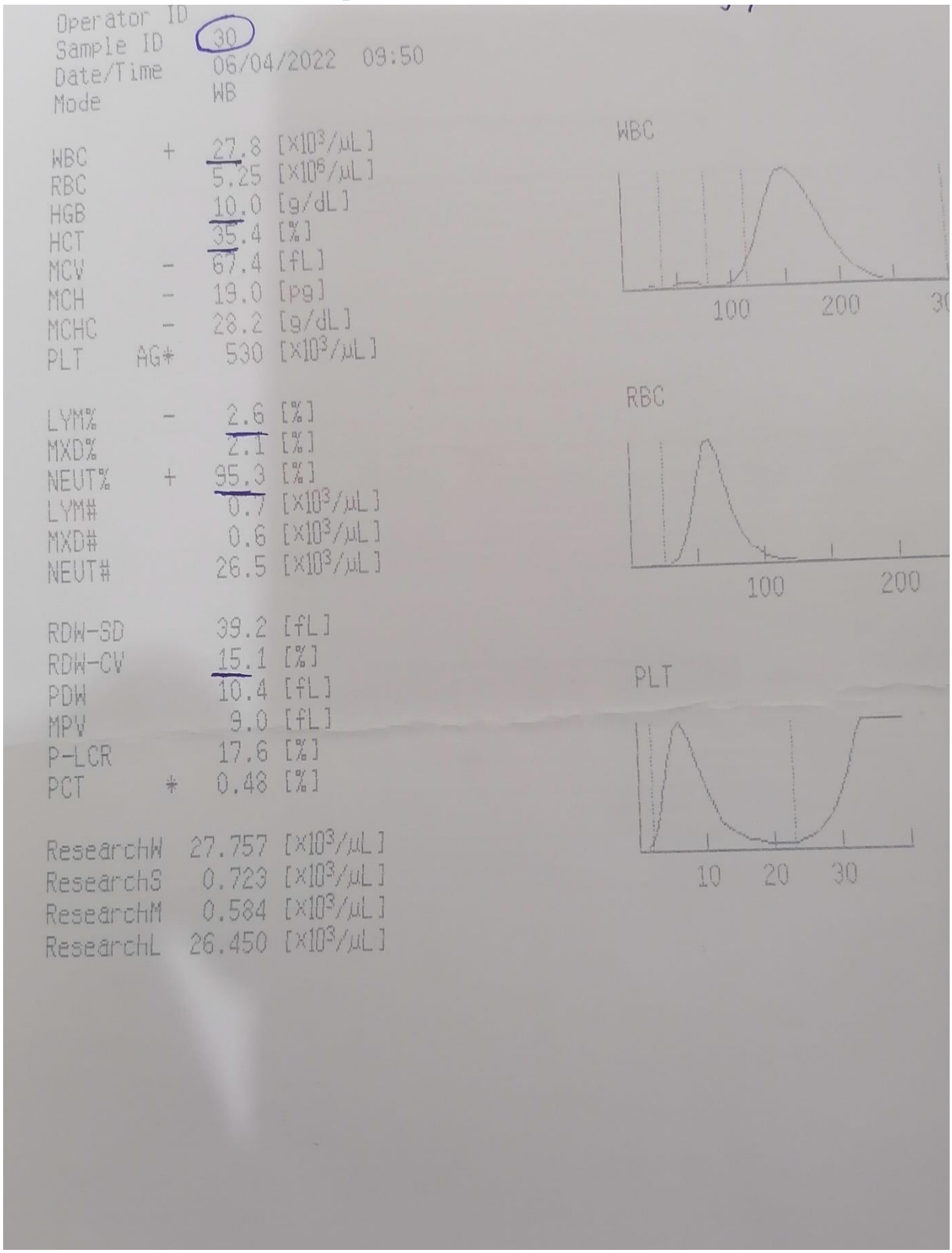
Appendix 2: A male patient in the burn unit at Al-Sadiq Hospital has an abnormal complete blood count.



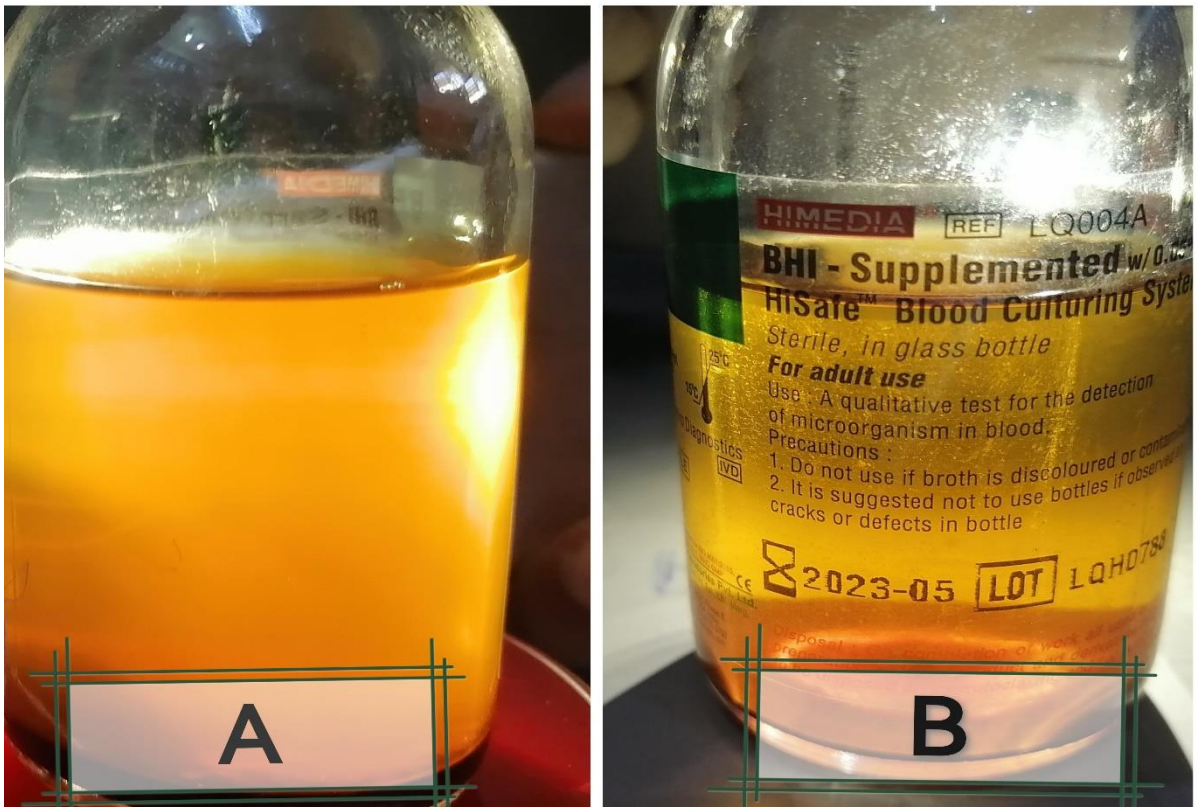
ID 1: m
ID 2:
Seq.: 18348
Date: 2022.04.20
Time: 10:55 AM
Prof.: Blood
Asp.: Open Tube
Oper.:
Notes:

				Ranges		
WBC	14.1	▲	10^9/l	4.0	:	11.0
LYM	0.6	▼	10^9/l	0.9	:	5.0
LYM%	4.7	▼	%	15.0	:	50.0
MID	0.2		10^9/l	0.1	:	1.5
MID%	1.7	▼	%	2.0	:	15.0
GRA	13.3	▲	10^9/l	1.2	:	8.0
GRA%	93.6	▲	%	35.0	:	80.0
RBC	4.07	▼	10^12/l	4.20	:	6.10
HGB	12.0		g/dl	12.0	:	17.0
HCT	34.7	▼	%	36.0	:	50.0
MCV	85.1		fl	81.0	:	101.0
MCH	29.4		pg	25.0	:	35.0
MCHC	34.5		g/dl	31.0	:	38.0
RDWa	56.8		fl	0.1	:	250.0
RDW%	11.7		%	11.0	:	16.0
PLT	137	▼	10^9/l	150	:	400
MPV	10.4		fl	6.5	:	11.0
PDWa	13.6		fl	0.1	:	30.0
PDW%	46.8		%	0.1	:	99.9
PCT	0.14		%	0.01	:	9.99
P-LCR	30.3		%	0.1	:	99.9
P-LCC	41		10^9/l	1	:	1999

Appendix 3: A female patient in the RCU at Marjan Medical City has an abnormal complete blood count.

