THE DETECTION OF MULTIDRUG-RESISTANT *Klebsiella pneumoniae* USING POLYMERASE CHAIN REACTION

MOHAMMAD ARAFATUDDIN FARUQUE

UNIVERSITI SAINS MALAYSIA

2025

THE DETECTION OF MULTIDRUG-RESISTANT *Klebsiella pneumoniae* USING POLYMERASE CHAIN REACTION

by

MOHAMMAD ARAFATUDDIN FARUQUE

Dissertation submitted in partial fulfilment of

the requirements of the degree of

Bachelor of Health Science (Biomedicine)

January 2025

DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

……

(MOHAMMAD ARAFATUDDIN FARUQUE)

Date: 28 January 2025

ACKNOWLEDGEMENT

I praise God, the Almighty for providing me this opportunity and granting me the capability, to proceed successfully. I am grateful to God for giving me strength and guidance, as well as making my journey easier in completing this final year research project. I would like to take this opportunity to acknowledge in writing to those who have been inspirational, generous, and very helpful in assisting me to complete this project.

First and foremost, I would like to express my sincerest gratitude to my supervisor and my co-supervisor, Dr. Nik Yusnoraini Yusof and Dr. Wan Nor Amilah Bt Wan Abdul Wahab, who willingly accepted the responsibility to support and give excellent guidance throughout the research project, as well as during thesis writing with patience and encouragement. I would also like to extend my gratitude to the research assistants, postgraduate students for spending their time and giving their sincerest efforts to guide and assist me throughout this project. Not forgetting, I would also like to appreciate the staff of the INFORMM, Universiti Sains Malaysia, my final year project colleagues, my classmates and friends for their valuable support and help, directly or indirectly.

Finally, I would like to thank my precious family members, especially my parents and my siblings and my entire family for their continuous support, love and understanding.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS, ABBREVIATIONS AND SYMBOLS

Symbol	Abbreviation
°C	Degree Celsius
%	Percentage
L	Litre
µL	Microlitre
mL	Mililiter
nM	nanometer
µg/mL	Microgram per mililiter
µg/µL	Microgram per microlitre
bp	Base pair
g	Gram
min	Minute
pH	Potential of hydrogen
sec	Seconds
Ta	Annealing temperature
V	Volt
A	Adenine
T	Thymine
C	Cytosine
G	Guanine
blaTEM	Beta-Lactamase TEM
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ESBL	Extended-Spectrum Beta-Lactamase
Kp.	*Klebsiella pneumoniae*

MDR-*kp*	Multidrug resistant *Klebsiella pneumoniae*
$MgCl_2$	Magnesium chloride
NA	Nutrient agar
NaCl	Sodium chloride
NB	Nutrient broth
NC	Negative control
PCR	Polymerase Chain Reaction
TAE	Tris-Acetate-EDTA
Tris-HCL	Tris(hydroxyl methyl)aminomethane hydrochloride
WHO	World Health Organization
CTXM-1	Cefotaximase-M-1
NDM-1	New Delhi Metallo-Beta-Lactamase 1
16s RNA	16s ribosomal ribonucleic acid

PENGESANAN *Klebsiella pneumoniae* RINTANG PELBAGAI UBAT MENGGUNAKAN TINDAK BALAS BERANTAI POLIMERASE

ABSTRAK

Klebsiella pneumoniae (MDR-*Kp*) yang rintang pelbagai ubat adalah kebimbangan perubatan utama di seluruh dunia, terutamanya dalam tetapan penjagaan kesihatan kerana ia tahan terhadap pelbagai antibiotik. Rintangan ini disebabkan oleh enzim seperti beta-laktamase dan karbapenem, yang menghadkan pilihan rawatan dan meningkatkan kadar kematian. Tindakbalas Rantaian Polymerase (PCR) telah digunakan dalam kajian ini sebagai cara yang cepat dan tepat untuk mengesan penanda genetik untuk pengenalpastian bakteria dan gen rintangan. Kajian ini bertujuan untuk menilai PCR untuk mengesan RNA ribosom 16S (16sRNA), beta-laktamase TEM (blaTEM), cefotaximase-M-1(CTXM-1) dan New Delhi metallo-beta-laktamase 1(NDM-1) dalam *K.pneumoniae* . Sampel yang diarkibkan dikumpul daripada INFORMM Biobank, dibiakkan di bawah keadaan aseptik dan DNAnya diekstrak menggunakan kaedah mendidih. PCR dilakukan dengan primer khusus dan elektroforesis gel agarose 2% digunakan untuk menggambarkan produk yang diperkuatkan. Gen blaTEM dan CTXM-1 telah dikesan dalam 78.9% dan 89.4% daripada isolat *K. pneumoniae,* masing-masing. Sementara itu, jalur tidak spesifik telah diperhatikan semasa amplifikasi NDM-1 bagi majoriti isolat (69%). Kajian menunjukkan bahawa PCR adalah kaedah yang berkesan untuk mengenal pasti MDP-kp dan gen rintangannya dengan pantas.

