MOHAMMAD SAAD ALZAAREER MASTER OF SCIENCE 2024

CHARACTERIZATION OF OPPORTUNISTIC GRAM-NEGATIVE BACTERIA STRAINS ISOLATED FROM INTENSIVE CARE UNIT PATIENTS IN RIYADH, SAUDI ARABIA

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Thesis Submitted in Fulfilment of the Requirement for the Master of Science
in the Institute of Climate Adaptation and Marine Biotechnology
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DEDICATION

Dedicated this thesis to:

My beloved parents, siblings and my family

My project supervisor,

For all their sacrifice, moral support, and endless love

I owe all of you, big time.

Thanks, a lot

May Allah bless.

Abstract of thesis presented to the Senate of Universiti Malaysia Terengganu in fulfilment of the requirements for the degree of Master of Science

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Main Supervisor : Professor Dr. Noraznawati Ismail, Ph.D

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The prevalence of opportunistic bacterial infections is a notable concern in healthcare facilities, including Saudi Arabia. Therefore, this study aimed to characterize opportunistic bacterial strains prevalent in healthcare settings within Saudi Arabia. The descriptive cross-sectional study included 36 samples, collected from ICU patients of six hospitals in Saudi Arabia. The isolates were processed for the identification of bacterial strains by the VITEK 2 system. Phylogenetic analyses were performed to explore relationships among various bacterial species based on their 16S rRNA gene sequences. Ten methanolic extracts from natural crude were tested *in vitro* for antibacterial activity. All isolated bacteria were Gram-negative and according to VITEK 2 analyzer the most prevalent bacteria was *Klebsiella pneumonia* representing (30.6%,) followed by *Pseudomonas aeruginosa* (25%), *Proteus mirabilis* (19.4%), *Escherichia coli* (13.9%), *Serratia marcescens* (8.3%) and *Citrobacter koseri* (2.8%). Results showed that all samples examined had DNA that was pure, with a purity ratio of 1.8 to 2.0. Phylogenetic tree analysis revealed various similarities in the nucleotide base of different bacterial strains. Only four extracts (*Melaleuca cajuputi, Acanthaster planci, Stylissa carteri, and Sonneratia*

lanceolata) showed antibacterial activity, which displayed various degrees of inhibition against all isolated bacteria using the disc diffusion method. The current study demonstrated that all isolated bacteria were Gram-negative, and the most frequent bacteria found was *Klebsiella pneumonia*. Also, different similarities were observed in the nucleotide base composition among different microorganisms specially *Escherichia* sp. strain and the *Citrobacter koseri* strain that displayed the most similarity among isolated samples. Furthermore, the potential efficacy of four extracts from natural resources could be the best potential as future antibiotics.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Malaysia Terengganu sebagai memenuhi keperluan untuk Sarjana Sains

PENCIRIAN STRAIN BAKTERIA GRAM-NEGATIF OPORTUNISTIK YANG DIPENCILKAN DARIPADA PESAKIT UNIT PENJAGAAN INTENSIF DI RIYADH, ARAB SAUDI

MOHAMMAD SAAD ALZAAREER

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Kelaziman jangkitan bakteria oportunistik adalah kebimbangan yang ketara dalam kemudahan penjagaan kesihatan, termasuk Arab Saudi. Oleh itu, kajian ini bertujuan untuk mencirikan strain bakteria oportunistik yang lazim dalam tetapan penjagaan kesihatan di Arab Saudi. Kajian keratan rentas deskriptif termasuk 36 sampel, dikumpulkan daripada pesakit ICU di enam hospital di Arab Saudi. Pemencilan isolat telah dilakukan untuk mengenalpasti strain bakteria oleh sistem VITEK 2. Analisis filogenetik dilakukan untuk meneroka hubungan antara pelbagai spesies bakteria berdasarkan urutan gen 16S rRNA mereka. Sepuluh ekstrak methanol daripada minyak mentah semulajadi telah diuji secara *in vitro* untuk aktiviti antibakteria. Semua bakteria terpencil adalah Gram-negatif dan menurut penganalisis VITEK 2 bakteria yang paling lazim adalah *Klebsiella pneumonia* mewakili (30.6%,) diikuti oleh *Pseudomonas aeruginosa* (25%), *Proteus mirabilis* (19.4%), *Escherichia coli* (13.9%), *Serratia marcescens* (8.3%) dan *Citrobacter koseri* (2.8%). Keputusan menunjukkan bahawa semua sampel yang diperiksa mempunyai DNA yang tulen, dengan nisbah ketulenan 1.8 hingga 2.0. Analisis pokok filogenetik mendedahkan

pelbagai persamaan dalam asas nukleotida bagi strain bakteria yang berbeza. Empat ekstrak (*Melaleuca cajuputi, Acanthaster planci, Stylissa carteri, dan Sonneratia lanceolata*) menunjukkan aktiviti antibakteria, yang memaparkan pelbagai darjah perencatan terhadap semua bakteria terpencil menggunakan kaedah penyebaran cakera. Kajian semasa menunjukkan bahawa semua bakteria terpencil adalah dari Gram-negatif, dan bakteria yang paling kerap ditemui ialah *K. pneumonia*. Perbezaan diperhatikan dalam komposisi asas nukleotida antara mikroorganisma yang berbeza khususnya strain *Escherichia* sp. dan strain *C.koseri* yang menunjukkan persamaan yang paling banyak di antara sampel terpencil. Potensi keberkesanan empat sebatian dari sumber semula jadi mempunyai potensi terbaik sebagai sumber antibiotik pada masa hadapan.

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APPROVALS

I certify that an Examination Committee has met on 16th January 2024 to conduct the final examination of Mohammad Saad Alzaareer, on his Master of Science thesis entitled **"Characterization of Opportunistic Gram-negative Bacteria Strains Isolated from Intensive Care Unit Patients in Riyadh, Saudi Arabia"** in accordance with the regulations approved by the Senate of Universiti Malaysia Terengganu. The Committee recommends that the candidate be awarded the relevant degree. The members of the Examination Committee are as follows:

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Date

This thesis has been accepted by the Senate of Universiti Malaysia Terengganu in fulfilment of the requirement for the degree of Master of Science.

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UMT or other institutions.

_____

MOHAMMAD SAAD ALZAAREER

Date:

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

HAIs	Healthcare-associated infections
KSA	Kingdom of Saudi Arabia
AFR	African Regional Office of the World Health Organization
AIDS	Human immunodeficiency virus infection and acquired immune deficiency syndrome
HIV	Human immunodeficiency viruses
RSV	Respiratory Syncytial Virus
MRSA	methicillin-resistant *Staphylococcus aureus*
ICU	Intensive Care Unit
E. coli	*Escherichia coli*
A baumannii	*Acinetobacter baumannii*
C.difficile	*Clostridium difficile*
P aeruginosa	*Pseudomonas aeruginosa,*
K pneumoniae	*Klebsiella pneumoniae*
SARS-CoV2	Severe acute respiratory syndrome coronavirus 2.
PICU	Paediatric intensive care unit
MDRGNB	Multi-Drug Resistant Gram-Negative Bacilli
GCC	Hospitals in the Gulf Cooperation Council countries
CRPA	Carbapenem-resistant *Pseudomonas aeruginosa*
ST	Sequence-type
KKUH	King Khalid University Hospital
NICUs	Neonatal intensive care units
MIC	Minimum inhibitory concentration
MDS	Multidrug-susceptible strains.

ESBL	Extended-spectrum beta-lactamase
NIs	Nosocomial infections
WHO	World Health Organization
CDC	The Centers for Disease Control and Prevention
PFGE	Pulsed-field gel electrophoresis
ESBL-EC	Extended-spectrum-lactamase-producing *Escherichia coli*
CF	Cystic fibrosis
CRPA	Patterns of *Pseudomonas aeruginosa* that exhibit resistance to carbapenem antibiotics
LAB	lactic acid bacteria
PCR	Polymerase chain reaction
HH	Hand hygiene
HCWs	Healthcare workers
MH	Mueller and Hinton agar
CV	Crystal violet
UV	Ultraviolet light
PA	Pairwise alignment
GT	Guide tree
MA	Multiple alignments

LIST OF APPENDIX

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Opportunistic microorganisms refer to germs that often do not cause disease in individuals with intact and functional host defence systems. Nevertheless, they have the potential to induce severe illnesses in numerous patients who are hospitalised or have impaired immune systems. Hence, it is likely that almost any bacterium capable of prolonged proliferation in humans can more easily induce illness in those with pre-existing chronic conditions or impaired immune systems (Sikora & Zahra, 2023). Healthcare-associated infections (HAIs) are illnesses that patients may get while their medical treatment in a healthcare facility (Gaid et al., 2018). HAIs have become a significant threat to the safety of patients who often have weakened immune systems, such as people on immune-suppressing therapies (Ahn & Simonne, 2018) and those with HIV/AIDS, notably in the Kingdom of Saudi Arabia (KSA), HIV is increasingly recognized as a significant health problem (Baadani et al., 2020a). Opportunistic bacterial infections pose a significant risk to public health, especially in hospital environments where patients may be more vulnerable to infection due to preexisting medical conditions or invasive medical procedures (Bicheiro, 2019).

Among patients receiving medical care, opportunistic bacterial infections are a major cause of mortality and morbidity. Like other nations, Saudi Arabia has seen an increase in the frequency of diseases brought by opportunistic bacteria, requiring a greater understanding of their epidemiology and characteristics. The prevalence of these illnesses is rising worldwide, including in Saudi Arabia, which emphasizes the

need for improved knowledge control over these pathogens (Bazaid et al., 2022). The rise of antibiotic-resistant strains is one of the main issues with opportunistic bacterial infections. This happens when bacteria develop defiance mechanisms against antibiotics, making them challenging to cure and raising the risk of treatment failure and patient fatality (La Rosa et al., 2022).

The contraction of Healthcare-Associated Infections (HAIs) by patients upon admission to healthcare facilities is a significant problem. These infections not only affect patient health but also have a substantial impact on patient morbidity and mortality rates. Additionally, they lead to prolonged hospital stays and increased healthcare expenses due to ineffective treatments (Voidazan et al., 2020The impact on human health varies depending on the toxicity of the substances, the duration of exposure, the amount of microorganisms present, and the immune system of the affected persons (Voidazan et al., 2020). The incidence of infections caused by microorganisms varies based on the geographical location of the healthcare facility, the type of healthcare setting, and the characteristics of the patient population. Bacteria are the predominant pathogens, followed by fungi and viruses (Kollef et al., 2021). An article published in 2021 stated that the prevailing Gram-positive organisms consist of coagulase-negative *Staphylococci* sp., *Staphylococcus aureus*, *Streptococcus* sp., and *Enterococcus* sp. (e.g. *Faecalis* sp., *faecium*). *Clostridium difficile*, also known as *C. difficile*, is the most frequently reported pathogen in US hospitals among all pathogens associated with humans, accounting for 15% of all reported illnesses caused by pathogens. Gram-negative bacteria commonly seen include several species from the Enterobacteriaceae family such as *Klebsiella pneumoniae* and *Klebsiella oxytoca, Escherichia coli, Proteus mirabilis, and Enterobacter* species. Additionally, *Pseudomonas aeruginosa, Acinetobacter baumannii, and Burkholderia cepacia*are also frequently encountered. *A. baumanii* is linked to a high death rate in the intensive care unit because to its natural resistance to multiple drugs (Kollef et al., 2021).

In the hospital setting, the primary concerns regarding airborne transmission involve respiratory infections, such as tuberculosis, measles, varicella, influenza, respiratory syncytial virus (RSV), *Bordetella pertussis*, as well as non-respiratory

infections like norovirus, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Clostridium difficile* (Monteiro et al., 2021). In addition, patients in the intensive care unit (ICU) are susceptible to acquiring nosocomial infections. Antimicrobials are frequently recommended in intensive care units (ICUs), particularly those with broad-spectrum properties. The inadequate practise exacerbates antimicrobial resistance and amplifies the adverse effects of such medications. Conversely, it results in inflated medical expenses for the patients (Alharthi et al., 2019). A study conducted in Abha, Saudi Arabia in 2020 revealed that *K. pneumoniae* is a prevalent organism identified in cases of bloodstream infection, followed by Coagulase-negative *Staphylococcus* and *P. aeruginosa*. *Pseudomonas aeruginosa*, followed by *Staphylococcus aureus,* is the predominant pathogen associated with healthcare-associated infections (HAIs) originating from lower respiratory tract infections. Moreover, the predominant microorganisms responsible for urinary tract infections are *Escherichia coli*, followed by *Candida albicans* (Almasadi et al., 2020; Al Bshabshe et al., 2020). In 2018, a separate study conducted in Saudi Arabia confirmed that the Infectious Diseases Society of America has classified *A. baumannii* as one of the six most critical multidrug-resistant bacteria seen in hospitals worldwide. *A. baumannii* is known to be the causative agent of various serious hospital-acquired infections, such as pneumonia, skin and soft tissue infections, wound infections, urinary tract infections, and pneumonia associated with ventilators and hospitals. These infections are particularly common among patients in the intensive care unit and have a high mortality rate (Aljindan et al., 2018).

One of the primary methods we employed to distinguish and characterize the bacterial isolates was16S ribosomal RNA sequencing (Faniyan et al., 2023). This method which is frequently used in microbiology and infectious disease research, is a useful method for identifying and characterizing bacterial strains (Gates et al., 2021). The process of amplifying and sequencing the 16S rRNA gene has been widely employed to study the evolutionary relationships and classification of bacteria. This has led to the creation of enormous public databases. They were able to determine the precise bacterial species present in our samples by sequencing the 16S rRNA gene of each bacterial isolate, this information was subsequently used to better characterize the bacterial strains and comprehend their epidemiology and antibiotic

resistance profiles (Gates et al., 2021). The 16S rRNA gene possesses multiple characteristics that render it the "ultimate molecular chronometer", the predominant genetic marker for essential cellular functions, and hence, a highly valuable focus for clinical diagnosis and phylogenetic analysis (Idris et al., 2020). The hospital environment is a crucial hub for the spread of opportunistic and antibiotic-resistant bacteria, which are known to contribute to healthcare-associated infections (HAIs). The implementation of enhanced environmental cleaning protocols has successfully decreased the occurrence of such diseases (Chng et al., 2020).

Adhering to recommended practises can effectively hinder the transmission of pathogens. These practises involve the identification of bacteria, thorough hand hygiene, the use of gowns and gloves, and proper cleaning of both medical equipment and patient care areas (Alshammari, 2021). Additionally, Clark et al. (2015) added to the prior statement that, opportunistic bacterial infections must be prevented and treated using a multifaceted strategy that includes measures to support patients' immune systems and general health as well as infection control procedures in order to halt the transmission of illnesses and the emergence of antibiotic-resistant variants. Furthermore, mentioned by (Edgar et al., 2019) hand hygiene, patient isolation, environmental sanitation, and the utilisation of personal protective equipment exemplify infection control measures (Iseppi et al., 2021) indicated that antibiotic stewardship programmers are also essential for ensuring that antibiotics are used responsibly and only, when necessary, in order to mitigate the development of antibiotic-resistant microorganisms.