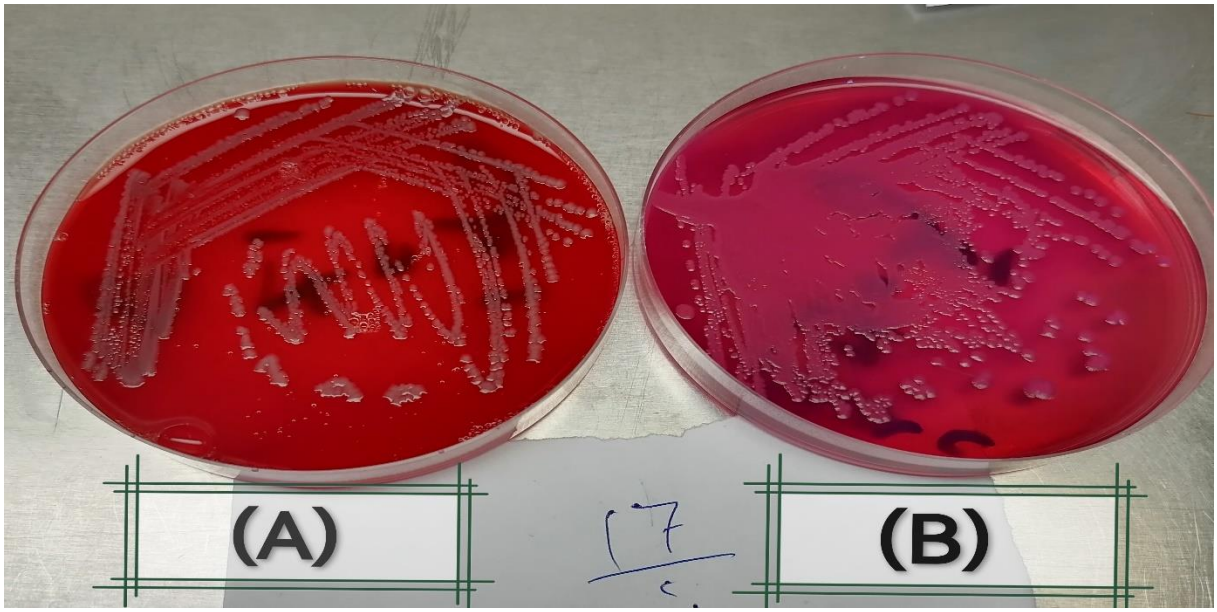


Appendix 4: The image shows vials of Brain Heart Infusion Broth (BHI) after they were inoculated with 5 ml of blood from the patients of interest, and incubated for 1–10 days at 37°C. The turbid vial at left **(A)** represents positive culture, while the clear vial at right **(B)** is negative culture.

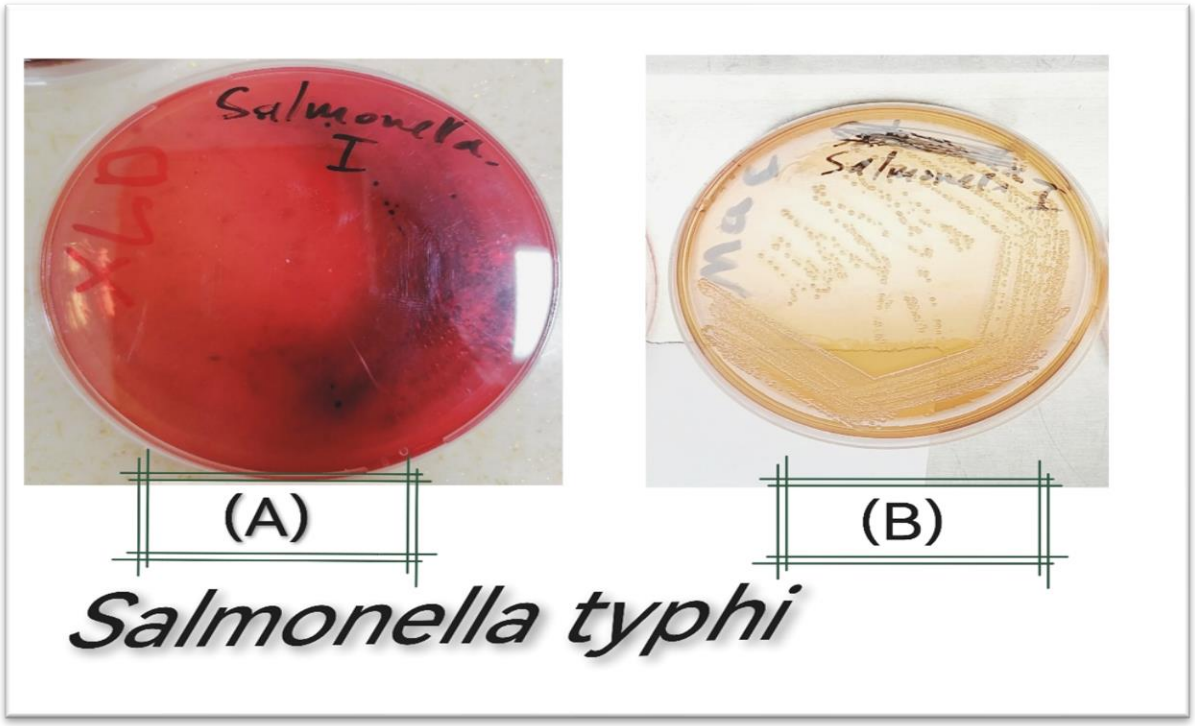


Appendix 5: The image shows growth of *Staphylococcus* spp. **(A)** growth of hemolytic species of *staphylococcus* on the blood agar. **(B)** growth of coagulase positive and coagulase negative species on the mannitol salt agar.

Appendix 6: This image shows growth of gram- negative bacteria. **(A)** large, circular, and grayish colonies on the blood agar. **(B)** pink colonies of lactose-fermenting bacteria on the MacConkey agar (MAC).