THE DETECTION OF MULTIDRUG-RESISTANT *Klebsiella pneumoniae* USING POLYMERASE CHAIN REACTION

ABSTRACT

Multidrug-resistant *Klebsiella pneumoniae* (MDR-*Kp*) is a major worldwide medical concern, particularly in healthcare settings because it is resistant to multiple antibiotics. This resistance is caused by enzymes such as beta-lactamases and carbapenems, which limit treatment options and increase mortality rates. Polymerase chain reaction (PCR) was used in this study as a fast and accurate way to detect genetic markers for bacterial identification and resistance genes. The study aimed to use PCR for detection of 16S ribosomal RNA (16sRNA), beta-lactamase TEM (blaTEM), cefotaximase-M-1(CTXM-1) and New Delhi metallo-beta-lactamase 1(NDM-1) in *K. pneumoniae.* Archived samples were collected from the INFORMM Biobank, cultured under aseptic conditions and their DNA was extracted using the boiling method. PCR was performed with specific primers and 2% agarose gel electrophoresis was used to visualize the amplified products. The results showed successful amplification of 16sRNA gene (100%) in all isolates confirming the bacterial identity. The blaTEM and CTXM-1 genes were detected in 78.9% and 89.4% of *K. pnuemoniae* isolates respectively, while non-specific bands were observed during NDM-1 amplification for majority (69%) of the isolates. The study demonstrates that PCR is an effective method for rapidly identifying MDP-*Kp* and its resistance genes.

CHAPTER 1

INTRODUCTION

1.1 Background

Klebsiella pneumoniae, a Gram-negative bacterium, is a leading cause of fatal infections such as pneumonia, urinary tract infections, and bloodstream infections, with numerous cases occurring in healthcare settings (Pei et al., 2022). This opportunistic, encapsulated, nonmotile bacterium can survive in hospitals, persist on environmental surface and colonize human skin, respiratory tract and bowels. *Klebsiella* can be transmitted via personal contact, contaminated environments and medical equipment (Hou et al., 2015). The bacterium generally infects the mucosal surfaces of the oropharynx and gastrointestinal tract in humans. Upon entering the body, the bacterium may exhibit significant virulence and resistance to antibiotics. Currently, *K. pneumoniae* is regarded as the predominant cause of hospital-acquired pneumonia in the United States, with the organism responsible for 3% to 8% of all nosocomial bacterial infections (Jondle et al., 2018).

Due to the high rates of resistance to antibiotics, *K. pneumoniae* was considered as one of the most frequent agents of infectious diseases and significant menace to public health (Sakkas et al., 2019). Resistance to antibiotics reflects evolution in action, related to the continuous exposure to antibiotics. The phrase "multidrug resistance (MDR)" refers to bacteria's ability to survive the effects of several antibiotics that would normally be effective against them. In the case of *K. pneumoniae*, resistance is primarily caused by the synthesis of enzymes such as β -lactamases, carbapenemases, and extended-spectrum β-lactamases (ESBLs), which render many conventional

antibiotics ineffective (Hsu et al., 2019). The estimated pooled prevalence of MDR and ESBL *K. pneumoniae* in South-East Asia was 55% and 27% of isolated pneumoniae strains, respectively (Salawudeen et al., 2023). The increased resistance to essential antibiotic families such as beta-lactams and carbapenems has significantly reduced treatment options, resulting in longer illnesses, greater mortality rates, and increased expenses for healthcare (Kao et al., 2023). The resistance of *K. pneumoniae* to numerous antibiotics arises from various mechanisms, including modification of antibiotic target sites, alteration of metabolic pathways, activation of efflux pump systems, changes in membrane permeability, and the release of antibiotic-inactivating enzymes (Adekunle, 2012).

Enterobacteria that produce ESBL significantly contribute to prolonged hospitalization, healthcare-associated infections, and elevated mortality and morbidity rates (Capelo-Martínez & Igrejas, 2019). ESBL plasmid-encoded enzymes are inhibited by clavulanic acid and possess the capability to hydrolyze β-lactam ring bonds in antibiotics such as penicillins, cephalosporins, and aztreonam. Currently, over 600 ESBL variants have been identified, predominantly linked to Temoneira (TEM), Sulfhydryl Variable (SHV), and Cefotaxime hydrolyzing capabilities (CTX-M) (Shahid et al., 2023). A new type of B enzyme, New Delhi metallo-β-lactamase (NDM-1), was identified in a *K. pneumoniae* isolate from Sweden, which appears to have been imported from India (Yong et al., 2009). The emergence of bla_{NDM-1}-harboring Enterobacteriaceae is alarming due to their resistance to commonly utilized antibiotics for gram-negative infections (β-lactams, fluoroquinolones, and aminoglycosides) and their rapid transmission (Karthikeyan et al., 2010).