Since many hospitals are faced with such a severe threat, this study is intended to give insight into the current situation, therefore, to implement strategies to combat opportunistic pathogens in order to mitigate the subsequent emergence and dissemination of these strains to lower the rate of HAIs and other microbial organisms, and to economies on avoidable healthcare expenses. However, this research also focused on the antibiotic resistance profiles, pathogenicity characteristics, and genetic diversity of the bacterial strains (B. Potić et al., 2020).

1.2 Problem Statement

The prevalence of opportunistic bacterial infections is a notable concern in healthcare facilities, including Saudi Arabia. The infections are instigated by bacteria that are usually innocuous in individuals with sound health but can results in maladies in those with compromised immune systems, such as geriatric individuals, hospital patients, and those with persistent illnesses.

This data would be of significant worth in comprehending the mechanisms by which these bacteria induce infections and devising tactics to avoid and manage them. Furthermore, this could entail an inquiry into the origins of these bacteria within healthcare facilities and the identification of plausible modes of transmission. Thus, characterizing opportunistic bacterial strains in Saudi Arabia's healthcare settings is crucial to mitigating healthcare-associated infections and enhancing patient outcomes.

1.3 Significances of the study

This research was to investigate the prevalence of aggressive pathogens and their resistance to antimicrobial drugs in intensive care unit (ICU) patients. The study was conducted in six governmental hospitals in Riyadh, Saudi Arabia, and focused on identifying the types of microbes and their resistance patterns in various infection sites of ICU patients. The combination of infection surveillance and efficient infection management programmes could lead to a decrease in the occurrence and frequency of Healthcare-Associated Infections (HAI). Hence, this study aims to ascertain the pattern and frequency of Healthcare-Associated Infections (HAI) in Intensive Care Units across several federal institutions.

1.4 Objectives

The objectives of this research are;

1. To characterize the bacteria collected from the intensive care units in Saudi Arabia using biochemical tests and 16s rDNA analysis.
2. To determine the distribution and abundance of opportunistic bacteria from the isolation process via phylogenetic tree construction.
3. To screen the antagonistic activities of the isolated bacteria and anti-bacterial activities of plant extract from UMT.

CHAPTER 2

LITERATURE REVIEW

2.1 Opportunistic bacteria

In the medical literature, opportunistic pathogens are often defined as organisms that can develop into pathogenic after being perturbed by their host (such as a disease, wound, medical care, prior infection, immunodeficiency, and aging). These opportunists' bacteria can appear among the symbionts that are typically commensal such as *Streptococcus pneumoniae* and *Staphylococcus aureus* or from bacteria that have been acquired by environmental exposure such as *Pseudomonas aeruginosa* and *Burkholderia cepacian* (Brown et al., 2012). One of the most common opportunistic infections is commensal bacteria. However, using antibiotics, which often also kill commensals and pathogens, led to an increase in illnesses caused by environmental microbes exhibiting reduced susceptibility to antibiotics (Martínez, 2014). In healthy individuals, these bacterial pathogens do not typically result in illnesses. Both the invasion of host commensal bacteria and the entry of bacteria from the environment into host body tissue are major initiators of opportunistic bacterial infections. Nevertheless, these bacteria with opportunistic tendencies primarily focus on and induce life-threatening infections in individuals with weakened immune systems, such as those who are HIV/AIDS positive, cancer patients undergoing immunosuppressive treatment or corticosteroid therapy, hospitalised patients preparing for surgery, individuals with underlying conditions like cystic fibrosis or diabetes, and patients with cancer (Das, 2021).

Escherichia coli, Klebsiella pneumoniae, Candida albicans, Enterococcus faecium, Staphylococcus aureus, methicillin-resistant Staphylococcus aureus

(MRSA), *Clostridium difficile*, and *A.baumannii* species are common opportunistic pathogens that significantly contribute to various types of nosocomial infections during hospital stays (Das, 2021; Nimer, 2022).

Previous studies would significantly contribute to the study of infectious diseases by informing clinical procedures and public health guidelines to lower the risk of healthcare-associated infections (Ling et al., 2015; Zare et al., 2018; Chelkeba et al., 2021; Chahal, 2021). Opportunistic bacteria pose a severe risk in hospital settings because they can infect people with weakened immune systems. Numerous studies have described the opportunistic bacteria strains in world healthcare facilities, and Saudi Arabia is no exception (Aldrazi et al., 2020; Abdoli et al., 2022; Odoyo et al., 2023).

Furthermore, studies have looked at the genetic traits of these bacteria and determined the frequency of various opportunistic bacteria strains. Whole-genome sequencing, as an illustration, has been utilized to pinpoint the genetic pathways behind antibiotic resistance in these bacteria (Shelenkov, 2021). Risk factors for opportunistic bacterial transmission in healthcare facilities have also been investigated. Some examples are overcrowding, poor hand hygiene habits, and intrusive medical devices like catheters and ventilators (Lompo et al., 2023). It is crucial to comprehend these risk factors to create efficient infection control measures and stop the spread of opportunistic bacteria in healthcare settings (Khater et al., 2020).

Several inquiries have been carried out to as certain he most prevalent opportunistic bacterial strains in Saudi Arabian healthcare institutions (Banawas et al., 2023; Jalal et al., 2023; Alhazmi et al., 2023; Jehad et al., 2023). Several bacteria, including *A.baumannii*, *P.aeruginosa*, *K.lebsiella pneumoniae*, and Enterobacter sp., have been discovered. These bacteria pose a challenge to treatment because to their high resistance to multiple medications (Feretzakis et al., 2019). Moreover, studies have been conducted on the clinical effects of infections by opportunistic microorganisms in Saudi Arabia. These results may include rates of illness and death, inpatient stay time, and medical expenses. Identifying how these infections

affect the clinical setting can assist in guiding treatment choices and enhance patient outcomes (Alanazi et al., 2022).

2.2 Nosocomial diseases

Nosocomial infections, often known as healthcare-associated infections (HAI), are illnesses or infections that occur following medical treatment but were not present upon admission. Nurse practitioners can be found in various healthcare settings, such as outpatient clinics, hospitals, nursing homes, and post-discharge locations (Dasgupta et al., 2015; Wang et al. 2023). Furthermore, they encompass occupational infections among the medical personnel. Infections are commonly linked to the use of invasive medical devices, such as catheters and ventilators, in modern healthcare (Khan et al., 2017).

The most often reported types of nosocomial infections include urinary tract infections (UTI), surgical-wound infections, pneumonia, and bloodstream infections (BSI). Most microbes, including viruses, bacteria, and fungi are quite likely to result in nosocomial infections; however, some of them have been discovered to do so more frequently than others. *Staphylococcus aureus*, CoNS, *P. aeruginosa*, *K. pneumoniae*, *E.coli*, *C. difficile*, Enterobacter spp., *Enterococcus* sp., and *Acinetobacter* sp. are the most frequent bacterial pathogens that have been found to cause nosocomial infections (Khan et al., 2018; Mohamad et al., 2020).

2.2.1 Nosocomial Infection in the Intensive Care Unit

Hospital-acquired infections (HAIs) are believed to be the primary cause of illness and death in intensive care units (ICUs) and neonatal intensive care units (NICUs). Frequently, the organisms on surrounding surfaces cause these diseases. There is limited information available regarding the microbial profiles linked to HAI in these settings because different ICUs may have different bacteria (Ribeiro et al., 2019).

Intensive care units have significantly enhanced the chances of survival for patients with trauma, shock states, and other life-threatening conditions, while having a noticeably higher likelihood of acquiring infections within the hospital (nosocomial infections). Hospitalised patients who require advanced life support for longer than a week have nosocomial infection rates that are three to five times greater compared to those who do not require advanced life support. In patients who survive a catastrophic injury or full-thickness burns, infection is the most frequent cause of death, either directly or indirectly. This infection is commonly acquired in a hospital setting, known as nosocomial infection. Moreover, it is well recognised as the primary cause of multiple-organ dysfunction syndrome (Stuart and Cairns, 2019).

2.3 Prevalence of hospital-acquired infections

More than 100 million patients experience nosocomial infections annually on a global scale. The burden of HAIs in developed nations indicates that more than four million patients are impacted annually. According to a prior meta-analysis study, 7.6 occurrences of infections related to healthcare occur for every 100 patients. However, the prevalence of NIs (nosocomial infections) ranged from 3 to 15% in emerging nations (Taye et al., 2023). The current meta-analysis included a total of 220 publications, out of which only 14 were from Africa. The incidence rate of nosocomial infections was found to be 7.4 infections per 100 patients (Taye et al., 2023).

A recent international study conducted a systematic review and meta-analysis to investigate the prevalence of healthcare-associated infections (HAIs). The prevalence of HAIs worldwide was 0.14 %. The annual rate of HAIs is rising by 0.06%. The AFR (African Regional Office of the World Health Organization) has the greatest rate of HAIs (Raoofi et al., 2023).

2.4 Causes of Healthcare-Associated Infections (HCAI)

Several healthcare-associated infections (HCAIs) result from the presence of implants and prostheses. Additionally, infections can arise from cross-contamination between patients and medical personnel, as well as from patients with compromised immune systems who are more susceptible to common infections. Furthermore, surgical site infections (SSIs) can also contribute to HCAIs. These encompass ventilator-associated pneumonia (VAP), catheter-associated urinary tract infections (UTIs), and central line-associated bloodstream infections (CLABSIs) (Haque et al., 2018).

Within the realm of contemporary healthcare, numerous infections are associated with intrusive interventions such as surgical procedures, indwelling medical apparatus, and prosthetic devices. During their hospitalisation, patients are susceptible to infections originating from various sources, such as the surrounding environment, healthcare personnel, and other infected patients. The most common types of nosocomial infections are those that impact surgical wounds, the respiratory system, the genitourinary system, and the gastrointestinal system. Untreated, these infections can lead to more consequential health complications. A wide range of nosocomial illnesses, including urinary tract infections, lung pneumonia, surgical site infections, bacteremia, gastrointestinal infections, and skin infections, are prevalent (Brennan, 2022).

2.5 Risk factors associated with HAIs.

Several risk factors have been discovered in Saudi Arabia that enhance the probability of opportunistic bacterial infections. These include immunosuppression, chronic diseases, advanced age, malnutrition, and hospitalization (Baadani et al., 2020; Voidazan et al., 2020). Additionally, environmental factors such as poor sanitation and hygiene practices, exposure to contaminated water or food, and close contact with infected individuals can also increase the risk. Individuals with these risk factors must take appropriate precautions to prevent opportunistic bacterial infections (Alshammari et al., 2021).

Nosocomial infections (NIs) are a significant problem in the Middle East, caused by various bacteria, viruses, and fungi. The Middle East has witnessed an increasing apprehension regarding the menace of antibiotic resistance in recent years. This phenomenon can result in prolonged hospitalisation, escalated expenses, and even fatalities (Micek et al., 2016). Multiple factors contribute to the issue of antibiotic resistance in the Middle East, such as overuse of antibiotics, poor infection control practices, and lack of access to new antibiotics (Al-Shami& Al-Haimi, 2018). These factors can lead to the spread of antibiotic-resistant bacteria, making it more challenging to treat these infections and leading to more extended hospital stays, higher costs, and even death. The problem of antibiotic resistance is a severe threat to public health in the Middle East, and it is essential to take steps to address it (Nimer, 2022).

A previous study examined the pathogen burden of patients in the ICU of a tertiary care hospital in Hail, Saudi Arabia. Five hundred ninety-one hospital-acquired infections that were clinically suspected were examined, resulting in the acquisition of 163 bacterial isolates from various clinical specimens. The most common isolates were *K. pneumonia* (39, 24%), followed by *A. baumannii* (35, 21.5%), *P. aeruginosa* (25, 15.3%), and *Proteus* sp. (23, 14%). Among the highly prevalent bacterial isolates, ESBL was predominant (42 42.4%). The study results indicate a significant prevalence of multidrug-resistant infections among patients in the intensive care unit (ICU), emphasising the necessity for enhanced infection prevention and control protocols in ICU environments. These measures should include hand hygiene, personal protective equipment use, cleaning and disinfecting equipment and surfaces, appropriate antibiotic use, and surveillance for HAI (Saleem et al., 2023).

According to the authors Church & McKillip, 2021, the emergence of drug-resistant bacteria can be attributed to the excessive and inappropriate utilization of antibiotics, coupled with the dearth of novel drug discovery. There is a demand for heightened investment in developing novel drugs, enhanced management of antibiotics, and disseminating knowledge to the public regarding the significance of judicious use of antibiotics.

2.6 Epidemiology of healthcare-associated infections

Due to their importance in preventing and managing healthcare-associated infections (HAIs), microbiology and epidemiology are closely intertwined (McLaws, 2014). Healthcare practitioners may create efficient infection control strategies and enhance patient outcomes by researching the microbiology and epidemiology of HAIs (Weber & Rutala, 2013).Healthcare-associated infections (HAIs) caused by multidrug-resistant organisms (MDROs) have significant consequences in terms of illness, death, and expenses. Various pathogens, particularly multidrug-resistant organisms (MDROs), can be involved in these illnesses. MDROs possess the capability to transmit across patients and readily develop resistance to antibiotics (Salmanov et al., 2023).

As per an article that examines the epidemiology of multi-drug resistance (MDR) Gram-negative bacteria (GNB) in the Middle East. The authors employ a "One Health" methodology, which considers the interdependence of human, animal, and environmental health, in order to investigate the factors that contribute to the dissemination of Multi drug resistance GNB. The authors identify various factors that contribute to the dissemination of Multi drug resistant GNB in the Middle East. These factors include the excessive use of antibiotics in both human and animal populations, insufficient implementation of infection control measures in healthcare facilities and related settings, the movement of people and animals across countries, and the presence of Multi drug resistant GNB in the environment. The authors state that the proliferation of multi-drug resistant Gram-negative bacteria (MDR GNB) in the Middle East is a grave issue that demands immediate attention. They advocate for a one Health approach to effectively prevent and manage the development of MDR GNB (Dandachi et al., 2019).

A previous study assessed the antibiotic resistance profile demonstrated by MRSA strains isolated from patients who were treated at King Fahd Hospital in Madinah, Kingdom of Saudi Arabia. The study's findings suggest that the MRSA strains isolated from patients exhibited resistance to a wide range of medications. The resistance patterns most commonly identified were for amoxicillin (99.5%), daptomycin (98.8%), linezolid (98.0%), clindamycin (91.3%), erythromycin

(90.8%), cotrimoxazole (84.4%), and vancomycin (37.2%). Their findings indicated that the summer season had the highest incidence of MRSA infections, accounting for 51% of cases, followed by autumn (20.7%), winter (18.5%), and spring (9.8%). The study's results indicate that MRSA poses a significant risk to public health in Saudi Arabia. Further investigation is necessary to comprehend the underlying factors that facilitate the dissemination of MRSA and devise novel approaches to prevent and manage MRSA infection (Samah et al., 2018).