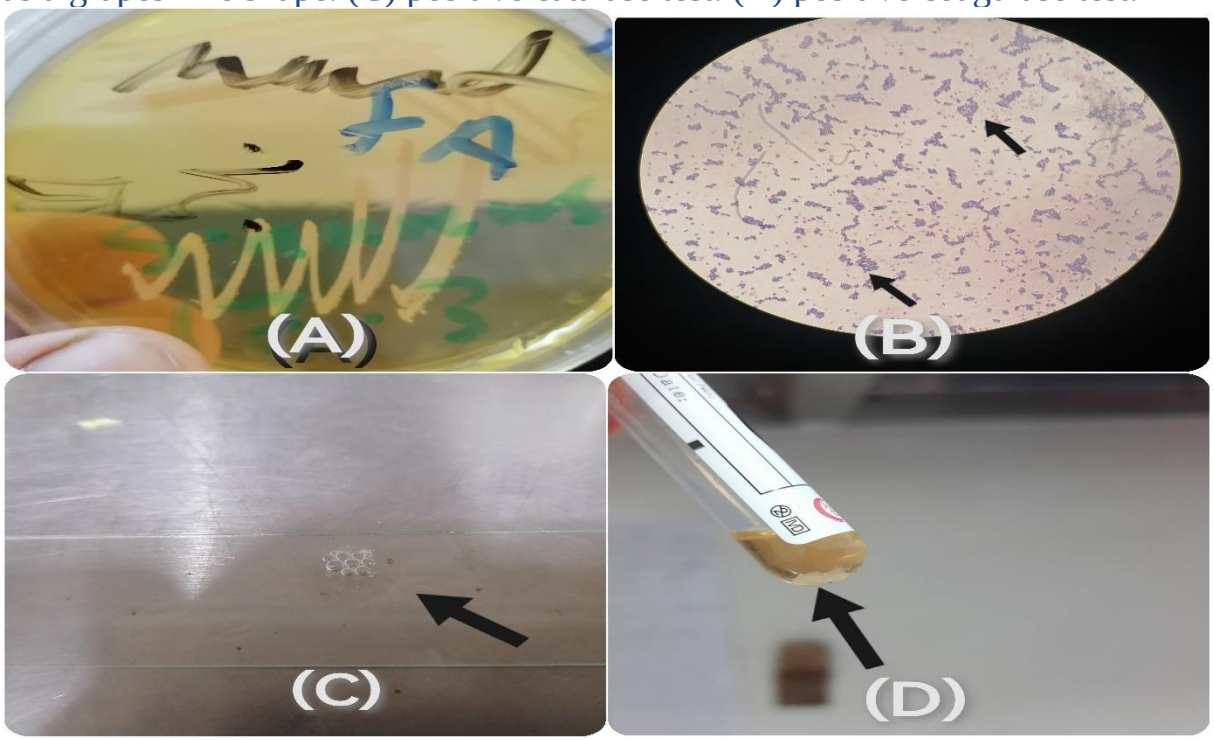


Appendix 7: Growth of *Salmonella typhi*. **(A)** Hydrogen sulfide production results in changing the color of the indicator to black on the XLD agar. **(B)** Growth of colorless colonies on MacConkey agar because *S. typhi* does not utilize lactose.

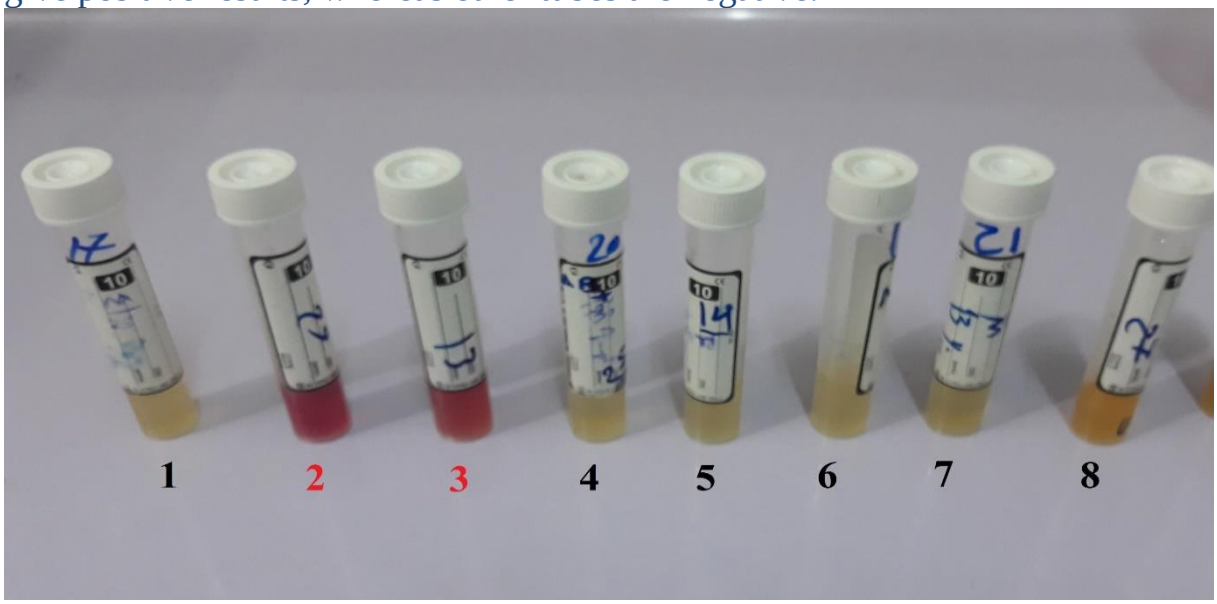


Appendix 8: Growth of *Escherichia coli* on the Eosin methylene blue (EMB) agar. As shown in this image, colonies of *E. coli* produce a metallic green sheen due to the color change of the indicator (methylene blue) in the medium when lactose is fermented by these bacteria.

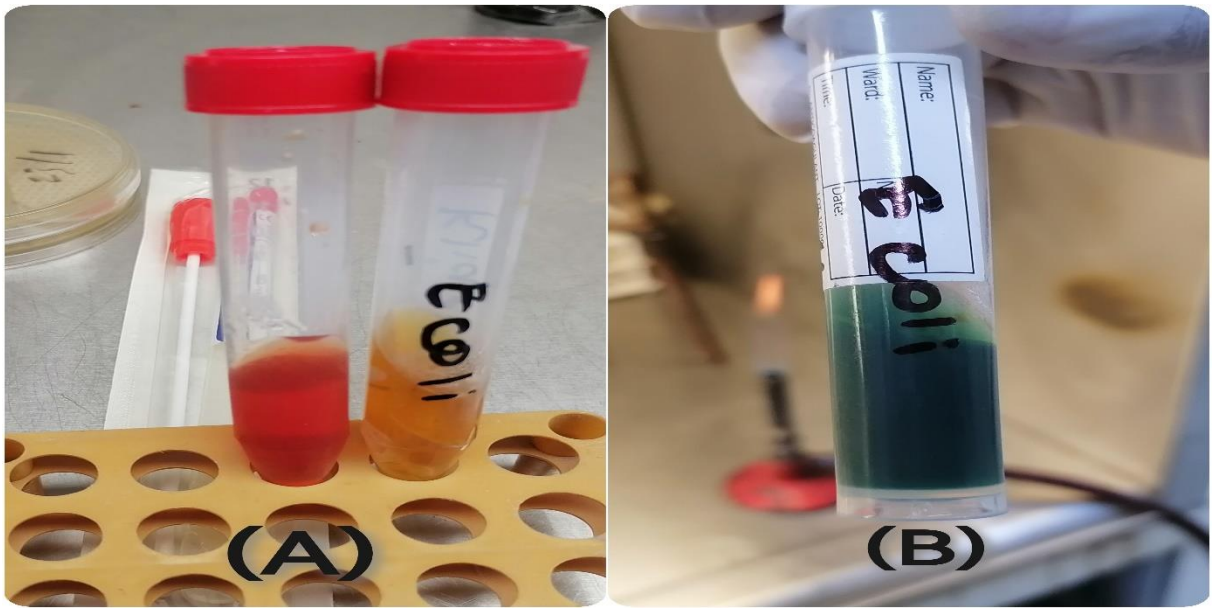
Appendix 9: Manual methods for species identifications. (A) mannitol salt agar is positive. (B) microscopic image of the gram-positive bacteria, which appears as a grapes-like shape. (C) positive catalase test. (D) positive coagulase test.



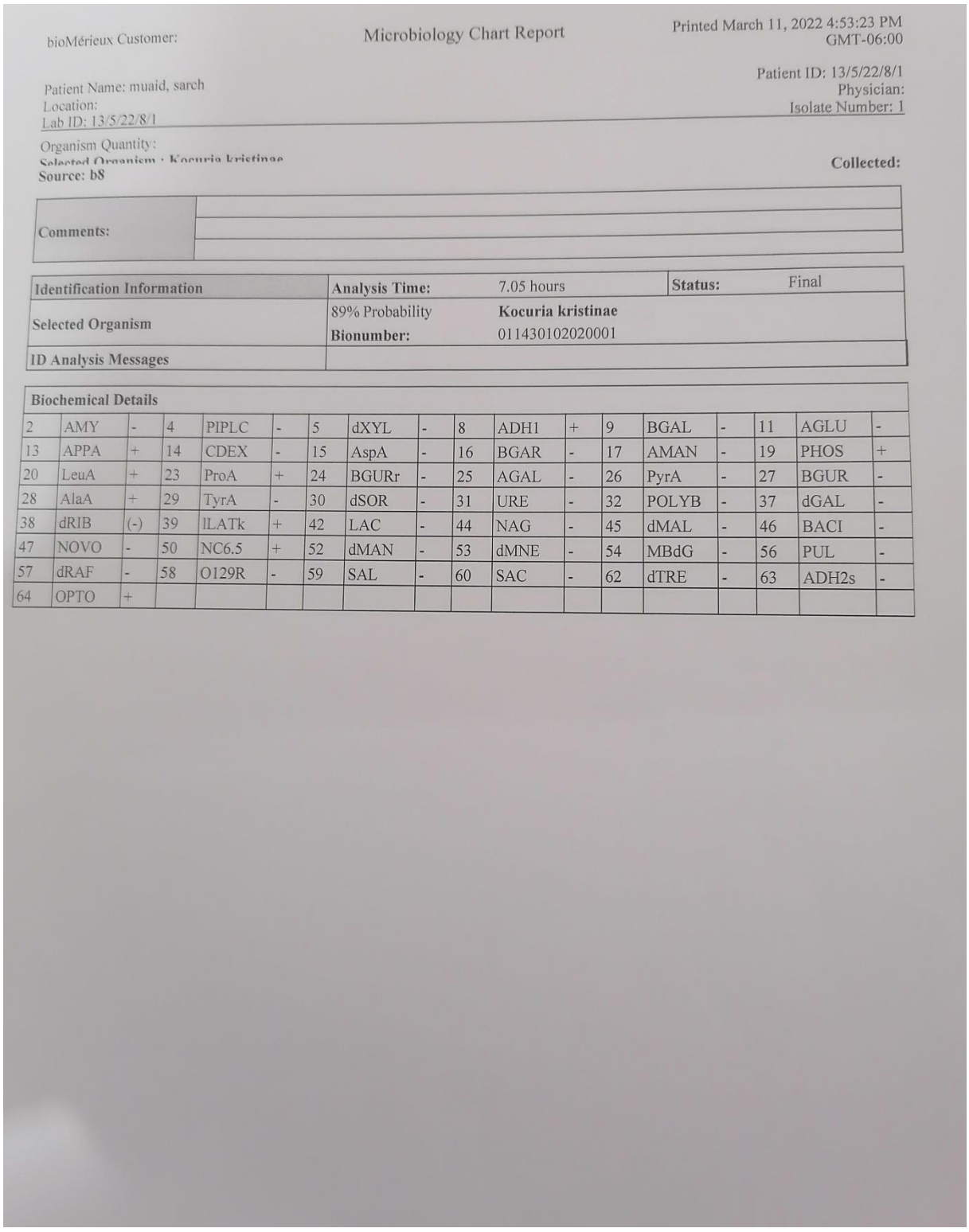
Appendix 10: Urease test. Yellow is the original color of urea agar. When an isolate produces urease, the enzyme breaks down urea into ammonia (NH3) and carbon dioxide (CO2). Ammonia production causes the medium to become alkaline and changes the color to pink. As shown in this image, tubes 2 and 3 give positive results, whereas other tubes are negative.



Appendix 11: Kligler iron and Simmons citrate agar for species identification. **(A)** The medium (Alkaline slant/Alkaline butt) is present in the left red tube. The right yellow tube exhibits bacterial growth; this bacterium turned the medium into acid slant/acid butt, with no H2S, indicating that the growth used lactose and glucose components of the medium. **(B)** tube contains Simmons citrate agar inoculated with the same bacterial growth; this bacterium cannot utilize citrate; thus, the medium remains green (negative result).



Appendix 12: This image shows the Vitek 2 report for species identification. The result is *Kocuria kristinae*.



bioMérieux Customer:

Microbiology Chart Report

Printed March 11, 2022 4:53:23 PM GMT-06:00

Patient Name: muaid, sarch
Location:
Lab ID: 13/5/22/8/1

Patient ID: 13/5/22/8/1
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Kocuria kristinae
Source: b8

Collected:

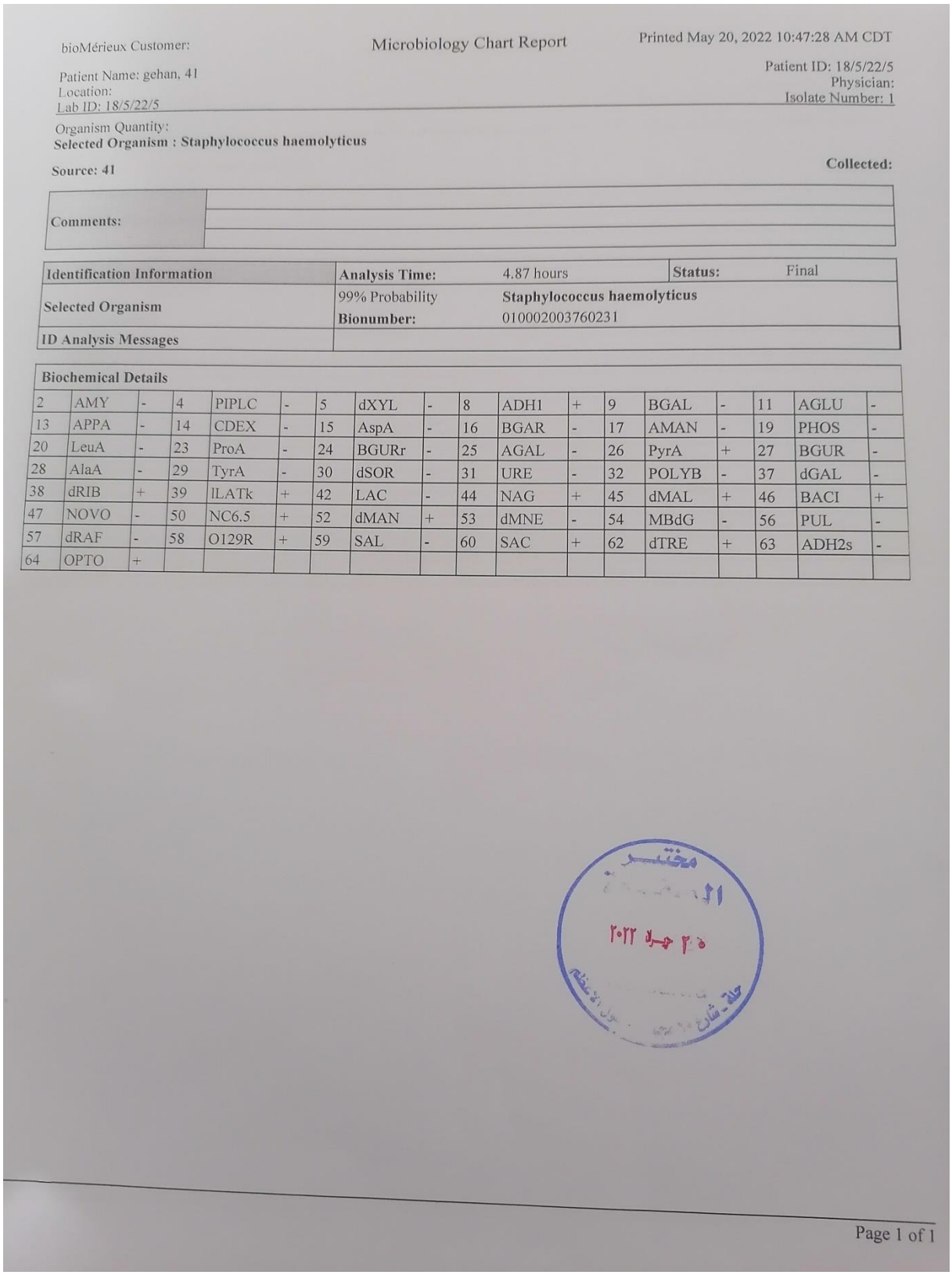
Comments:	