An earlier study examined the molecular epidemiology and mechanisms of resistance to carbapenem in *Pseudomonas aeruginosa*, a bacterium, in hospitals located in the countries of the Gulf Cooperation Council (GCC). A total of ninety-five distinct CRPA isolates were obtained from hospitals located in Saudi Arabia, the United Arab Emirates, Oman, Qatar, Bahrain, and Kuwait. The most common gene encoding carbapenems was blaVIM-type, which was detected in 37 out of 95 isolates, accounting for 39%. Out of the total of 14 sequence-type (ST) clusters, four were found to be present in multiple countries. The data indicate that CRPA is prevalent in the GCC countries and that high-risk clones are the predominant strains in the region. Additional investigation is required to comprehend the determinants behind the dissemination of CRPA in the GCC region and to formulate efficient approaches for prevention and control (Zowawi et al., 2018).

2.7 Distribution of opportunistic Pathogens Associated with Nosocomial Infections

The National Healthcare Safety Network identifies *Staphylococcus aureus,* coagulase-negative *Staphylococcus, Enterococcus, Escherichia coli, Pseudomonas aeruginosa, Enterobacter,* and *Klebsiella pneumoniae* as the most prevalent pathogens causing surgical site infections (SSI). While, *Staph aureus* and *Pseudomonas aeruginosa* are the most prevalent pathogens for HAP and VAP, but *E coli* and *Klebsiella pneumoniae* are more prevalent in paediatric populations. However, *Candida* spp. (adult ICU), *Enterobacteriaceae* (adult wards, paediatric ICU and wards, and oncology wards), and *staph aureus* are typical CLABSI species. The pathogens *Enterococcus, Pseudomonas, Klebsiella, Staphylococcus aureus,*

Proteus, and *Candida* are commonly associated with the occurrence of CAUTI (Monegro et al.,2023).

Numerous pathogenic bacteria are frequently found in airborne microorganisms, and their presence poses a serious risk to human health. For instance, airborne transmission of *Acinetobacter baumannii* in hospitals can cause meningitis, bacteraemia, and other illnesses such as respiratory infections. *Staphylococcus* is a significant human pathogen that is widely prevalent in wastewater treatment plant bioaerosols and that can infect humans and cause bacteraemia and skin diseases (Yan et al., 2021).

An earlier retrospective observational study identified the associated risk factors and evaluated the prevalence of NI in three private hospitals. The study encompassed all patients who were admitted to the Intensive Care Unit (ICU) and surgical ward and remained hospitalised between 2017 and 2018. *E-Coli* alone or in combination was the most prevalent organism in 6 (40.1%) cases, followed by *Klebsiella pneumoniae* and *Staph aureus* in similar amounts in 3 (20.0%) (Ahmed Enani et al., 2019).

2.8 Current issue related to opportunistic bacteria.

2.8.1 Multidrug-Resistant Gram-Negative Bacteria

Antibiotic resistance is a significant concern in treating HAIs caused by opportunistic bacteria. Studies have reported high rates of antibiotic resistance in bacterial strains separated from HAIs in Saudi Arabia, including resistance to carbapenems and other last-resort antibiotics (Al-Said et al., 2023). The attribution of the emergence of antibiotic-resistant bacteria has been attributed to the widespread use of an antibiotics in healthcare settings and the horizontal transfer of antibiotic-resistance genes between bacterial strains (Soto, 2023).

The proliferation of antibiotic-resistant variants has been ascribed to the extensive utilization of antibiotics in healthcare environments and the lateral transmission of genes conferring antibiotic resistance among bacterial strains (Gajdács & Albericio, 2019). Another study showed a significant prevalence of opportunistic bacteria that are resistant to antibiotics, *K. pneumoniae* is the predominant ESBL-secreting Enterobacteriaceae, accounting for 58.33% of such strains, while *E. coli* and *E. cloacae* represent 25% and 8.33%, respectively, the prevalence of ESBL-producing Enterobacteriaceae in various infectious sites has been studied, revealing that 60% of such isolates are associated with urinary tract infections. The incidence of ESBL in urinary tract infections is relatively lower in previous research investigation (LemineOuld Salem, 2022).

A case-control study was conducted at a tertiary care hospital in Saudi Arabia to explore possible risk variables linked to the acquisition of infections caused by MDR GNB. The study findings suggest a strong correlation between infection caused by MDR GNB, previous antibiotic usage, admission to the ICU, and the presence of medical devices inserted into the body. Patients who were older, had longer hospital stays, and had more serious underlying medical conditions were more likely to be infected with MDR GNB. The findings of the study emphasise the importance of applying infection control measures to reduce the spread of MDR GNB. The prevailing MDR GNB species were *A. baumannii* (38%), *K. pneumoniae* (31%), *P. aeruginosa* (20%), *and E. coli* (11%). The investigation was carried out over a period spanning from 2015 to 2017, and the findings of the research exhibited statistical significance. The study's discoveries hold significance for healthcare practitioners in Saudi Arabia and other nations, as they underscore the necessity for heightened monitoring of MDR GNB and adopting efficacious infection control measures to impede the dissemination of these microorganisms (AlThaqafi et al., 2019).

Based on a previous investigation, a total of seventy samples were obtained from different parts of the body. Among them, 25 (35.7%) were identified as *P. aeruginosa*, 23 (32.9%) as *K. pneumoniae*, 16 (22.9%) as *E. coli*, and 6 (8.6%) as *A. baumannii* were confirmed to be ESBL-positive using the double-disk diffusion

technique. Every strain of *P.aeruginosa* and *A.baumannii* shown resistance to carbapenem antibiotics. MBL was detected in all strains of *P. aeruginosa* and *A. baumannii*, as well as in 4 out of 23.4% strains of *K. pneumoniae*, however it was not found in *E. coli*. The strains studied exhibited ESBL and MBL as the main mechanisms of resistance (Azim et al., 2019).

The study titled "Phenotypic and genotypic characterization of clinical *P. aeruginosa"* was published in the Journal of Taibah University Medical Sciences in 2022. It aimed to examine the phenotypic and genotypic traits of *P. aeruginosa* strains obtained from clinical samples. The study revealed a positive correlation between a greater quantity of MDR strains and their proficiency in biofilm production. Additionally, these strains exhibited the presence of the modA and pslA genes, as well as demonstrated all three forms of motility: swimming, swarming, and twitching. The colistin minimum inhibitory concentration (MIC) exhibited a notable increase in MDR strains compared to multidrug-susceptible (MDS) strains. These data indicate that biofilm development, drug resistance, and motility are all crucial elements contributing to the pathogenicity of *P. aeruginosa*. Additional research is required to examine the impact of these parameters on the pathogenicity of multi-drug resistant *P. aeruginosa* strains (Mallikarjuna & Dhanashree, 2023).

2.9 Incidence of ESBL-Producing *Enterobacteriaceae*

The increasing prevalence of Enterobacteriaceae developing extended-spectrum beta-lactamase (ESBL) poses a worldwide public health concern (Sangare et al., 2015) Antibiotic overuse and abuse selection pressure have caused the evolution of antibiotic resistance. Effective initiatives are required, including steps to encourage optimal antibiotic usage and infection control and develop novel medicines and alternative treatment options (Tetz&Tetz, 2022).

The rise of antibiotic-resistant bacteria, which are becoming increasingly difficult to cure, has been linked to the excessive and inappropriate use of antibiotics, as well as the lack of new treatment discovery. As per the Centres for Disease

Control and Prevention (CDC), it is projected that by the year 2050, approximately 10 million individuals will succumb to infections that are resistant to antibiotics. The antibiotic resistance crisis presents many challenges and imperatives that require attention, including but not limited to the overuse and misuse of antibiotics, insufficient development of new drugs, the emergence of novel resistant bacteria, enhanced antibiotic stewardship, augmented investment in new drug development, and heightened public education (Church & McKillip, 2021).

A previous investigation aimed to examine the prevalence and distribution of ESBL encoding genes in multidrug-resistant Gram-negative pathogens obtained from three distinct nations, namely Egypt, Saudi Arabia, and Sudan. This study obtained a sample size of 292 clinical isolates, with a majority of 85.6% exhibiting resistance to over three antibiotic classes. The predominant genes encoding ESBLs were identified as blaCTX-M and blaTEM, with a 100% and 66.7% detection rate among the isolates, respectively. The prevalence of ESBL resistance was observed to be highest among *E.coli*, *K. pneumoniae*, and *P. aeruginosa*. The research emphasizes the escalating incidence of ESBL-producing microorganisms in emerging nations and underscores the imperative for prompt measures to impede the dissemination of these pathogens (Azab et al., 2021). The article investigated the prevalence and molecular characterization of ESBL-producing *A. baumannii* isolates from clinical settings in Saudi Arabia by Alyamani. A total of 107 *A. baumannii* isolates were obtained from the ICUs of three hospitals in Makkah. The findings indicated that 94% of the *A. baumannii* isolates exhibited ESBL production. The prevailing ESBL genes identified were TEM, OXA-51-like, and OXA-23-like.

2.10 Clinical manifestation and diagnosis

Diagnosing infection in patients in the ICU can present difficulties. The clinical manifestations of certain organ infections, as well as the symptoms of systemic inflammatory response syndrome (SIRS), such as hypotension or fever, may be concealed by organ support. Additionally, SIRS frequently has a non-infectious origin. Clinical history and examination could be scant, and diagnostics

sometimes take a while to complete compared to how quickly a therapeutic strategy decision needs to be made. Although prediction methods have been created to aid in the early detection of sepsis, their overall effectiveness is still often constrained (Rawson et al., 2023).

Patients in the ICU frequently exhibit non-specific classic indications of infection, such as pyrexia and tachycardia. To avoid missing an infectious condition that can be treated, treatment of infection must be approached in the ICU in order to avoid neglecting an infection. This strategy entails giving broad-spectrum antibiotics to sick individuals in large, rather indiscriminate doses (Heffernan & Denny, 2021).

2.10.1 Identification of opportunistic bacteria in the laboratory

To facilitate therapy, healthcare practitioners should promptly obtain cultures while simultaneously emphasising the importance of timely administration of antibiotics. Obtaining cultures prior to the administration of antibiotics can help clinicians identify the offending bacterium, potentially enabling de-escalation through effective therapy. If blood cultures are taken after taking antibiotics, the yield of such samples may decline, which could raise the patient's costs and length of stay (Bayot & Bragg, 2022).

Cultures can be obtained from sites that are either sterile or colonised by bacteria. Individuals who have bacterial colonisation are at risk of becoming infected by their own natural microorganisms, which could lead to inaccurate outcomes. According to (Giuliano et al., 2019), when analysing blood, respiratory, urine, skin, soft tissue, bone, and joint cultures, it is important to consider that biological sites such as blood, pericardial fluid, and cerebral spinal fluid are commonly regarded as devoid of microorganisms. The pollution of sputum and nasal passageways is well-known. Improper culture-collection methods can further heighten the danger of contamination. In case of blood cultures, it’s critical to distinguish contamination from bacteremia while assessing blood cultures and it is essential to take at least two samples from different parts of the body to make sure that the bacteremia resolves.

Also Giuliano et al., (2019), indicated that urine cultures should only be drawn when infection is suspected, on the other hand, obtaining one stool culture is sufficient and can detect the pathogen 87–94% of the time.

Bacteria are primarily classified according to their morphological characteristics, such as substrate utilisation, Gram staining, flagella morphology, and the presence or lack of flagella. The development pattern on the solid media is a crucial characteristic, since it can result in significantly varied colony forms among various species. Examining the morphology of individual cells or the characteristics of colonies remains a dependable method for identifying bacterial species (Rodrigues et al., 2023).

Culture media are nutrient- and mineral-rich substrates that promote the growth of microorganisms in laboratory settings. Due to the distinct characteristics, features, habitats, and nutritional requirements of each microbe variety, it is challenging to cultivate them using a uniform culture medium (Bonnet et al., 2020). Culturing microorganisms is essential for diagnosing infectious diseases, extracting antigens, developing serological assays for vaccines, conducting genetic studies, and identifying microbial species. Furthermore, it is important for the preservation of cultural resources, analysis of biochemical reactions, identification of microbiological contamination, assessment of the efficacy of antimicrobial agents and preservatives, quantification of living organisms, and evaluation of antibiotic susceptibility (Kheirabadi & Macia, 2022).

2.10.2 Gram staining

Gram staining is a highly notable technique in the field of microbiology. The Danish bacteriologist Hans Christian Gram designed it in 1882 largely for the purpose of identifying pneumonia-causing bacteria. Gram staining, typically the initial diagnostic procedure, employs crystal violet or methylene blue as the primary dye. Gram-positive organisms are characterised by their ability to retain their initial colour and appear as purple-brown when observed under a microscope. When

observed under a microscope, Gram-negative organisms that do not absorb the initial dye can be seen as red (Wu & Yang, 2020). The primary step in the gram staining procedure involves applying crystal violet dye to the slide for initial staining. The last phase, commonly known as dye fixation, involves the utilisation of iodine to form a compound of crystal violet and iodine, so preventing the colour from being easily removed. The dye is subsequently eliminated using a decolorizer, commonly consisting of a solvent composed of ethanol and acetone. The underlying principle of gram staining is on the bacterial cell wall's capacity to retain the crystal violet dye even after solvent treatment. Gram-negative organisms possess a greater amount of lipids compared to Gram-positive microorganisms, while Gram-positive germs have a higher amount of peptidoglycan (Yoshimura et al., 2022).

Gram's method has been modified numerous times, and the literature brought up an unexpectedly large number of distinct techniques referred to as the Gram method (Savadori et al., 2023). The Gram stain differentiates between different bacteria by exploiting the diverse staining properties of bacterial cell walls.

2.10.3 Biochemical and Molecular identification

For the identification and characterizing of bacteria isolated from healthcare facilities in Saudi Arabia and other parts of the world, biochemical testing and 16S rDNA analysis are frequently utilized techniques. Performing a series of tests on bacterial isolates to ascertain their metabolic capacities and other qualities, such as their capacity to ferment particular sugars or create particular enzymes, is known as a biochemical test (Winand et al., 2020). These assays can help identify bacterial isolates more precisely and reveal details about their physiology.

The utilization of rRNA sequences, specifically the 16S rRNA gene, is employed for the purpose of taxonomic classification and identification of prokaryotic organisms. The universal distribution of these entities facilitates the evaluation of phylogenetic relationships among extant organisms. Their conserved nature serves as a robust foundation for evolutionary lineages, while their remarkably

conserved regions allow for the development of nearly universal PCR primers within the bacterial domain (Lawalata et al., 2020). The 16S rRNA gene, a specific portion of the bacterial genome present in all bacteria, is sequenced as part of a process known as 16S rDNA analysis (Johnson et al., 2019). Based on the relatedness of several bacterial isolates, as shown by their 16S rDNA sequences, a phylogenetic tree can be created using this sequence (Church et al., 2020). When trying to identify bacterial isolates that are challenging to identify using conventional biochemical assays, this method can be beneficial (Promega Corporation, 2019).

Utilising 16S rDNA sequencing, the process of molecular identification and phylogenetic analysis of multidrug-resistant (MDR) bacteria serves as a potent method for comprehending antibiotic resistance processes and formulating innovative approaches to address this escalating menace to human well-being. This technique has been used to study a variety of MDR bacteria, including those that cause infections in humans, animals, and plants (Vivas et al., 2019). The results of these studies have shown that MDR bacteria are widespread and are constantly evolving, and the transfer of MDR genes among different bacterial species is a significant concern (White & Hughes, 2019). Utilising molecular techniques to identify and analyse the phylogeny of multidrug-resistant (MDR) bacteria can facilitate the monitoring of resistance dissemination and the detection of origins for novel resistance genes. This research has important implications for developing new strategies for combating antibiotic resistance, by understanding the mechanisms of resistance and how it spreads; we can develop new approaches to prevent the development and spread of MDR bacteria (Ullah H et al., 2022).