Identification Information	Analysis Time:	7.05 hours	Status:	Final
Selected Organism	89% Probability	Kocuria kristinae		
	Bionumber:	011430102020001		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	-
13	APPA	+	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	+	24	BGURr	-	25	AGAL	-	26	PyrA	-	27	BGUR	-
28	AlaA	+	29	TyrA	-	30	dSOR	-	31	URE	-	32	POLYB	-	37	dGAL	-
38	dRIB	(-)	39	lLATk	+	42	LAC	-	44	NAG	-	45	dMAL	-	46	BACI	-
47	NOVO	-	50	NC6.5	+	52	dMAN	-	53	dMNE	-	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	-	59	SAL	-	60	SAC	-	62	dTRE	-	63	ADH2s	-
64	OPTO	+															

Appendix 13: This image shows the Vitek 2 report for species identification. The result is *Staphylococcus haemolyticus*.



bioMérieux Customer:

Microbiology Chart Report

Printed May 20, 2022 10:47:28 AM CDT

Patient Name: gehan, 41
Location:
Lab ID: 18/5/22/5

Patient ID: 18/5/22/5
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Staphylococcus haemolyticus

Source: 41

Collected:

Comments:	

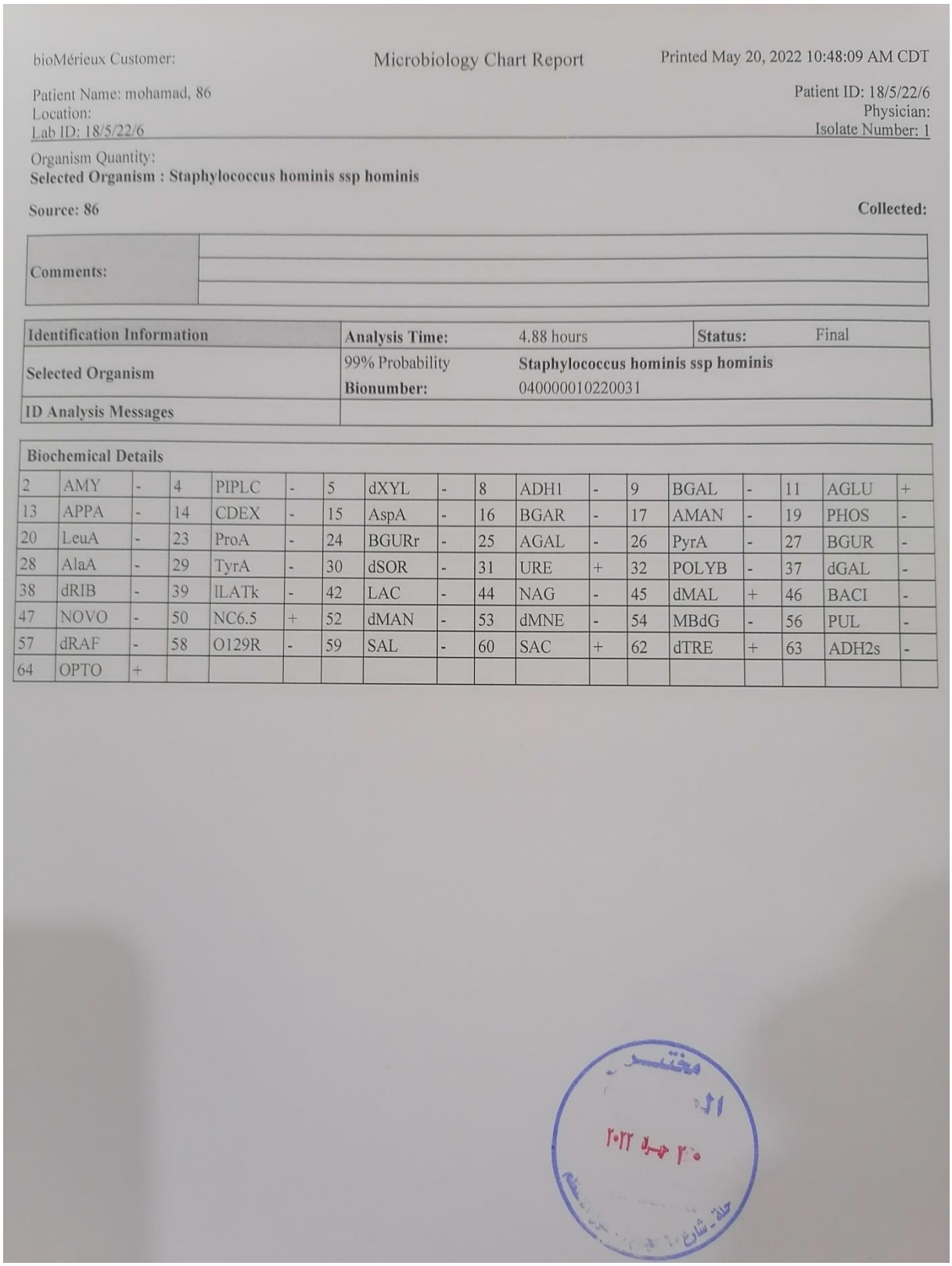
Identification Information	**Analysis Time:**	4.87 hours	**Status:**	Final
Selected Organism	99% Probability	**Staphylococcus haemolyticus**		
	Bionumber:	010002003760231		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	-	32	POLYB	-	37	dGAL	-
38	dRIB	+	39	lLATk	+	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	-	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Page 1 of 1

Appendix 14: This image shows the Vitek 2 report for species identification. The result is *Staphylococcus hominis*.



bioMérieux Customer:

Microbiology Chart Report

Printed May 20, 2022 10:48:09 AM CDT

Patient Name: mohamad, 86
Location:
Lab ID: 18/5/22/6

Patient ID: 18/5/22/6
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Staphylococcus hominis ssp hominis

Source: 86

Collected:

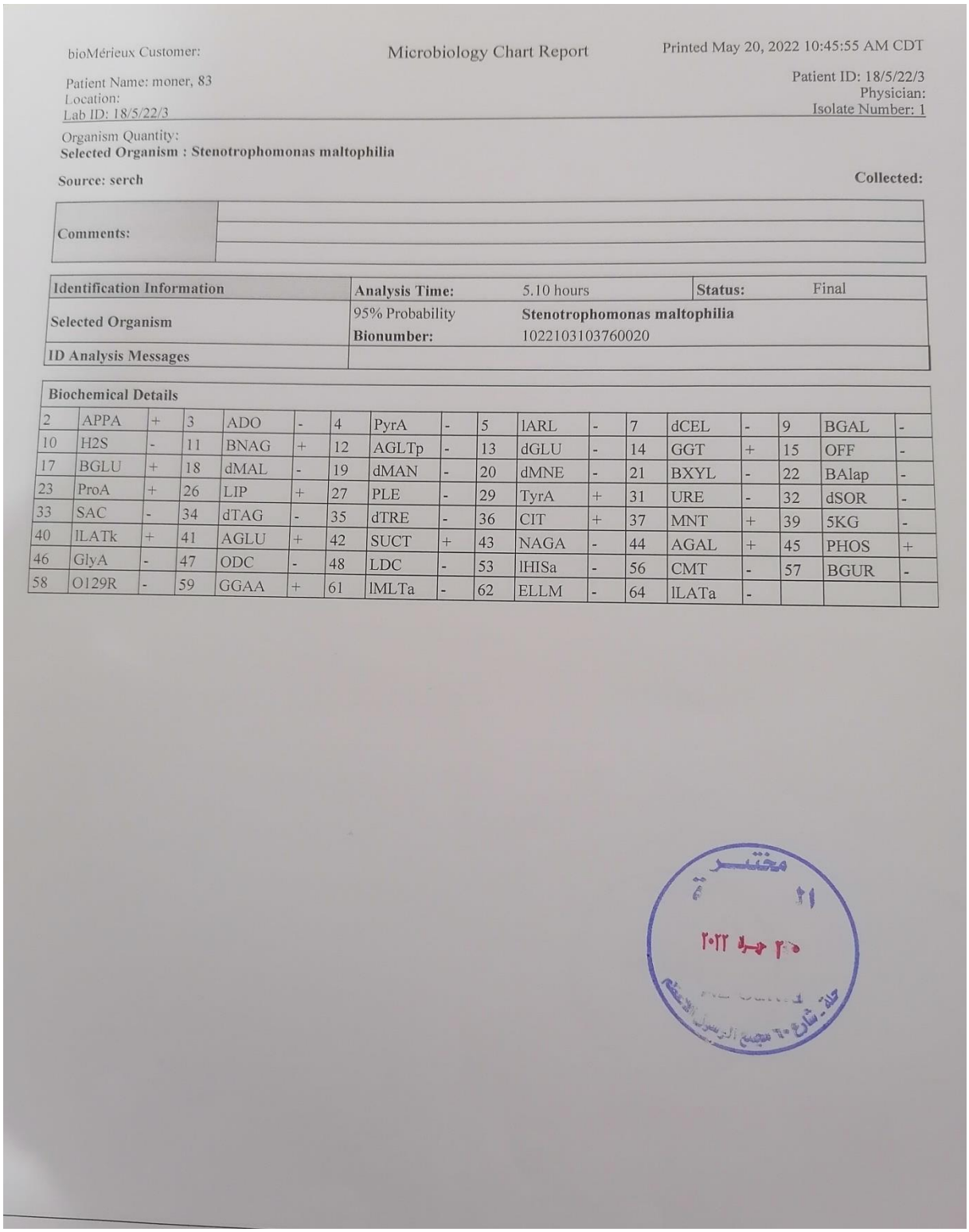
Comments:	

Identification Information	**Analysis Time:**	4.88 hours	**Status:** Final
Selected Organism	99% Probability	**Staphylococcus hominis ssp hominis**	
	Bionumber:	040000010220031	
ID Analysis Messages			

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	-	9	BGAL	-	11	AGLU	+
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	-	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	+	32	POLYB	-	37	dGAL	-
38	dRIB	-	39	lLATk	-	42	LAC	-	44	NAG	-	45	dMAL	+	46	BACI	-
47	NOVO	-	50	NC6.5	+	52	dMAN	-	53	dMNE	-	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	-	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Appendix 15: This image shows the Vitek 2 report for species identification. The result is *Stenotrophomonas maltophilia*.



bioMérieux Customer:

Microbiology Chart Report

Printed May 20, 2022 10:45:55 AM CDT

Patient Name: moner, 83
Location:
Lab ID: 18/5/22/3

Patient ID: 18/5/22/3
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Stenotrophomonas maltophilia

Source: serch

Collected:

Comments:	

Identification Information	**Analysis Time:**	5.10 hours	**Status:**	Final
Selected Organism	95% Probability	**Stenotrophomonas maltophilia**		
	Bionumber:	1022103103760020		
ID Analysis Messages				

Biochemical Details

2	APPA	+	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	+	12	AGLTp	-	13	dGLU	-	14	GGT	+	15	OFF	-
17	BGLU	+	18	dMAL	-	19	dMAN	-	20	dMNE	-	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	lLATk	+	41	AGLU	+	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	-	47	ODC	-	48	LDC	-	53	lHISa	-	56	CMT	-	57	BGUR	-
58	O129R	-	59	GGAA	+	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

Appendix 16: This image shows the Vitek 2 report for species identification. The result is *Morganella morganii*.

bioMérieux Customer:
System #:
Patient Name: muaid, sarch
Isolate: 14/5/22/5-1 (Approved)
Card Type: GN Bar Code: 2411807103367297 Testing Instrument: 00000B4E1D71 (1830)
Card Type: AST-N222 Bar Code: 6221819203491310 Testing Instrument: 00000B4E1D71 (1830)
Setup Technologist: Laboratory Administrator(Labadmin)

Laboratory Report

Printed by: Labadmin
Patient ID: 14/5/22/5

Bionumber: 0017010340542211
Organism Quantity:

Selected Organism: Morganella morganii ssp sibonii

Comments:	

Identification Information	**Card:** GN	**Lot Number:** 2411807103	**Expires:** Nov 7, 2022 12:00 GMT-06:00
	Status: Final	**Analysis Time:** 5.83 hours	**Completed:** Mar 12, 2022 01:52 GMT-06:00
Organism Origin	VITEK 2		
Selected Organism	95% Probability **Morganella morganii ssp sibonii** Bionumber: 0017010340542211 Confidence: Very good identification		
Analysis Organisms and Tests to Separate:			
Analysis Messages: The following antibiotic(s) are not claimed: Rifampicin,			
Contraindicating Typical Biopattern(s) Morganella morganii ssp sibonii H2S(1),			

Susceptibility Information	**Card:** AST-N222	**Lot Number:** 6221819203	**Expires:** Nov 19, 2022 12:00 GMT-06:00
	Status: Final	**Analysis Time:** 11.22 hours	**Completed:** Mar 12, 2022 07:15 GMT-06:00

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ticarcillin	>= 128	R	Amikacin	16	*I
Ticarcillin/Clavulanic Acid	32	I	Gentamicin	8	I
Piperacillin	>= 128	R	Tobramycin	>= 16	R
Piperacillin/Tazobactam	8	S	Ciprofloxacin	>= 4	R
Ceftazidime	>= 64	R	Pefloxacin		
Cefepime	32	R	Minocycline	>= 16	R
Aztreonam	16	R	Colistin		
Imipenem	2	I	Rifampicin		
Meropenem	<= 0.25	S	Trimethoprim/ Sulfamethoxazole	>= 320	R

*= AES modified **= User modified

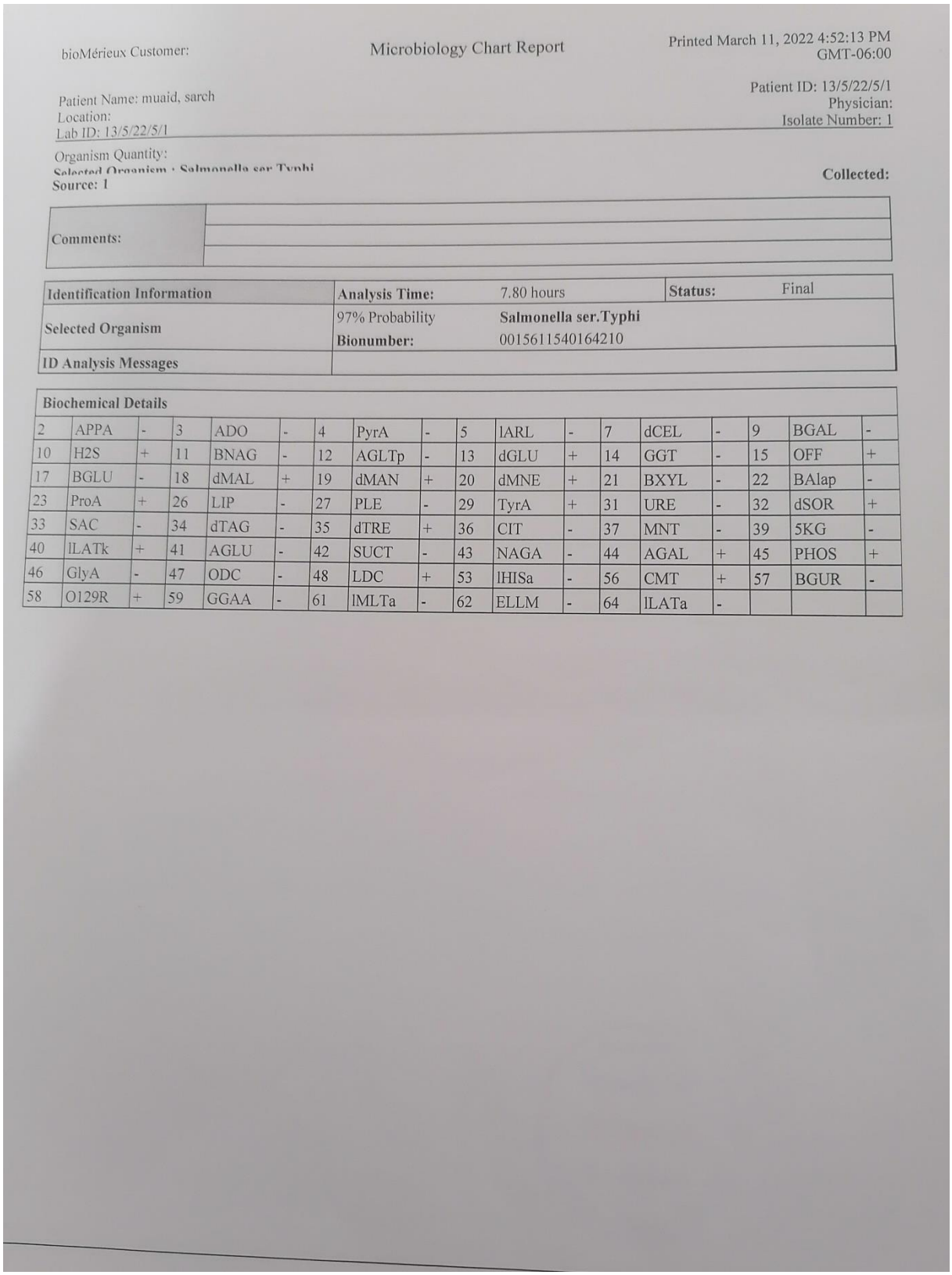
AES Findings:	**Last Modified:** Feb 11, 2021 09:22 GMT-06:00	**Parameter Set:** Global CLSI-based +Phenotypic 2019
Confidence Level:	Consistent	

Installed VITEK 2 Systems Version: 9.02
MIC Interpretation Guideline: Global CLSI-based 2019
AES Parameter Set Name: Global CLSI-based+Phenotypic 2019

Therapeutic Interpretation Guideline: PHENOTYPIC 2019
AES Parameter Last Modified: Feb 11, 2021 09:22 GMT-06:00

Page 1 of 2

Appendix 17: This image shows the Vitek 2 report for species identification. The result is *Salmonella typhi*.



bioMérieux Customer:

Microbiology Chart Report

Printed March 11, 2022 4:52:13 PM
GMT-06:00

Patient Name: muaid, sarch
Location:
Lab ID: 13/5/22/5/1

Patient ID: 13/5/22/5/1
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Salmonella ser Typhi
Source: I

Collected:

Comments:	

Identification Information	Analysis Time:	7.80 hours	Status:	Final
Selected Organism	97% Probability Bionumber:	Salmonella ser.Typhi 0015611540164210		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	-
10	H2S	+	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	-	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	lLATk	+	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	-	47	ODC	-	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

Appendix 18: This image shows the Vitek 2 report for species identification. The result is *Pseudomonas stutzeri*.

bioMérieux Customer:

Microbiology Chart Report

Printed June 1, 2022 5:33:37 PM CDT

Patient Name: eida, search
Location:
Lab ID: 31/5/22/3

Patient ID: 31/5/22/3
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas stutzeri

Source: swab

Collected:

Comments:	

Identification Information	**Analysis Time:**	7.78 hours	**Status:** Final
Selected Organism	97% Probability	**Pseudomonas stutzeri**	
	Bionumber:	0001201100000200	
ID Analysis Messages			

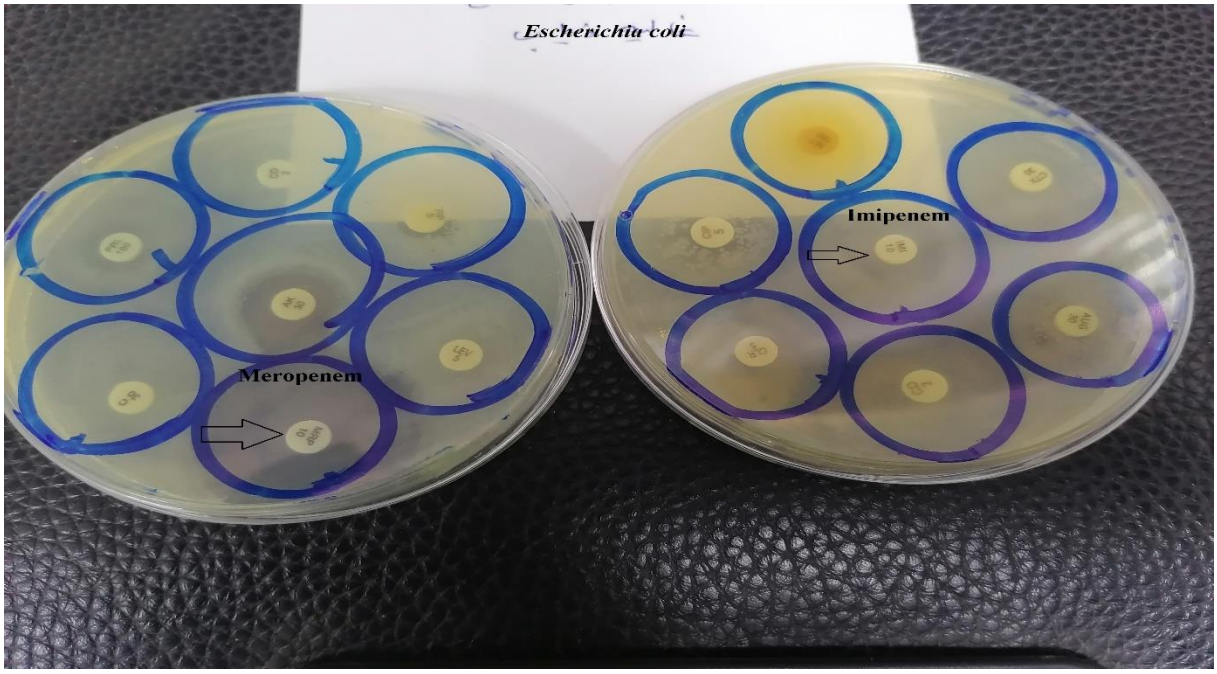
Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	-
17	BGLU	-	18	dMAL	+	19	dMAN	-	20	dMNE	-	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	-	37	MNT	-	39	5KG	-
40	lLATk	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	-	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

Appendix 19: Testing for antibiotic susceptibility using the disk diffusion method. This image illustrates a *Staphylococcus hemolyticus* growth that has demonstrated sensitivity to both doxycycline and vancomycin, and intermediate sensitivity to levofloxacin and amikacin but resistance to others.

Appendix 20: Testing for antibiotic susceptibility using the disk diffusion method. This image illustrates a *Kocuria kristinae* growth that has demonstrated sensitivity to chloramphenicol and levofloxacin but resistance to others.

Appendix 21: Disk diffusion method for antibiotic susceptibility testing. This image illustrates a certain strain of *E. coli* that has demonstrated sensitivity to meropenem and imipenem but resistance to others.



Appendix 22: Human Pentraxin 3 ELISA kit. **(A)** kit components. **(B)** wells that are ready for reading. **(C)** the reader for reading the optical density of protein. **(D)** report contains the optical density of protein for each sample.