Studies that characterize the bacteria isolated from Saudi Arabian healthcare facilities using biochemical assays and 16SrDNA analysis can reveal important details about the kinds of bacteria present there and their antibiotic resistance patterns. By applying this understanding, one can create infection control and preventive techniques to halt the transmission of antibiotic-resistant microorganisms. (Alsanie et al., 2018) conducted a study in which they identified and collected 30 multi-drug resistant bacterial strains from multiple hospitals in the Taif province of Saudi Arabia. The 16S rDNA gene was subjected to sequencing, and the GenBank

databases were utilised to provide comprehensive identification results. The analysis of the consensus sequences from 21 species, including *Bacillus cereus, Bacillus subtilis, Bacillus tequilensis, Caldimonasmanganoxidans, Citrobacter freundii, Enterococcus faecium, Escherichia ferguson, K. pneumoniae,* and *Lactobacillus plantarum*, revealed nucleotide identities ranging from 76% to 100%. The isolates were categorised into three categories according to phylogenetic analysis. The assessment of nucleotide diversity indicated that all examined sequences exhibited variability at individual sites and had significant levels of nucleotide diversity, with values ranging from 0.17 to 0.94. All isolates exhibited areas that were significantly preserved ($p< 0.05$). Ultimately, alterations in the genetic makeup of the targeted bacterial strains can be associated with the development of antibiotic resistance and the exchange of genes between different bacterial strains inside the hospital setting. Further investigation of antibiotic-resistance gene sequences is required (Alsanie et al., 2018).

2.10.3.1 16s rDNA and Phylogenetic Analysis

Molecular technologies have made it possible for researchers to examine human microbiota more thoroughly and sensitively than has been possible with culture testing (Simon, 2022). Phylogenetic tree building is constructing a tree that represents the evolutionary relationships between a set of organisms. Traditional methods are based on comparing the sequences of individual genes, but with the advent of next-generation sequencing; it is now possible to sequence the entire genomes of multiple organisms. New methods have been developed to address these challenges and build accurate phylogenetic trees (Kapli et al., 2020).

The approach of molecular systematic and phylogenetic analysis, specifically based on 16S rRNA gene analysis, can be employed to identify and characterise indigenous bacterial isolates that possess the capability to digest contaminants. These bacteria have the potential to serve as effective bioremediation agents. It is a relatively rapid and inexpensive technique that can be used to identify bacteria not easily cultured in the laboratory and to identify bacteria present in complex microbial communities (Iskandar et al., 2021).

The present investigation by (Al-Zahrani & Al-Ahmadi, 2020) examined the genetic variability and distribution patterns of *P. aeruginosa* that exhibit resistance to carbapenem antibiotics (CRPA) within a tertiary and quaternary healthcare facility in Makkah, Saudi Arabia. The study's findings indicate that the CRPA isolates exhibited genetic diversity, comprising 20 distinct sequence types. However, it was observed that two clones, namely ST235 and ST654, were responsible for 31.4% of the total isolates. The researchers additionally discovered that the CRPA isolates were distributed throughout the hospital, as evidenced by 11 out of the 35 isolates being identified in patients admitted to the same ward. The authors suggest that hospitals in the area establish surveillance initiatives to oversee the dissemination of CRPA and enforce infection control measures to curb the transmission of these microorganisms (Al-Zahrani & Al-Ahmadi, 2020). Moreover (Lagha et al., 2021) performed a study entitled " Molecular characterization of multidrug-resistant *Klebsiella pneumoniae* clinical isolates recovered from King Abdulaziz Specialist Hospital at Taif City, Saudi Arabia", which employed diverse techniques to delineate the genetic constitution of MDR *K. pneumoniae* isolates. Thirty *K. pneumoniae* strains were isolated from the hospital and subjected to antibiotic susceptibility testing. The genetic variability of the isolates was assessed by conducting PCR analysis targeting genes that encode porins and efflux pumps, as well as (GTG)5 and BOX repetitive sequences. *K. pneumoniae* shown variability in antibiotic resistance dependent on gender or specimen type. In addition, among the 30 isolates, 25 distinct profiles were identified, with a prevalence of multidrug resistance seen in 83.33% of the cases. The PCR analysis identified seven distinct genotypes associated with the genes responsible for porins and efflux pumps. Furthermore, a significant link was seen between the resistance to antibiotics and the presence of these specific genes. PCR genomic fingerprinting revealed the extensive genetic variability of K. pneumoniae. BOX-PCR and (GTG)5 produced 18 and 19 clusters, respectively, with discriminating indices of 0.97 and 0.98 at a similarity level of 80%. *K. pneumoniae* clinical isolates exhibit significant phenotypic and genetic diversity, with many strains potentially circulating concurrently (Lagha *et al*., 2021).

2.11 Antagonistic Activity of Bacteria and antibacterial effect

For the public health systems to maintain their level of infection control, new antimicrobial options are crucial. Drug-resistant infections come in a variety of forms, including different bacterial and fungal species. Numerous outbreaks of infection have been documented within this multidrug-resistant (MDR) diversity of bacteria, including *Enterobacter cloacae*, which has become an important nosocomial pathogen in neonatal facilities, *Salmonella enterica*; *Staphylococcus aureus*; and *Streptococcus mutans*. All of these species are now challenging to treat with traditional antibiotics (Amer et al., 2021). MDR has increased substantially over the course of the past ten years. Even though MDR is a natural process, it causes disease-causing organisms to become resistant to numerous chemotherapeutic drugs, increasing patient mortality. Thus, fewer, and fewer medicinal compounds are linked to MDR. It is anticipated that conventional herbal medicines could replace the usage of chemical substances. Therefore, a new approach to treating bacterial infections is needed (Klewica et al., 2023). Microbe populations can interact in a variety of ways, either synergistically or antagonistically. Bacteria are always competing with one another for nutrients and niche spaces in order to endure the challenges and coexist with other microorganisms. A producer strain in the context of bacterial antagonism is one that is able to create toxic compounds that prevent the growth of other non-producing strains, which are typically sensitive to the toxin (Cesa-Luna et al., 2020).

Lactic acid bacteria (LAB) have garnered the interest of researchers as a potential alternate approach for combating harmful pathogens. Research has demonstrated that LAB species produce a range of chemicals that exhibit antagonistic effects. Many scientists have employed these antagonistic microbes to combat plant and human infections, as well as to prevent the growth of food spoilage microorganisms in the presence of harmful bacteria (Dejene et al., 2021; Leska et al., 2021). Microbial Antagonism is a method of inhibiting pathogenic microorganisms by secreting substances that interfere with the life cycle of the microorganism. The mechanisms of antimicrobial activity of LAB species may be through the production of several substances such as organic acid, hydrogen peroxide, antibiotics and bacteriocins (Ghydaa et al.,2020)

There is currently a strong emphasis on re-evaluating herbal preparations in the pharmaceutical and therapeutic fields. This involves gaining a comprehensive understanding of the physio-chemical and therapeutic capabilities of the active compounds found in medicinal plants, as well as developing extraction procedures and quality control systems. Extractive solutions in pharmaceutical formulations employ solvents to extract active compounds, along with varied quantities of less active components and impurities. The extraction process involves employing specific solvents following established protocols to isolate the therapeutically advantageous constituents of the plant tissues from the inactive or inert constituents (Șachir et al., 2022). The fact that some medicinal plants are widely available and have beneficial effects on specific types of bacteria that infect humans allows others to research their potential benefits and consider alternative methods of studying various plant species to evaluate and test their constituents that could be used as a therapeutic dose for patients with no or minimal side effects in comparison to drugs (Al-Qudaha, 2022).

The article reported by Yang et al. (2020) examines the existing research on the antibiotic properties and resistance of lactic acid bacteria (LAB) and other microorganisms that produce bacteriocins with antagonistic effects. Lactic acid is generated by LAB through fermentation, leading to a decrease in the pH of the surroundings and the suppression of detrimental bacterial proliferation. LAB synthesises many antimicrobial substances including as organic acids, hydrogen peroxide, bacteriocins, and diacetyl, which possess the ability to impede the proliferation of a broad spectrum of bacteria. Additional investigation is required to gain a more comprehensive understanding of the antibiotic activity and resistance of LAB and other bacteriocin-producing microorganisms that exhibit antagonistic properties. This research has the potential to contribute to the development of novel approaches for the prevention and treatment of food-borne diseases, as well as the creation of new probiotic products that offer potential health advantages (Yang et al., 2020).

Pallavali et al ., (2019) presented an article that contains the data on the antimicrobial activity of methanol extracts of plant leaves. The data included 11

natural plant species which are widely used as folk medicine against multi drug resistant bacteria, which was isolated of septic wound infections. According to the data, it showed that among 11 plant methanol leaf extracts; *Punica granatum* and *Syzigium cumini* have the potential antibacterial activity against the predominant bacterial isolates of septic wounds that are MDR-*P. aeruginosa, S. aureus, K. pneumoniae* and *E. coli.*

Table 2.1: Effect of methanol plant extracts on the MDR-bacterial isolates (Pallavali et al ., 2019).

	Leaf extracts of medicinal plants										
Bacterial isolates											
	PG	**SC**	**DE**	**DM**	**EH**	**GA**	**MO**	**PS**	**AA**	**HA**	**JG**
S. aureus	+	+	–	–	–	–	–	–	–	–	–
P. aeruginosa	+	+	–	–	–	–	–	–	–	–	–
K. pneumoniae	+	+	–	–	–	–	–	–	–	–	–
E. coli	+	+	–	–	–	–	–	–	–	–	–

Keys: (+) inhibition zone, (−) no inhibition zone, *Punica granatum* (PG), *Syzygium cumini* (SC), *Delonix elata* (DE), *Digera muricata* (DM), *Jatropha gasipifed* (JG), *Maeua oblongifolia* (MO), *Pterocarpus santalinus* (PS), *Gyrocaspus americana* (GA), *Acalipha alinifolia* (AA), *Hygrophilia auriculata* (HA)*Euphorbia heterophilla* (EH). *Punica granatum* (PG), *Syzygium cumini* showed the lytic activity and forms the zone around the disc against the MDR-bacteria of septic wound infections.

2.12 Infection Control Strategies and Prevention

Epidemic occurrences are singular incidents that necessitate expeditious inquiries and interventions by public health authorities. Research endeavours enhance the comprehension of the inherent characteristics of diseases, causative agents, modes of transmission, and oversights that precipitate epidemics. Reports on outbreaks frequently exhibit inadequacies stemming from delayed notification, unavailability of clinical specimens, unsuitability of laboratories, inadequate resources, and insufficiently trained personnel. The Ministry of Health in Saudi Arabia has created manuals that outline protocols for the surveillance and prevention of healthcare-associated infections and communicable diseases (Sharaheeli et al, 2022). Healthcare providers should be aware of the increasing threat of Gram negative bacterial infections, patients at risk should be closely monitored for signs and symptoms of infection, effective infection control measures should be implemented, and research is needed to develop new antimicrobial agents and strategies for infection control (Care et al., 2021).

Characterization of opportunistic bacteria strains from Saudi Arabian healthcare settings is critical for creating effective control techniques to avoid HAI transmission. Infection control practices and antimicrobial stewardship are prevention methods (Khater et al., 2020), whereas antimicrobial therapy and supportive care are treatment measures. Genotyping methods can be used to identify outbreaks and track the spread of bacterial diseases. Prevention, treatment, and focused control techniques should be prioritized in control initiatives (Armstrong et al., 2019).Maintaining proper hand hygiene (HH) is a fundamental aspect of mitigating healthcare-associated infections and curtailing the proliferation of antimicrobial resistance. The research conducted emphasizes the critical need for enhanced infection control practices and antibiotic stewardship initiatives in Saudi Arabian healthcare institutions. Despite the undisputed advantages of hand hygiene, adherence to hand hygiene protocols remains below optimal levels. The level of adherence to HH protocols is believed to be influenced by the extent to which healthcare workers (HCWs) are cognizant of being under surveillance (Alslamah & Abalkhail, 2022).

Opportunistic bacteria strain identification in Saudi Arabian healthcare settings is a significant area of research that can guide clinical practices and public health policies to lower the incidence of HAIs (Elbehiry et al., 2022). These studies underline the significance of continuing surveillance and investigation to track the prevalence and features of opportunistic bacteria strains in Saudi Arabian healthcare settings and to create efficient preventative, diagnostic, and therapeutic approaches (Abalkhail et al., 2022). These initiatives are essential for enhancing patient outcomes and lowering the incidence of infections linked to healthcare in the nation (Zhou et al., 2020).

Another study sought to discover microbiological contaminants in neonatal intensive care units (NICUs) to enhance disinfection and decrease healthcare-associated illnesses in infants by Al- Jabir. Surfaces, equipment, and nurses' hands were sampled, and out of 169 samples, only 122 bacterial isolates were detected. Bacterial genetic testing showed 11 genera, including common microorganisms such as *Staphylococcus* sp., *Enterococcus* sp., *Pseudomonas* sp., *Klebsiella* sp. And *B. cereus* was the most often isolated bacterium, followed by *S. epidermidis*. Some bacterial isolates tested positive for antibiotic resistance, underscoring the potential risk to hospitalized newborns. Improving disinfection and cleaning methods may aid in the reduction of neonatal healthcare-associated illnesses (Al-Jabri et al., 2022).

Hence, the aim of this investigation was to delineate the opportunistic bacterial strains identified in healthcare-associated infections in Saudi Arabia. In order to recognize and analyze the bacterial strains, we collected bacterial isolates from patients in a variety of healthcare settings, including hospitals and long-term care facilities (Jegede et al., 2022), at an effort to address this problem, our study gathered bacterial isolates from patients who had infections related to healthcare at various healthcare facilities Saudi Arabia. Therefore, we can develop more effective techniques for the prevention, diagnosis, and treatment of these bacteria by identifying strains and their virulence factors and antibiotic resistance profiles.