Appendix 23: PNEUMOBACT kit for detecting serum IgM and IgG against *L. pneumophila, M. pneumoniae, C. burnetiid, C. pneumoniae, and C. psittaci* using an indirect immunofluorescent assay.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية العلوم
قسم علوم الحياة

دراسة بكتريولوجية ومناعية لبعض المؤشرات الحيوية للإنتان لدى مرضى تجرثم الدم في مدينة الحلة

اطروحة مقدمة الى
مجلس كلية العلوم - جامعة بابل كجزء من متطلبات نيل
درجة دكتوراه فلسفة في علوم الحياة

من قبل
مؤيد مهدي كاظم حسن
بكالوريوس علوم حياة/كلية العلوم/جامعة بابل
١٩٩٨
ماجستير احياء مجهرية - كلية العلوم للبنات جامعة بابل
٢٠١٩
بإشراف

الأستاذ الدكتور حسين عليوي مطلب الدهموشي

١٤٤٤هـ ٢٠٢٣ م

الخلاصة

تجرثم الدم هو عدوى بكتيرية انتشرت من الموقع الرئيسي للعدوى ووصلت إلى مجرى الدم. يمكن أن تسبب العوامل البكتيرية الموجودة باستمرار داخل الدورة الدموية أمراضا خطيرة في الجسم تؤدي إلى الإنتان، خاصة عندما تفشل الإجراءات المختبرية في التعرف على العوامل المسببة الفعلية.

الإنتان هو استجابة التهابية معقدة ترتبط ارتباطا وثيقا بخلل وظيفي في الأعضاء المتعددة وتعتبر سببا رئيسيا للاعتلال والوفيات في جميع أنحاء العالم. تأكيد تجرثم الدم والإنتان يمكن تحقيقه باستخدام العديد من الفحوصات بما في ذلك الطرق ا الزرعية والمناعية والجزيئية.

تهدف الدراسة الحالية إلى تقييم العلامات الحيوية المحتملة للإنتان بين تجرثم الدم القابل للزراعة وغير القابل للزراعة لمرضى الإنتان المشتبه بهم سريريا. تم استخدام مزرعة الدم واختبار الفلورة المناعية غير المباشرة للكشف عن العوامل المسببة لعدوى مجرى الدم القابلة للزراعة وغير القابلة للزراعة. تم استخدام تعداد الدم الكامل (CBC) والبروتين التفاعلي (CRP) ونازعة هيدروجين اللاكتات (LDH) كاختبار فحص روتيني قيم لتجرثم الدم / تسمم الدم بينما تم استخدام البروكالسيتونين (PCT) و البريسبسين (PSN) و البنتراكسين -3 (PTX3) كثلاثة مؤشرات حيوية محتملة للإنتان.

تم جمع ما مجموعه 100 عينة دم من المرضى الذين يعانون من أعراض الإنتان والذين يقيمون في مستشفيات مدينة الحلة وكذلك تم جمع 25 عينة من الأشخاص الأصحاء كمجموعة مراقبة. تم تقسيم كل عينة دم إلى ثلاث حاويات: 2 مل في أنبوب EDTA لاختبار CBC، و 3 مل في أنبوب طرد مركزي لفصل المصل لاختبار CRP و LDH و PCT و PSN و PTX3 و IFA، و 5 مل في وسط نقيع القلب والدماغ BHI)) لزراعة الدم.

أظهرت النتائج أن 65/100 (65٪) عينة لديها اختبارات فحص غير طبيعية (معلمات CBC ، CRP و LDH مرتفعة) ، في حين أن 25/65 (38.4٪) منها فقط اعطت نتائج إيجابية لزرع الدم. جميع الأشخاص في المجموعة الضابطة لديهم نتائج طبيعية من CBC و CRP و LDH وأعطوا نتائج سلبية لزراعة الدم. العزلات البكتيرية ال 25 التي حددها نظام Vitek 2 المدمج هي: المكورات العنقودية الذهبية 6 (24٪) ، المكورات العنقودية الجلدية 4 (16٪) ، ستينوتروفوموناس مالتوفيليا 2 (8٪)،

الإشريكية القولونية 2 (8٪)، العقدية الرئوية 2 (8٪)، السالمونيلا التيفية 1 (4٪)، المكورات العنقودية الانحلالية 1 (4٪)، الكلبسيلة الرئوية 1 (4٪)، مورغانيلة مورغانية 1 (4٪)، الزائفة الزنجارية 1 (4٪)، المكورات العنقودية البشرية 1 (4٪)، كوكوريا كريستينا 1 (4٪) ، امعائية مرياحة 1 (4٪) ، والزائفة الشتوتزرية 1 (4٪). كانت معظم هذه العزلات متعددة المقاومة للمضادات الحيوية عند اختبارها باستخدام العوامل المضادة للميكروبات المختارة.

فيما يتعلق باختبارات الفحص الروتينية (CRP، CBC وLDH)، يظهر التحليل الإحصائي فرقا معنويا كبيرا (مستوى الاحتمالية $P \leq 0.001$) بين المرضى ($n = 65$) والمجموعة الضابطة ($n = 25$). لم يلاحظ أي فرق معنوي في هذه المعلمات بين المرضى الإيجابيين للزرع والمرضى سلبيي الزرع (مستوى الاحتمالية $P > 0.05$). من ناحية أخرى، كان لهذه المعلمات ارتباط قوي كبير فيما بينهم. بالإضافة إلى ذلك، لم تكن هناك فروق ذات دلالة إحصائية بين الذكور والإناث فيما يتعلق بقيم CBC، CRP وLDH لأولئك الذين أعطوا زراعة إيجابية (مستوى الاحتمالية $P > 0.05$). كما أن الذكور والإناث الذين أعطوا الزراعة سلبية ايضا لم يختلفوا بشكل كبير ($P> 0.05$).

تم تحديد مستويات PCT، PSN وPTX3 في المصل بواسطة اختبار ELISA خلال الجزء التالي من الدراسة. يكشف التحليل الاحصائي (اختبار دونكان) ان المجموعة الضابطة لها اختلاف معنوي كبير عن المرضى (الانتان الإيجابي للزرع والانتان السلبي للزرع) فيما يتعلق بتركيز PSN وPCT (وجود أحرف صغيرة مختلفة في صف واحد)، بينما لم يكن الفرق معنويا بالنسبة لتركيز PTX3 بين المرضى والمجموعة الضابطة (وجود نفس الاحرف الصغيرة في صف واحد). علاوة على ذلك، لم تختلف تركيزات PSN وPCT بشكل كبير بين المجموعات الإيجابية وسلبية الزراعة للإنتان. تراكيز PSN و PCT لها ارتباط إيجابي قوي و معنوي فيما بينهما ($P <0.001; r = 0.500$).

كشفت نتائج الفحص المناعي الفلوري غير المباشر أن 36 (90٪) من أصل 40 عينة مصل سلبية لزرع الدم تحتوي على أجسام مضادة للبكتيريا تم تحديدها بناء على هذه الطريقة، وهذه الأجسام المضادة تعود ل: الفيلقية الرئوية، الكلاميديا الرئوية، الميكوبلازما الرئوية، كوكسيلا بورنيتي، الكلاميديا بسيتاسي. كانت العدوى المشتركة موجودة (أعطى كل اختبار ردود فعل إيجابية لأجسام مضادة مختلفة تنتمي إلى أكثر من كائن حي دقيق).

الخلاصة

كشفت نتائج جميع المعلمات التي تم فحصها أنه لا توجد معلمة واحدة مثالية بما يكفي لتحديد الإنتان، وعلى الرغم من أن مزرعة الدم تستخدم بشكل شائع كطريقة تشخيصية للكشف عن تجرثم الدم، إلا أن هذه الطريقة ليست حساسة بما فيه الكفاية وحدها. وبالتالي، يجب استخدام طرق متعددة من اجل تحديد المصدر الفعلي للإنتان.