CHAPTER 3

METHODOLOGY

3.1 Experimental design

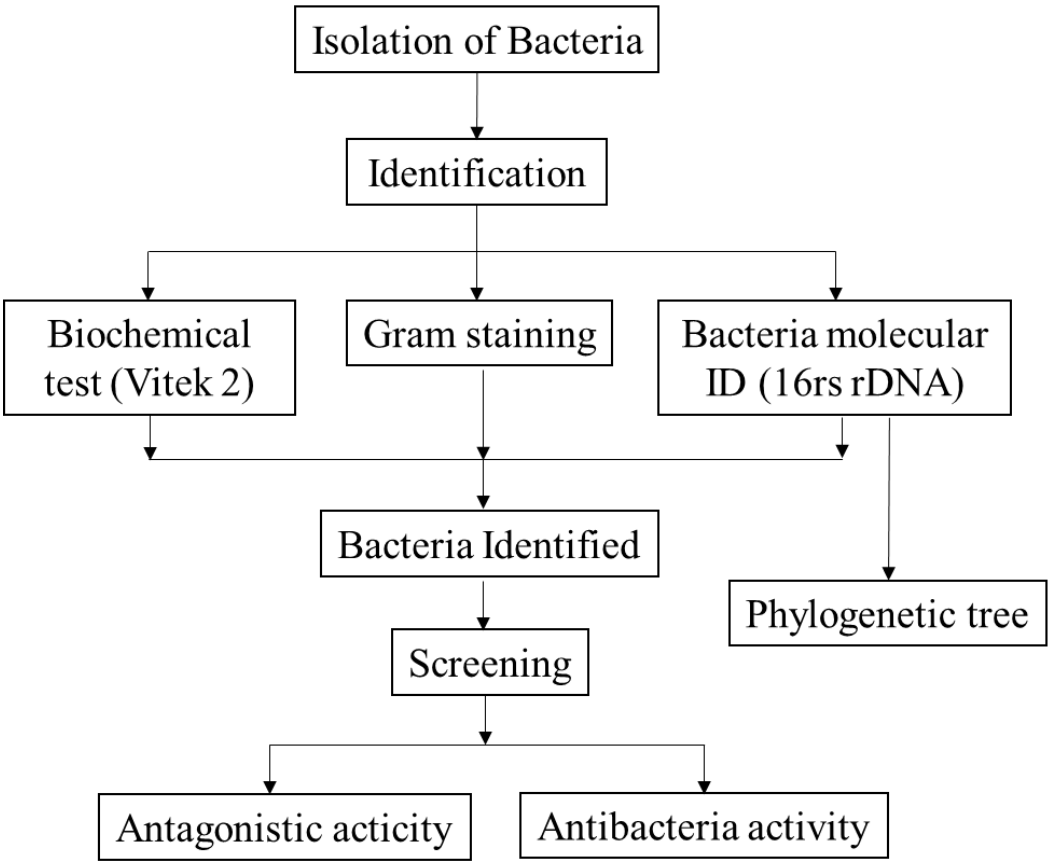

Figure 3.1 Bacterial identification of isolated bacteria

3.2 Sampling sites

The research was carried out in the intensive care unit (ICU) of four public hospitals and two private hospitals in Riyadh, Saudi Arabia. The government hospitals were King Saud Medical City (KSMC), King Khalid University Hospital (KKUH), King Faisal Specialized Hospital and Research Center (KFSH), and King Khalid National Guard Hospital (KKNGH). The private hospitals were Anfas Hospital and Al-Hammadi Hospital. Based on medical performance, data relating to the standard of care, hygiene measures, and patient safety that are taken, these governmental hospitals were considered the biggest and regarded as the top hospitals in Saudi Arabia. The study was conducted from July 2021 to September 2022.

3.3 Sample Size and Study Population

A total of 36 samples were isolated from ICU patients, consisting of 19 males and 17 females. These samples were obtained as part of normal laboratory diagnosis. The study included only patients who were admitted to the ICU for a duration of more than 48 hours throughout the trial period and were at least 18 years old. The patient's personal data and the origin of the sample collection were acquired. Furthermore, the ICU stay involved recording the duration of stay, invasive medical procedures such as intubation, central venous catheterization, arterial catheterization, and urinary catheterization, as well as the length of mechanical ventilation, sedation, use and duration of antibiotics, duration of isolation techniques, and ICU mortality.

3.4 Sample collection and bacteria identification

Only one sample was taken from each participant in the trial, either a sample of urine, sputum, or wound, depending on the sample availability at the time of collection. To prevent contaminating patients with urinary catheters, urine samples were taken with a syringe from fresh catheters and then transferred to sterile specimen tubes and stored in the refrigerator at 4 °C until used (Odoki et al., 2019). On the other hand, wound samples were collected from diabetic patients who had not been treated with topical or systemic antibiotics within two weeks prior through a semi-solid bacterial transport medium (Price et al., 2009). Sputum samples were collected in sterile containers under the supervision of the nurse in charge (Tunney et al., 2008).

3.5 Culture of bacteria

The Mueller Hinton agar (MHA) was poured into all plates and was left to harden before using it. Then the loop was heated until it turned red and let to cool down. A loop from each sample was streaked using the 4 quadrant method, this step was repeated to all plates and samples. After that, the plates were sealed and incubated at 37 ℃. The bacteria grew in 18 hours to 24 hours (Healing, 1993; Alshammari, 2021).

3.6 Gram staining of bacterial culture

The Gram staining technique was employed as a method of differential staining to classify bacteria into two groups, Gram-positive or Gram-negative, based on the chemical and physical characteristics of their cell walls. Bacteria are distinguished via a sequence of staining and decolorization procedures. Gram-positive cells exhibit a purple stain, while Gram-negative cells display a red to pink stain.

A clean microscope slide was prepared and labelled with the sample. A thin smear of bacterial culture was created on the slide and fixed by flame several times. The slide was immersed in crystal violet (CV) solution and allowed to incubate for a duration of 1 minute. Subsequently, it was delicately rinsed with water to eliminate any surplus discoloration. Iodine was applied and left for 1 minute, after which it was rinsed gently with water to eliminate any remaining stain. The smear was decolorized using a small amount of 95% ethanol, and the slide was promptly rinsed with water to halt the decolorization process. The smear was treated with safranin as a counterstain and allowed to incubate for a duration of 2 minutes. Safranin, known for its ability to stain Gram-negative cells pink or red, was then gently washed off with water to eliminate any excess. The slide was permitted to thoroughly dry in the open air. Finally, the stained bacteria were observed using a light microscope and oil immersion to visualize. Gram-positive bacteria showed (purple), thick peptidoglycan, while Gram-negative showed (red/pink), thin peptidoglycan (Beveridge, 2009; Coico, 2006).

3.7 Biochemical tests

The isolates were identified using standard microbiological techniques, including microscopy, culture characteristics, catalase, oxidase, aesculin hydrolysis, lysine decarboxylase, and DNase tests. These tests were performed using the BioMerieux® system, followed by the manufacturer's recommended protocols. The bacterial isolates were identified via commercial biochemical test kits manufactured by BioMerieux®.The test was conducted in accordance with the instructional instructions provided by the manufacturer in each package(API®, 2022).

The VITEK 2 system, produced by BioMerieux®, utilises the ID-GNB card to verify the identity of bacteria. This card is specifically developed for the automated identification of the majority of medically important bacteria that can ferment or not ferment. This card contains a total of 41 fluorescent biochemical tests. These tests include 18 enzymatic testing for aminopeptidases and -osidases, 18 fermentation tests, 2 decarboxylase tests, and 3 miscellaneous tests. A standard

method followed by manufacturer instructions (VITEK® 2: Healthcare | BioMérieux, n.d.).

3.8 DNA Extraction

A clean and sterile workplace was prepared, and 15 mL of falcon tubes were sterilized using Ultraviolet light (UV). The MH Broth was poured into 10 mL of each of the falcon tubes. An optional step was too sterile the 15 mL of falcon tube by UV whereby the ready MH broth was inside again. A pure colony was selected from the plate and transferred to the MH culture broth. The culture was incubated at 37℃ with shaking incubators. After 24 hours it was stored in the fridge before extraction.

One millilitre of the initial culture was put into a centrifuge tube with a capacity of 1.5 millilitres, and the liquid portion above the sediment was separated by spinning it in a centrifuge. After that, it was centrifuged and proceeded. The manual was referred to for Gram-positive or Gram-negative bacteria. Gram-positive require treatment with, Lysozyme, prelysis buffer, and Proteinase RNase treatment, while Gram-negative needs treatment with Proteinase K.

3.8.1 Nanodrop Spectrophotometer

The using nano of concentration and purity of DNA, double-stranded DNA (DeNovix DS-11) was selected for analysis. In the absence of double-stranded DNA, single-stranded DNA was utilized. Next, a volume of 1ul of deionized water, serving as a blank, was introduced into the spectrophotometer, The button labelled as "Blank" was depressed, followed by a wiping action. One microliter of the samples was loaded and organized based on their numbering before being sealed. The measure was initiated by pressing the measure button or by clicking on the autorun option. Until the final sample, the process involved wiping and repeating. The concentration was measured and recorded by determining the ratios of 260/280 nm.

3.8.2 PCR Test for bacterial identification

Master-mix reagents for PCR were formulated in following. The initial step, denoted as Forward (8F), involves dispensing 1 µL of samples, resulting in a total volume of 40 µLwhen multiplied by the number of samples (40). The subsequent step is not specified. The obtained volume of 40 µL was calculated by multiplying 1 µL of the sample by a factor of 40, using the reverse technique (1492R). A volume of 12.5 µL of 2x MyTAQ was added to a sample of 40, resulting in a total volume of 500 µL. Multiplying 8.5 µL of deionized water or double distilled water by 40 results in a total volume of 340 µL. The DNA template utilized in this study consisted of either 1 µL or 2 µLfrom each sample. The total volume of the master mix is 920 µL, which is then divided by 40 to yield 23 µL for each PCR tube. An additional 2 µL of sample is added to each tube. Consequently, the total volume of the mixture, comprising both the master mix and samples, in each PCR tube is 25 µL. All the PCR products and samples were loaded onto the ice. The negative control was generated by combining 2 µl of deionized water with 23 µL of the master mix (Weisburg et al., 1991).

The initial step of the protocol involves subjecting the sample to a temperature of 94 ℃ for 4 minutes, for a single cycle. Subsequently, the denaturation procedure was conducted at a temperature of 95 ℃ for 1 minute, and this was iterated for a total of 35 cycles. Subsequently, the annealing process was conducted within a temperature range of 53-65 ℃ for 30 seconds, and this procedure was repeated for a total of 35 cycles. The extension phase of the PCR protocol involves subjecting the reaction mixture to a temperature range of 53 ℃- 65 ℃, with a recommended temperature of 72 ℃, for 20 seconds. This process is repeated for a range of 30-45 cycles, with a recommended cycle count of 35. The final extension step involves subjecting the sample to a temperature of 72 ℃ for 7 minutes. The PCR process was conducted for approximately two hours.

3.8.3 Gel Electrophoresis

Gel agarose 2%, Agarose powder 0.8 g, and TAE buffer 40 mL were all prepared, using a cylinder flask and measuring cylinder. Then using a microwave, it was heated for 30 seconds. It was poured on the gel tray until hardend for 30 minutes. The sample 4 µL was prepared and the dye 1 µL (5 µL) was loaded. After 30 minutes, the gel tray was set in its place. After that, TAE buffer was poured into the gel electrophoresis. 5 µL of 1 kb Hyperladder was used, and a pipette at the end of the right or end of the left gel well, the sample was loaded into the wells. The wires were all attached to the power source, which was subsequently switched on. The voltage was then adjusted to 90V, and a timer was set for a duration of 50 minutes. The machine subjected the band to UV screening.

3.9 Phylogenetic tree construction

Determination and the distribution of opportunistic bacteria from the isolation process via Phylogenetic tree construction, 16s rDNA analysis gave the identity of the bacteria with homology similarity of at least 95%. After the confirmation of the bacteria ID, then the phylogenetic tree was constructed accordingly. The tree's construction used 1000 bootstrap by Mega 11 software (Georgiev, 2017).

Clustal-W computed the optimal alignment for the chosen sequences and arranged them in a way that displays their similarities, differences, and identities. The Clustal W algorithm operates in three distinct stages: pairwise alignment (PA), guide tree construction (GT), and multiple sequence alignments (MA). The phases of ClustalW are rather autonomous. Every stage generated intermediate data, which serves as input for the subsequent stage. The execution time was highly contingent on the quantity and magnitude of the sequences (Georgiev, 2017).

3.10 Antagonistic activity and anti-bacterial test

3.10.1 Antagonistic Activity

Five stock cultures of bacteria was obtained from the Institute climate adaption and Marine Biotechnology, University Malaysia Terengganu (UMT), including *E. coli, P. aeruginosa* (P.A.), *B.cereus*(B.C.), *Streptococcus uberis* (S.U.) and *Vibrio parahaemolyticus* (V.A.). Six strains of bacteria collected from health care in Saudi Arabia, including *K. pneumonia, P. aeruginosa, C.koseri, E.coli,P. mirabilis, and Serratia marcescens* were collected from ICU hospitals in Saudi Arabia.

All species were grown in shake tubes for 24 hours at 37 °C, and 150 rpm with 10 mL of MH-Broth (Muller Hinton) broth. After that all species were transferred to MH agar plates for another 24 hours of incubation at 37 °C and stored in the fridge. On MH agar, indicator bacteria were preserved at 4 °C. Then 1 mL of the culture was taken from each tube and spun at 14,000 g for 20 minutes to get the cells to stick together.

The culture broth (CB) was taken for the agar well diffusion test to see if the bacteria were fighting each other (Sci, 2008). However, some changes were made to Kivanc's instructions for making washed cells. Indicator bacteria isolated from IMB were grown for 24 hours and were sp. at 14,000 g for 15 minutes, and the supernatants were taken out supernatant for their used (Kivanç, 1990).

3.10.1.1 Cross streak method

The cross-streak method is a technique employed for the expeditious screening of microorganisms in relation to their antagonistic properties. The targeted isolated microbial strain from ICU of each hospital is inoculated through a solitary streak at the midpoint of the agar plate. After a variable period of time (overnight

incubation), which depends on the specific strain of microorganisms, the isolated bacteria from ICU were streaked from perpendicular to the middle streak. The antimicrobial interactions are assessed by measuring the size of the zone of inhibition after a prolonged incubation period of 24 hours (Balouiri et al., 2016).

3.10.2 Anti-bacterial test

All isolated bacteria were used for the anti-bacterial test. Fresh bacterial isolates were cultured overnight in Muller Hilton Agar and then incubated at an appropriate growth temperature overnight (37 °C). The procedure outlined by Yasmin et al., 2017b will be utilised to generate cell-free bacterial suspensions. The strains and test organisms were inoculated in MH broth and incubated in a shaking incubator at 37 °C until they reach their logarithmic growth phase. Upon reaching sufficient development for each strain, the cells were detached from the media following centrifugation at a speed of 9000 rpm for a duration of 10 minutes. The liquid portion was discarded, and the solid portion was mixed with sterilised distilled water for future use in disc diffusion procedures. The evaluation of antimicrobial activity relied on the measurement of zones of inhibition on the agar surface surrounding the well (Yasmin et al., 2017).

3.10.2.1 Disc diffusion assay

A 6 mm sterile disc were soaked with methanolic crudes extract collected from Terengganu (*Salix babylonica*, *Padina* sp., and *Ulva*sp)., which was established earlier at the Institute Of Climate Adaption and Marine Biotechnology, UMT. The drying and grinding of fresh leaves into a powder is part of the biota preparation process. The samples were divided into smaller pieces and processed using a grinder. The liquid samples were turned into powder by freeze -dried. The samples undergo trituration in methanol three times to get the raw extract, which was subsequently filtered using Whatman filter paper and concentrated using a vacuum rotary evaporator. A total of 0.5 g/mL of the extracts was used for this purpose. Incubation

of the plate was overnight at 37^{o}C. Anti-bacterial activity was determined against bacterial pathogens by the disc diffusion assay (Masood et al., 2021).

3.11 Statistical analysis:

The data analysis was conducted using the Statistical Package for Social Sciences software, specifically version 25.0 developed by IBM SPSS Inc. in Chicago, IL. At first, all data collected from patients' records was encoded into variables. The results were analysed using descriptive and inferential statistics, namely the Chi-square test. A p-value below 0.05 was considered statistically significant for every test. The phylogenetic tree was constructed using internet software named Mega version 11 cluster-W (Habibie et al., 2019).

3.12 Ethical consideration

Permission for carrying out the study was obtained in advance from all hospitals included in the study. Intensive care unit patients or their legal guardians were contacted in person and told about the purpose of the study and that their results will be kept anonymous. Also, verbal consent was obtained from all participant's guardians. Participants had the freedom to withdraw from the study at any time of their choosing and were assured that the obtained data would solely be used for the research aims and not for any other reason.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Samples Data analysis

The data were gathered from 36 adult patients with a mean age of 50.53 years (SD ±11.224 years) who were hospitalized in the intensive care unit. Male respondents accounted for 52.8% of the study's total patients, whereas female patients comprised up 47.2%.

The patients were categorised into two groups according to the duration of their hospital stay: less than 7 days and more than 7 days. 41.7% of those evaluated were hospitalized for more than 7 days, while 58.3% were admitted for less time. Moreover, among the total samples analyzed in the microbiology laboratory for culture, urine 52.8% followed by wound 30.6% and sputum 16.7%.

Five separated Saudi Arabian healthcare facilities provided the samples isolated for the investigation. Many samples were acquired from the KKNGH, accounting for 22.2% of all samples obtained, followed by Anfas hospital, Hammadi hospital, and KFSH accounting 19.4%. KKUH and KSMC provided the remaining 11.1% and 8.3%, respectively, (Table 4.1).

Table 4.1 Patient Characteristics and ICU Admission Data

Participants Age					
Age	N	Minimum	Maximum	Mean	Std. Deviation
36		30	66	50.53	11.224
Valid N (listwise)	36				
Participants Gender					
		Gender	Frequency	Percent	Valid percent
Valid		F	17	47.2	46.2
		M	19	52.8	52.8
		Total	36	100.0	100.0
Admission Time					
		Admission Time	Frequency	Percent	Valid percent
Valid		> 7 days	15	41.7	41.7
		< 7 days	21	58.3	58.3
		Total	36	100.0	100.0
Hospital					
		Hospital	Frequency	Percent	Valid percent
Valid		Anfas	7	19.4	19.4
		Hammadi	7	19.4	19.4
		KFSH	7	19.4	19.4
		KKNGH	8	22.2	22.2
		KKUH	4	11.1	11.1
		KSMC	3	8.3	8.3
		Total	36	100.0	100.0
Sample collected					
		Sample type	Frequency	Percent	Valid Percent
Valid		Sputum	6	16.7	16.7
		Urine	19	52.8	52.8
		Wound	11	30.6	30.6
		Total	36	100.0	100.0

4.2 Identification of isolated bacteria

The study analysed a total of 36 bacteria samples obtained from 36 different patients hospitalised in the ICU. These samples were analysed using light microscopy to identify the bacteria based on the structural features of their cell walls. It was based on Gram- staining that differentially classified all isolated bacteria (100% of sample collected) in the study as Gram negative bacteria, that showed pink-red color under microscope. 81% of stained bacteria occurred in pairs or in group of four, which indicate Gram- negative rod shaped bacillus. While 19% showed slightly curved rod-shaped bacteria that occurred singly were gram negative rods.

This finding aligns with a prior study that performed a comprehensive examination and statistical analysis to explore the worldwide occurrence of (HAIs)(Raoofi et al., 2023). The prevalence of HAIs worldwide was 0.14 %. The annual rate of HAIs is rising by 0.06 % (Raoofi et al., 2023). Furthermore, environmental factors such as poor sanitation and hygiene practices, exposure to contaminated water or food, and close contact with infected individuals can also increase the risk. Individuals with these risk factors must take appropriate precautions to prevent opportunistic bacterial infections (Alshammari, 2021). In addition, according to a recent study conducted in 2023 in Saudi Arabia, the adoption of insufficient preventive measures may be to blame for the exceptionally high rate of Gram-negative bacterial infections in ICU patients (Saleem et al.,2023). This was clarified by the prior study that found that the high prevalence of nosocomial infections is caused by unsanitary conditions in hospitals, staff incompetence, and non-compliance with antibiotic stewardship guidelines (Saleem et al.,2023)

According to a recent study conducted at King Abdulaziz Medical City in Jeddah, Saudi Arabia, which involved a three-phase pre-and post-intervention analysis. During a period of 26 months, the researchers collected 1090 MDR-GNB isolates from various locations throughout the hospital. The adult ICU had a total of 456 isolates, accounting for 42% of the cases. The incidence rate in the adult ICU was significantly higher at 22.8 isolates per 1000 patient-days compared to the combined regions of the remainder of the hospital, which had an incidence rate of 2.3

isolates per 1000 patient-days. During the duration of the investigation, the prevalence of MDR-GNBwas higher in adult ICUs (Althaqafi et al., 2023). Additionally, based on the results of this analysis, Gram-negative bacilli isolates were identified as the predominant pathogens in six years of epidemiological surveillance in Saudi Arabia for ventilator-associated pneumonia in a tertiary-care ICU. Surveillance programmes conducted in Europe and the United States have also identified a significant presence of Gram-negative bacilli in ICUs (Ibrahim, 2018).

4.3 VITEK 2 Analyzer Results

All samples were evaluated by modified method, direct identification and susceptibility testing using Vitek 2. Identification of bacteria by VITEK analyzer showed that, the most common bacteria isolated were *K pneumonia* representing (30.6%), followed by *P aeruginosa* (25%), *Proteus mirabilis* (19.4%), *E.coli* (13.9%) ,*Serratia marcescens* (8.3%) and *Citrobacter koseri* (2.8%). Figure 4.1 shows the identified organisms.

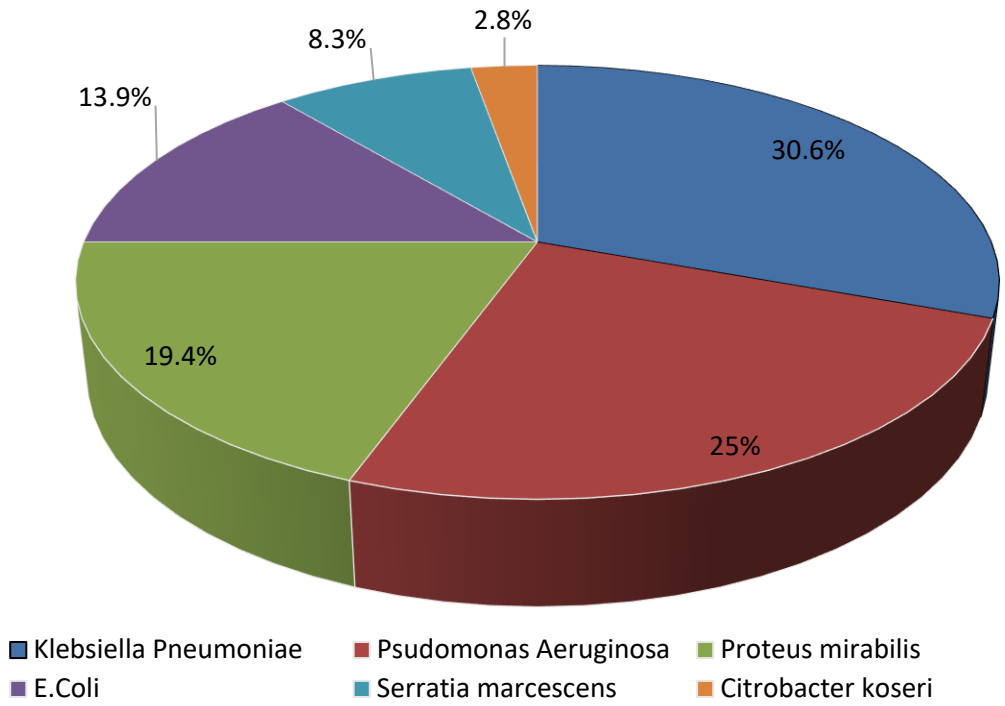

Figure 4.1Types of Bacteria isolated from different ICU hospitals in Saudi Arabia

Isolated bacteria were distributed across hospitals based on where they had been detected. The results of Vitek 2 showed that *K.pneumonia* had the highest prevalence of all the species found, however, it was mostly isolated (27.3%) from both hospitals; KKGH and KKUH. The second most prevalent organism, *P aeruginosa*, was isolated from each of ANFAS, Hammadi, and KSMC in equal amounts (22.2%). Most of the organisms (42%) in *Proteus*, occurred from the ANFAS hospital. Came next from KKNGH, (40%) *E. coli* was isolated. *(33.3%)* of *Serratia marcescens* were isolated from Hammadi, KFSH, and KKUH. *Citrobacter koseri*, for which just one sample was taken (2.8%), was the least isolated organism (Table 4.2).

Table 4.2 Distribution of isolated bacteria across different hospitals

BACTERIA		HOSPITAL					
		ANFAS	HAMMADI	KFSH	KKNGH	KKUH	KSMC
A.koseri r	Count	0	0	1	0	0	0
	% within Bacteria Isolated	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%
	% within Hospital	0.0%	0.0%	14.3%	0.0%	0.0%	0.0%
	% of Total	0.0%	0.0%	2.8%	0.0%	0.0%	0.0%
E.coli	Count	1	1	1	2	0	0
	% within Bacteria Isolated	20.0%	20.0%	20.0%	40.0%	0.0%	0.0%
	% within Hospital	14.3%	14.3%	14.3%	25.0%	0.0%	0.0%
	% of Total	2.8%	2.8%	2.8%	5.6%	0.0%	0.0%
K. pneumoniae	Count	1	2	2	3	3	0
	% within Bacteria Isolated	9.1%	18.2%	18.2%	27.3%	27.3%	0.0%
	% within Hospital	14.3%	28.6%	28.6%	37.5%	75.0%	0.0%

	% of Total	2.8%	5.6%	5.6%	8.3%	8.3%	0.0%
Proteus mirabilis	Count	3	1	0	2	0	1
	% within Bacteria Isolated	42.9%	14.3%	0.0%	28.6%	0.0%	14.3%
	% within Hospital	42.9%	14.3%	0.0%	25.0%	0.0%	33.3%
	% of Total	8.3%	2.8%	0.0%	5.6%	0.0%	2.8%
P. aeruginosa	Count	2	2	2	1	0	2
	% within Bacteria Isolated	22.2%	22.2%	22.2%	11.1%	0.0%	22.2%
	% within Hospital	28.6%	28.6%	28.6%	12.5%	0.0%	66.7%
	% of Total	5.6%	5.6%	5.6%	2.8%	0.0%	5.6%
Serratia marcescens	Count	0	1	1	0	1	0
	% within Bacteria Isolated	0.0%	33.3%	33.3%	0.0%	33.3%	0.0%
	% within Hospital	0.0%	14.3%	14.3%	0.0%	25.0%	0.0%
	% of Total	0.0%	2.8%	2.8%	0.0%	2.8%	0.0%

Total	Count	7	7	7	8	4	3
	% within Bacteria Isolated	19.4%	19.4%	19.4%	22.2%	11.1%	8.3%
	% within Hospital	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
	% of Total	19.4%	19.4%	19.4%	22.2%	11.1%	8.3%

Table 4.3 summarizes the distribution of Gram-negative bacteria isolated (n=36) recovered from various clinical specimens of ICU patients. *K. pneumoniae*is discovered as one of the prevalent microbes obtained from wounds representing 45.5%, while, *P. aeruginosa* were most pathogens identified from Sputum representing 50% and *E. coli, K pneumonia*, P*roteus mirabilis* and *P. aeruginosa* were the predominant isolates from urine samples all showing 21.1%.

The study's patients were divided into two groups based on the duration of their stay, either exceeding 7 days or less than 7 days. The data in Table3.4, shows the distribution of all Gram-negative organisms detected in the study in relation to the time of admission. However more organisms (58.3%) were isolated from patients who had been admitted for more than seven days and the most predominant organism was *K. pneumonia* (Table 4.4). Conversely, 41.7% of the overall bacteria detected in the study were from patients who spent less than 7 days in the ICU and most common organism found was *P. mirabilis* and *K. pneumonia*.

Table 4.3 The distribution of GNB (n=36) recovered from various clinical specimens of ICU patients:

BACTERIA ISOLATED		SAMPLE ORIGIN		
		SPUTUM	**URINE**	**WOUND**
A.koseri	Count	0	1	0
	% within Sample Origin	0.0%	5.3%	0.0%
E.coli	Count	1	4	0
	% within Sample Origin	16.7%	21.1%	0.0%
K. pneumonia	Count	2	4	5
	% within Sample Origin	33.3%	21.1%	45.5%
P. mirabilis	Count	0	4	3
	% within Sample Origin	0.0%	21.1%	27.3%
P. aeruginosa	Count	3	4	2
	% within Sample Origin	50.0%	21.1%	18.2%
S. marcescens	Count	0	2	1
	% within Sample Origin	0.0%	10.5%	9.1%
Total	Count	6	19	11
	% within Sample Origin	100.0%	100.0%	100.0%

Table 4.4 Types of bacteria isolated in relation to duration of admission

BACTERIAL ISOLATED		DURATION		TOTAL
		< 7 DAYS	> 7 DAYS	
A.koseri	Count	0	1	1
	% within Duration	0.0%	4.8%	2.8%
E. coli	Count	2	3	5
	% within Duration	13.3%	14.3%	13.9%
K. pneumonia	Count	4	7	11
	% within Duration	26.7%	33.3%	30.6%
P. mirabilis	Count	4	3	7
	% within Duration	26.7%	14.3%	19.4%
P. aeruginosa	Count	3	6	9
	% within Duration	20.0%	28.6%	25.0%
S. marcescens	Count	2	1	3
	% within Duration	13.3%	4.8%	8.3%
Total	Count	15	21	36
	% within Duration	41.7%	58.3%	100.0%

Al-Qasem., et al (2023), have provided description to the same pattern identified in this study. It was included in a retrospective cohort research with data from 2015 to 2019 that was carried out at Asir Central Hospital, Saudi Arabia. 69 patients out of 300 ICU patient samples were included. The study's findings showed that *K. pneumoniae* (27%), *P. aeruginosa* (14.7%), were most frequently isolated from the recovered samples.

Another study was done to find out how common healthcare-related infections were among patients hospitalized to the ICU. According to the study, most

of the isolates, were gram-negative pathogens (71.05%), with *Acinetobacter* sp. (29%) leading in this regard, followed by *Pseudomonas* sp. (15.8%) and *Klebsiella* sp. (13.2%) (Araújo et al., 2018).

Numerous bacteria, including *A.baumannii, P. aeruginosa*, *K. pneumoniae*, and *Enterobacter* sp., have been discovered and due to their strong resistance to numerous antibiotics, these bacteria were challenging to treat (Feretzakis, el al., 2019). During the patient's hospitalisation, there is a high likelihood of nosocomial infections spreading beyond the confines of the hospital. Nosocomial surgical site infections (SSIs), nosocomial urinary tract infections (UTIs), nosocomial bloodstream infections (NBSIs), and nosocomial pneumonia infections (NPNEUs) comprise 80% of all nosocomial infections (NIS) (Nimer, 2022). The patient immune condition is directly connected with the severity and frequency of infection. The most impact categories are newborns ICU patients, organ transplant patients, burn unit patients, and ICU patients. An escalation in infections is accompanied with a rise in the quantity of continuous hospitalisations, enduring disability, resistance to antibiotics, disturbance to socioeconomic conditions, and elevated mortality rates (Avershina et al.,2021).

4.3.1 Antibiotic testing

According to this study, six antibiotics—Cefuroxime, Amoxicillin, Gentamicin, Norfloxacin, Nitrofurantoin, and Trimethoprim—were examined for resistance using the Vitex 2 analyzer. These antibiotics exhibited a broad spectrum of resistance. Amoxicillin, Cefuroxime and Trimethoprim were the most effective antibiotics against all tested Gram-negative bacteria. In which, all six types of bacterial isolates (100%) from all those who participated showed resistance to Amoxicillin, while 77.7% of samples showed resistance to Cefuroxime. The next antibiotic tested was Trimethoprim/Sulfamethoxazole, to which 55.5% of samples tested positive for resistance. This is followed by Nitrofurantion representing 50% resistance to bacteria. Furthermore, Gentamicin and Norfloxacin, which both showed

the same outcome for resistance against 33.3% of total samples examined, were recognized to have the least resistance, Table 3.5

P. aeruginosa exhibited high resistance rates to various groups of antimicrobial drugs experimented in the study, such as Amoxicillin, Cefuroxime, Gentamicin, Trimethoprim/Sulfamethoxazole and Nitrofurantion. Wherase, *K. pneumoniae* which showed resistance against Amoxicillin, Cefuroxime and *Nitrofurantion*. *S. marcescens* and *A. koseri* demonstrated resistance to the same antibiotics, which are Amoxicillin, Cefuroxime, and Nitrofurantion, However *P. mirabilis* and *E coli* showed resistance to all antibiotics except Gentamicin and Norfloxcin respectively, Table 3.6.

Table 4.5 Bacteria resistance to antibiotic

ANTIBIOTICS		**TOTAL**
Amoxicillin	Count	36
	% within Antibiotics	100.0%
Cefuroxime	Count	28
	% within Antibiotics	77.7%
Gentamicin	Count	12
	% within Antibiotics	33.3.0%
Nitrofurantion	Count	18
	% within Antibiotics	50%
Norfloxacin	Count	12
	% within Antibiotics	33.3%
Trimethoprim / Sulfamethoxazole	Count	20
	% within Antibiotics	55.5%
Total	Count	36
	% within Antibiotics	100.0%
	% of Total	100.0%

Table 4.6 resistance of bacteria to different antibiotics

	Antibiotics	*Citrobacter*	*E.coli*	*K. pneumoniae*	*P. aeruginosa*	*Proteus*	*Serratia*
Amoxicillin	Count	1	5	11	9	7	3
	% within Antibiotics	2.8%	13.9%	30.6%	25.0%	19.4%	8.3%
Cefuroxime	Count	1	4	9	7	5	2
	% within Antibiotics	3.6%	14.3%	32.1%	25.0%	17.9%	7.1%
Gentamicin	Count	0	4	0	8	0	0
	% within Antibiotics	0.0%	33.3%	0.0%	66.7%	0.0%	0.0%
Nitrofurantion	Count	1	3	5	0	6	3
	% within Antibiotics	5.6%	16.7%	27.8%	0.0%	33.3%	16.7%
Norfloxacin	Count	0	0	0	6	6	0
	% within Antibiotics	0.0%	0.0%	0.0%	50.0%	50.0%	0.0%
Trimethoprim / Sulfamethoxazole	Count	0	4	0	9	7	0
	% within Antibiotics	0.0%	20.0%	0.0%	45.0%	35.0%	0.0%

A recent study demonstrated that MDR organisms' infection outbreaks in hospitals are increasingly prevalent in intensive care units (ICUs). Despite recent advances in critical care medicine, the rate of acquired infections in ICU wards remains much greater than in non-ICU hospitals (G.Y. Gu*et al*., 2023).In agreement with the study results, a prior study indicated that multidrug resistance in microorganisms is an increasing major problem in the health care sector.However, in line with a separate prior study carried out in Ethiopia, which identified a rise in multidrug-resistant (MDR) and extended-spectrum beta-lactamase (ESBL) strains among the isolated fermentative Gram-negative bacilli at the research location. Based on the information provided, there is a significant level of resistance observed against ampicillin, piperacillin, sulfamethoxazole-trimethoprim, and tetracycline. Therefore, it is not advisable to use these antibiotics for empirical treatment. Furthermore, our analysis specifically targeted Ethiopian healthcare and identified the presence of *Carbapenems-resistant K. pneumonia* (Beyene et al., 2019). Moreover, a prior investigation discovered a rise in infections caused by MR-GNB across all healthcare settings, with a notable increase in the ICU. The significant morbidity and mortality rates associated with MR-GNB require prompt and coordinated intervention from all medical providers to prevent infections. Previous research has demonstrated that antibiotic optimisation programmes (AOP) are a successful strategy for reducing rates of multidrug-resistant bacteria (MRB) in many healthcare environments, including community, hospital, and notably ICU. Based on the study, a meta-analysis of 24 studies (including only three randomised clinical trials), it was shown that there was a reduction in the usage of antimicrobials, resulting in reduced costs and shorter duration of use (Montero et al, 2021). A study was undertaken at the King Faisal Hospital in Makkah, Saudi Arabia to investigate the occurrence of ESBLs and carbapenem-resistant gram-negative bacteria (GNB) among 298 patients in the intensive care unit (ICU). The study found that 20.6% of the samples included ESBL-producing GNB, whereas 79.4% of the samples had GNB that were resistant to carbapenem. All ESBL-producing GNB and carbapenem-resistant GNB were shown to be bacteria that are resistant to many drugs (Kabrah, 2022).

4.4 Molecular results analysis

Microbial culture is the major component of the conventional pathogenic bacteriological detection approach. Culture-based procedures are widely used in laboratories because to their cost-effectiveness, ease of application, and high level of standardisation. The primary limitations of these techniques, however, consist in their inability to differentiate between the target and other non-target indigenous microorganisms within the same samples, the occurrence of false negative or positive outcomes, laborious procedures, and their inability to identify viable but non-culturable cells (Zhang et al.,2021).

As a result, recently created quantitative molecular techniques and technologies may be better suited for detecting various diseases. The advantages include the capacity to simultaneously identify multiple species of harmful organisms, great sensitivity, and effective species specificity. With the help of these method, numerous bacteria can be quickly detected in a single response (Zhang et al., 2021).

The findings of the Nanodrop instrument analysis following DNA extraction revealed that all separated samples exhibited a ratio of corrected absorbance at 260 nm to corrected absorbance at 280 nm. A DNA sample is considered pure when it has an A260/A280 purity ratio ranging from 1.8 to 2.0 (Figure 4.2).

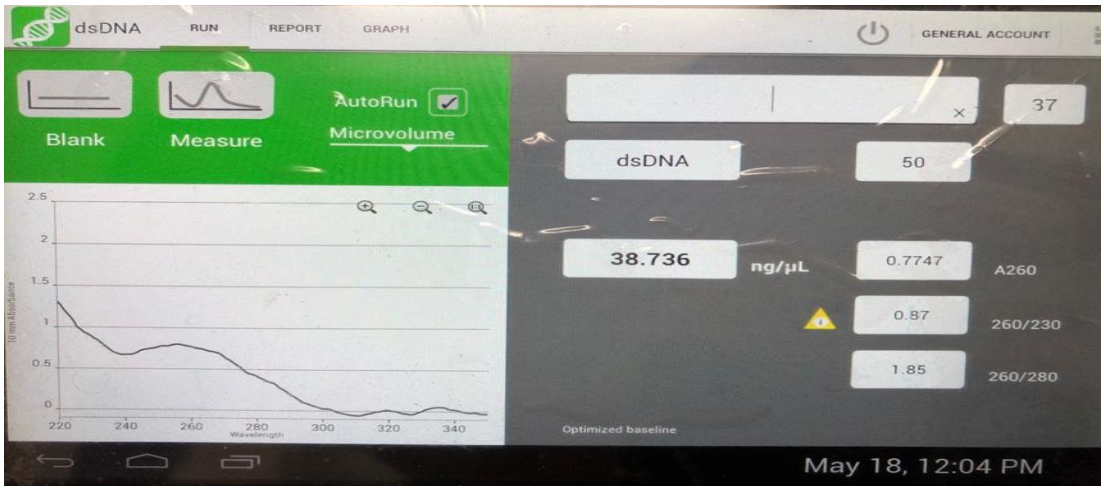

Figure 4.2 Nanodrop results after DNA extraction, optimal results for Intact DNA with OD reading ratio 260/280 (1.8 – 2.0).

Only six of the total number of samples collected were subjected to molecular typing. The PCR result was utilised for the sequencing reaction in order to characterise the strains at the strain level and to eliminate the possibility of numerous isolates from the same strain. The forward primer 8F and reverse sequencing primer 1492R were employed to sequence the complete extent of the double stranded DNA, as depicted in Figure 4.4. To validate the PCR products. The resulting DNA fragment from the aforementioned procedure was placed onto a gel made of agarose with a concentration of 1.5%. The ladder utilised had a size of 1 kilobase (kb) and was specifically the Invitrogen ladder. The anticipated nucleotide pairing of the amplicon was around 1500 base pairs. The sequencing reaction was also purified. Figure 4.3displays an image of the gel obtained with a digital camera in a dark room with the camera flash turned off, as well as labels for each lane created with the PowerPoint ™ program.

The purified product was placed into the gene analyzer manufactured by Applied Bio-systems to obtain the sequences. The results obtained from the NCBI BLAST gene bank analysis of the 16S rRNA gene sequences derived from bacterial isolates indicate a high degree of similarity, exceeding 99.8% with other strains belonging to the same species.

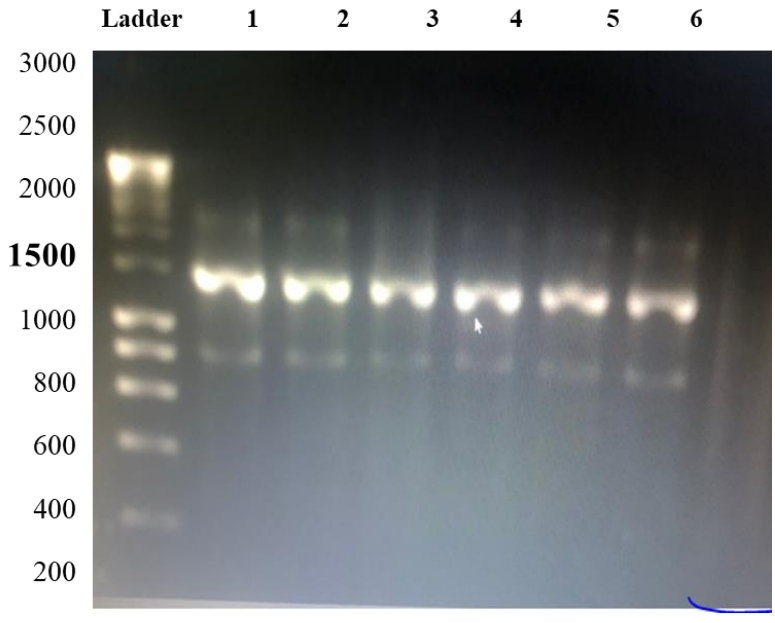

Figure 4.3Agarose gel electrophoresis (2%) for PCR product 16 S r RNA (1500bp). at 90 V for 50 minutes (Samples 1-6: for bacterial isolates)

4.5 Phylogenetic tree

For much biological research, understanding the evolutionary relationships among species is essential. An accurate phylogenetic tree is crucial for inferring the genesis of novel genes, detecting molecular adaptation, understanding the development of morphological traits, and reconstructing demographic changes in recently diverged species. Furthermore, it provides the fundamental basis for comprehending significant shifts in evolution, such as the development of novel anatomical structures or metabolic processes (Kapli et al.,2020).

A phylogenetic tree was created in this work using neighbor-joining analysis of 16S rRNA. The six isolates that were studied were grouped into three distinct clusters., Figure (4.4). Each bacteria forms a cluster with individuals of the same species but originating from different strains. Samples 5 and 10, which belonged to the *Citrobacter Koseri*strain, and the *Escherichia* sp. Strain formed a cluster, and showed the highest similarity among isolated samples. This was followed by sample (13,35,12) which formed a cluster as well, but showed less similarity. On the other hand, sample 9 that belongs to *P. aeruginosa* did not form a cluster with other bacterial strains, Figure 4.4.

Given the genetic diversity among living organisms, the challenge of reconstructing and visualising evolutionary relationships among them is inherently challenging. To differentiate between groups, early phylogenetic visualisations employed a solitary gene, such as the rRNA gene, which is adequately conserved to be found in all organisms (Carolina et al.,2021).

A previous study outlined a systematic approach for constructing a phylogenetic tree by concurrently grouping the whole proteomes of 360 bacterial species. 49 protein sequences, found in 99% of the organisms inside the homologous clusters, were used to form a tree. There were 47 sequences out of 49 that are homologous in both archaea and eukarya. Additionally, a network made from the clusters was utilized to identify bacteria having horizontally transferred genes from other phyla (Khaledian et al., 2020).

In another previous study, Sanger sequence procedure was performed for 16S ribosomal RNA and Molecular Evolutionary Genetics Analysis (MEGA-7) software for phylogenetic tree was build. The previous study indicated that *Escherichia coli, K pneumonia*, and *Staphylococcus* sp. accounted for 58%, 28%, and 14.0% respectively. The phylogenetic tree revealed that sample No. Ai (05) is highly similar to *E. coli* strain NBRC 102203 (NR 114042.1), with a 99% genetic match. Aii (23) exhibits a 99% resemblance to *K. pneumoniae* strain DSM 30104 (NR 117686.1), while Bi (48) is strongly associated with *S. aureus* strain NBRC 100910 (NR 113956.1) with a similarity of 90%. Phylogenetic research establishes that sequences are interconnected and highly valuable as they unveil the resemblances and disparities among isolates (Ullah et al.,2022).

In a previous study, Nakano et al. (2023) compared the genome-wide K-mer profiles of various bacterial species, including *E. coli*, *Shigella* sp., *Yersinia* sp., *Klebsiella* sp., and *Neisseria* sp., as well as different serotypes of *Listeria monocytogenes*. They used this data to construct phylogenetic trees. In order to distinguish between highly similar species, such as *Yersinia* species, *Shigella* sp., and *E. coli*, a pentanucleotide frequency analysis was conducted. This technique involved examining 512 patterns of 5 nucleotides each. *E. albertii* strains can be readily distinguished from both *E. coli* and *Shigella sp*., while they do exhibit a phylogenetic connection with enterohemorrhagic *E. coli*. Furthermore, the physical similarities mentioned earlier were supported by the phylogenetic tree of Ipomoea species, which was constructed using pentamer frequency data from chloroplast genomes (Nakano et al., 2023).

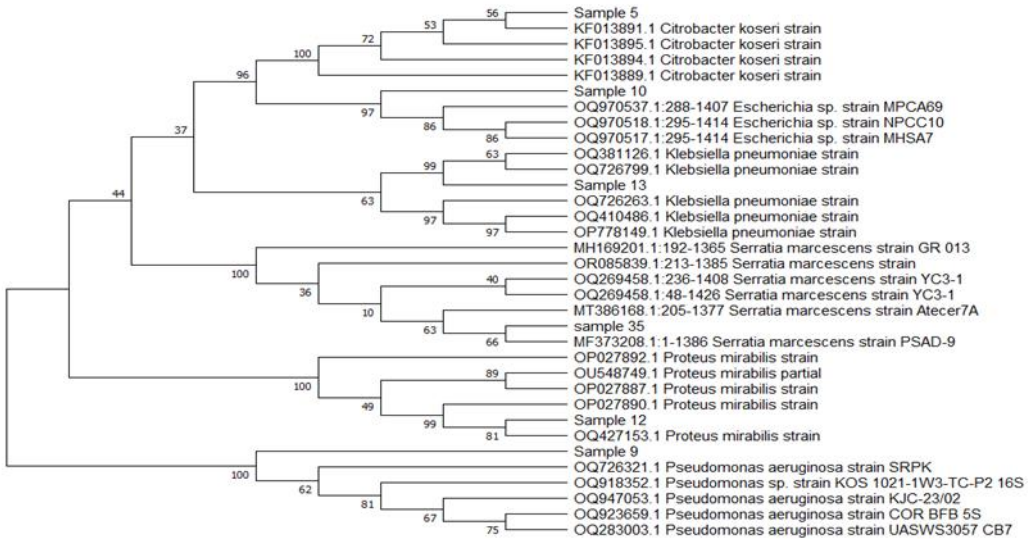

Figure 4.4 Phylogenetic tree construction based on neighbour-joining analysis of 16S rRNA

4.6 Cross streak method for antagonistic activity of bacteria

Cross-streak technique was employed for the expeditious screening of microorganisms in relation to their antagonistic properties. In measuring the antagonistic activity of the six isolated bacteria (*K pneumonia, P aeruginosa, P. Mirabilis, E.coli,, S. marcescens and C. Koseri*) from ICU patients against the five-stock culture of bacteria obtained from the Institute of marine biotechnology – UMT*E. coli*, PA (*P. aeruginosa*), B.C (*B.cereus*), S.U (*S. uberis*) and V.P (*Vibrio paraheamolytics*), it was found that all of them did not produce an inhibitory effect against the tested pathogens, and there was no inhibition zones produced (Figure 4.5).

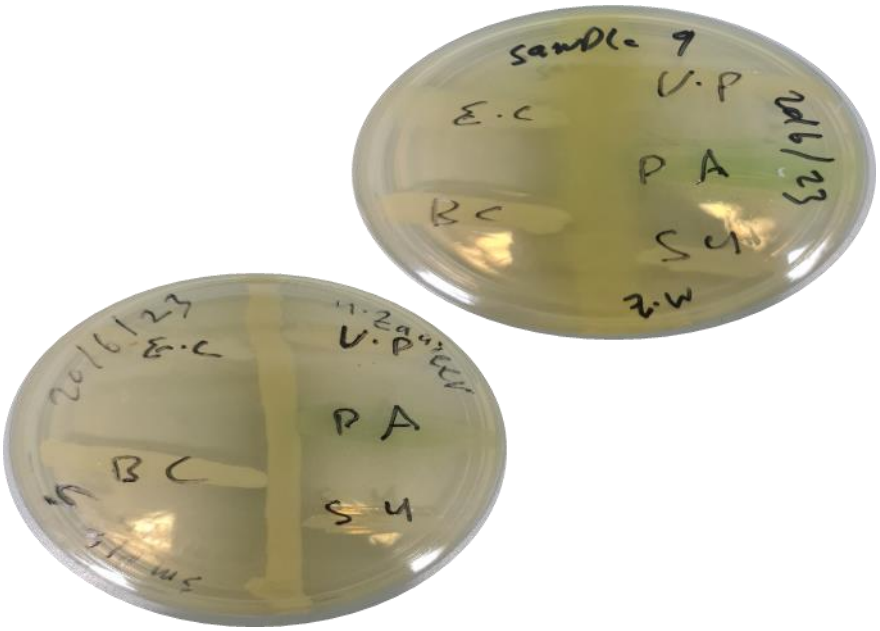

Figure 4.5. Cross streak method measuring the antagonistic activity of the six isolated bacteria from ICU patients against the five-stock culture of bacteria obtained from the Institute of marine biotechnology – UMT.

The results of this study contrast with those of a previous project that sought to collect *E. coli* antagonistic bacteria from leafy green vegetables and investigate their potential as biocontrol agents for altering the microbiota of plants in the field **(**Uhlig et al.,2021**)**. Upon identification of the 16S rRNA gene, the project discovered that most of the isolates with antagonistic properties belonged to *the Micrococcaceae, Bacillaceae, and Pseudomonadaceae* groupings. *Pseudomonas spp.* is recognised for their antagonistic characteristics against human infections, including *E. coli* O157:H7 **(**Uhlig et al.,2021**)**.

Furthermore, the literature examining interactions between Bacillus and *Pseudomonas* has primarily concentrated on how chemicals generated by one strain impact the other. It made emphasis on the fact that a number of *Pseudomonas* species are widely recognized for possessing a type VI secretion system (T6SS) that functions as a toxin and is triggered during cell-to-cell contact with competing species. *B. subtilis* and *P.chlororaphis* are co-cultured, and *P. chlororaphis* utilises a

T6SS to compete for space. This causes *B. subtilis* cells to sporulate as a defense mechanism (Lyng and Kovács, 2023).

Nonetheless, biological control utilizing antagonist microbes against pathogens is an alternative management technique to reduce damaging plants, as it was previously discussed. Antagonizing germs that are particularly effective include bacteria and fungi. Pathogen-inhibiting or parasitizing bacteria produce antibiotics or compete for nutrition (Trisawa et al., 2022).

4.7 Antibacterial activity of isolated bacteria

In this study, the methanolic extracts from ten Natural Crude were tested in vitro for antibacterial activity. Ten extracts tested were *Salix babylonica, M.cajuputi , Caulerpa taxfolia , A.planci, Ulva* sp, *Stylissacarteri , S. lanceolata, Avicennia alba, Sargasumabbottie, Padina* sp. The extracts were evaluated for their antibacterial activities using the disc diffusion method. Nevertheless, only four extracts (*M. cajuputi, A. planci, Stylissacarteri,* and *S. lanceolata*) exhibited various levels of inhibition against the six isolated bacterial strains when tested using the disc diffusion method. Microorganisms tested were found to be susceptive to the methanol extracts with diameter of inhibition zones that ranges between 7 to 12 mm and its growth was partially inhibited by the extracts. The lowest inhibition zones were found for among *Acanthaster planci* and *Melaleuca cajuputi* whereas the highest were from *Stylissacarteri* and *Sonneratia lanceolata*, (Table 4.7).

Methanolic extracts of *Acanthaster planci* and *Stylissacarteri* shown antibacterial activity against *Klebsiella pneumoniae* with inhibitory zones of 7 mm and 10 mm, respectively. *Melaleuca* and *Sonneratia lanceolata*, on the other hand, both showed inhibitory zones of 8 and 9 mm on *Pseudomonas aeruginosa* growth, respectively. Likewise, the same two extracts also inhibited *Proteus mirabilis*, with inhibition zones of 8 and 12 mm, respectively. *Stylissacarteri* displayed inhibition zone toward both *Ctrobacter koseri* (12 mm) and *E coli* (8 mm). While *Acanthaster planci* showed inhibition zone to *Serratia marcescens* (8 mm).

Touzout et al., (2023) stated that higher morbidity, death, and treatment costs are associated with a number of infectious diseases brought on by resistant bacteria. He mentioned that finding new compounds to use as prototypes for the creation of less harmful and more effective drugs for dealing with infectious diseases by restricting the multiplication of bacteria has become critically important in view of these expanding therapeutic challenges. Numerous bioactive compounds with strong antibacterial properties have been found to be within natural products derived from medicinal plants (Touzout et al., 2023). There have been increasingly published studies in recent years demonstrating the effectiveness of many traditional medicinal plants in combating MDR bacteria various organisations have been actively engaged for ten years in researching the antibacterial properties of plants found worldwide (Gonelimali et al, 2018; Machado et al, 2023; Ali et al.,2023)

An experiment was recently undertaken as part of a screening programme to identify Ethiopian medicinal herbs possessing antibacterial characteristics. Nine plants originating from the highlands of Chencha, Ethiopia, were selected for the study. The antimicrobial efficacy of plant extracts, containing secondary metabolites, dissolved in various organic solvents, was assessed against both standard bacterial strains and multidrug-resistant clinical isolates. All nine plant species that were evaluated showed in vitro antibacterial activity against at least one of the bacterial isolates during the antibacterial screening, using crude extracts. It was concluded that the antibacterial activity of different plant species varies widely, and for each plant type, it may change based on the genus of bacteria being tested as well as the extraction method utilized. The committee also advised that a more thorough examination is necessary, as well as a consideration of their whole range of efficacy (Manilal et al., 2023).

A recent paper publication mentioned that, as more resistance profiles may be adjusted, it is anticipated that the frequency of nosocomial infections caused by MDR bacteria will increase. Furthermore, this raises the possibility of an antibiotic shortage, which is concerning. As a result, new treatments or infection-treating strategies should be developed. Idris and Nadzir (2023) highlighted that, although numerous medications and treatments have been developed, it was noted that no

effective preventative strategies have yet been found. Moreover, to combat MDR-organisms, fresh alternative medications must be created. Utilizing plant extracts, which not only have been demonstrated to exert an inhibitory impact against the MDR bacterial infection but also have very few adverse effects (Idris and Nadzir, 2023).

However, a previous investigation analysed the antibacterial characteristics of ethanolic extracts derived from five herbal plants, specifically the leaves of guava (*Psidium guajava*), sage (*Salvia officinalis*), Rhamnus (*Ziziphusspinachristi*), mulberry (*Morus alba L.*), and olive (*Olea europaea L*). It was discovered that the ethanolic extract can function as a more effective antibacterial agent than the outcomes of the commercial antimicrobial agents used in the work (Hassan et al., 2020). Folk medicine provides a rich and underutilised resource for identifying and developing potential new medications to combat microbial infections, with the aim of reducing the development of resistance and minimising undesirable treatment effects. Additionally, the utilization of medicinal plants opens doors for underdeveloped nations because they may be more accessible affordable and readily available (Nigussie et al., 2021).

Table 4.7 Anti-bacterial properties of methanolic crude extracts against *K. pneumonia*, *P. aeruginosa*, *P.mirabilis*, *E. coli*, *S. marcescens* and *C. kose*

Test straint samples	*K. pneumonia*	*P. aeruginosa*	***P.mirabilis***	***E. coli***	*S. marcescens*	*C.* ***koseri***
Methanol (Negative control)	–	–	–	–	–	–
Gentamicin (Positive control)	+	+	+	+	+	+
M. cajuputi	–	+	+	–	–	–
A. planci	+	–	–	–	+	–
S. carteri	+++	–	–	+	–	++++
S. lanceolata	–	+	++	–	–	–
S. babylonica	–	–	–	–	–	–

C. taxfolia	–	–	–	–	–	–
Ulva sp.	–	–	–	–	–	–
A. alba	–	–	–	–	–	–
S. abbottie	–	–	–	–	–	–
Padina sp.	–	–	–	–	–	–

*(-) Zone of inhibition (inactive); (+): 7–8 mm zone of inhibition (mildly active); (++): 8–9 mm zone of inhibition (moderately active); (+++): 9–11 mm zone of inhibition (significantly active); (++++): 11-13 mm zone of inhibition (strongly active).

CHAPTER 5

CONCLUSION

In conclusion, this investigation indicated that Gram negative bacteria were detected in all isolated samples collected from ICU patients. *K. pneumonia* was the most prevalent bacteria found,followed in decreasing orderby *P. aeruginosa, P. mirabilis, E. coli, S. marcescens,* and *C. koseriare*.

Despite the fact that Amoxicillin was the most efficient antibiotic against all tested gram-negative bacteria, followed by Cefuroxime and Trimethoprim/Sulfamethoxazole, all six types of bacterial isolates recovered from all individuals shown resistance to this antibiotic. Following this are Nitrofurantion, Gentamicin, and Norfloxacin, which were found to have the least amount of resistance.

A phylogenetic tree was created using a neighbor-joining analysis of 16S rRNA, which grouped six examined isolates into three different clusters. The nucleotide base compositions of *C.koseri*strain and the *E. coli*sp. strain showed the highest similarity among isolated samples

The four above-mentioned plant extracts, (*Melaleuca cajuputi*,*Acanthasterplanci*, *Stylissacarteri,* and *Sonneratia lanceolata*) possess significant antibacterial activity that inhibits the growth of isolated bacteria. The disc diffusion approach revealed varying levels of inhibition against the six individual bacterial strains. The methanol crude extracts were shown to be effective against the

investigated microorganisms, partially inhibiting their growth and having inhibition zones with a diameter of 7 to 12 mm.

Further study needs to be done for more samples isolation and identification to indicate the numbers of contamination.

APPENDIX

APPENDIX 1

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Citrobacter koseri strain RCB871 16S ribosomal RNA gene, partial sequence	Citrobacter koseri	1038	1038	100%	0.0	100.00%	562	OR128488.1
Citrobacter koseri isolate 0123A_53_520, complete genome	Citrobacter koseri	1033	7178	100%	0.0	99.82%	4752495	CP017665.1
Citrobacter koseri strain RCB871 16S ribosomal RNA gene, partial sequence	Citrobacter koseri	1033	1033	100%	0.0	99.82%	1410	KT261083.1
Citrobacter koseri strain RCB781 16S ribosomal RNA gene, partial sequence	Citrobacter koseri	1033	1033	100%	0.0	99.82%	1238	KT260993.1
Citrobacter koseri strain RCB645 16S ribosomal RNA gene, partial sequence	Citrobacter koseri	1033	1033	100%	0.0	99.82%	1280	KT260857.1
Citrobacter koseri strain RCB643 16S ribosomal RNA gene, partial sequence	Citrobacter koseri	1033	1033	100%	0.0	99.82%	1409	KT260855.1
Citrobacter koseri strain RCB495 16S ribosomal RNA gene, partial sequence	Citrobacter koseri	1033	1033	100%	0.0	99.82%	1403	KT260707.1
Citrobacter koseri strain RCB386 16S ribosomal RNA gene, partial sequence	Citrobacter koseri	1033	1033	100%	0.0	99.82%	1460	KT260598.1
Citrobacter koseri strain RCB381 16S ribosomal RNA gene, partial sequence	Citrobacter koseri	1033	1033	100%	0.0	99.82%	1460	KT260593.1
Citrobacter koseri strain RCB263 16S ribosomal RNA gene, partial sequence	Citrobacter koseri	1033	1033	100%	0.0	99.82%	1447	KT260475.1

Appendix 1The results obtained from the NCBI BLAST gene bank analysis of the 16S rRNA gene sequences.

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