Traditional methods of detecting *K. pneumoniae* infections and assessing resistance profiles, such as culture-based approaches and antibiotic susceptibility testing, are useful but time-consuming, frequently taking several days to get results (Hsu et al., 2019). Additionally, these methods rely on phenotypic observations, which may not always be accurate or consistent in identifying resistance mechanisms (Russo & Marr, 2019). To address this challenges, molecular diagnostic techniques, particularly Polymerase Chain Reaction (PCR), offer a quick, sensitive, and precise alternative. PCR is a method for amplification of specific DNA sequences, which allows infections and resistance genes to be detected with great accuracy in just a few hours (Liu & Guo, 2019). By targeting genetic markers linked with antibiotic resistance, PCR avoids the delays associated with older procedures and offers a more reliable approach to early detection (Hsu et al., 2019).

This study evaluates the effectiveness of PCR as a diagnostic tool for detecting multidrug-resistant *K. pneumoniae*. It seeks to validate the existence of *K. pneumoniae* by targeting the 16s rRNA gene while also identifying important resistance genes, such as TEM, CTXM-1, and NDM-1, which are linked to beta-lactam and carbapenem resistance. Primers for PCR in this study were obtained from previous studies to ensure high accuracy, amplifying only the targeted genetic material to provide precise results. The key objective is to show that PCR can accurately identify *K. pneumoniae* and its resistance genes, making it an effective tool for diagnosis in clinical applications. The expected outcome is to establish PCR as a faster, more precise alternative to conventional diagnostic methods, allowing for the timely and successful treatment of MDR-*Kp* infections.

1.2 Problem Statement

The rising prevalence of MDR and ESBL *K. pneumoniae* is an important global public health challenge. Morbidity and mortality associated with this condition are alarming particularly in the developing regions of the world (Salawudeen et al., 2023). Furthermore, the frequency of highly virulent forms of *K. pneumonia* infection in Asia and developing countries remains high. Malaysia, similar to other Asian countries, is addressing the spread of MDR *K. pneumoniae*. MDR is associated with restricted therapeutic alternatives, prolonged hospitalizations, elevated healthcare expenses, and increased morbidity and mortality rates globally (Yakubu et al., 2021).

The emergence and spread of antibiotic-resistant genes diminish treatment efficacy, leading to increased mortality and morbidity among patients, prolonged hospital stays, and elevated healthcare costs. Consequently, it is essential to identify them promptly and implement measures to mitigate the prevalence of ESBLs. Conventional antimicrobial testing is often inadequate and time-consuming for the detection of multidrug resistance. Microorganisms frequently go unidentified as ESBL producers, allowing for their proliferation within hospital settings (Ghenea et al., 2022). Therefore, the identification of resistant phenotypes is essential, particularly in nations with high antibiotic consumption and inadequate infection control protocols. Thus, there is a need for a rapid and accurate diagnostic method to identify resistance gene in MDR-*Kp* strains. Additionally, there is no specific information available regarding the prevalence of resistance genes such as *blaTEM*, *CTX-M-1*, and *NDM-1* in the MDR *K. pneumoniae* isolates from our samples.

1.3 Rationale of Study

Beta-lactamase production represents the predominant mechanism underlying resistance to beta-lactam antibiotics. The emergence of ESBLs has led to an increase in resistance observed in medical practice. Plasmids that facilitate ESBL production often harbor resistance genes for antibiotics. Consequently, enzymes such as TEM, CTX_M, and NDM, commonly linked to antibiotic resistance in *K.pneumoniae*, are frequently studied. For example, a study conducted in Iran reported the presence of TEM, CTX-M-15, and NDM genes in 82.7%, 79.3%, and 6.9% of multidrug-resistant *K. pneumoniae* isolates, respectively (Farhadi et al., 2021). Moreover, studies conducted in the Middle East have demonstrated an increasing frequency of ESBL in *K. pneumoniae* over the past decade.

The discovery of a virulence plasmid in epidemic CR*Kp* strains raises concerns that these "hybrid" organisms, which are MDR and hypervirulent, may be highly transmissible and able to cause fatal hospital and community infections. Consequently, clinical and public health needs necessitate a swift and thorough molecular detection assay to identify and monitor the dissemination of these strains and furnish prompt infection control data. The molecular characterization of *K. pneumoniae* isolates provides information on drug resistance and identifies risk factors associated with patient colonization and infection. Diagnosing and identifying resistance genes including TEM, CTX-M-1, and NDM-1 in *K. pneumoniae* is essential for effective treatment, infection control, as well as public health management. PCR promises elevated precision and reliability by concentrating on a single gene at a time. Moreover, the application of PCR facilitates rapid detection, thereby supporting prompt treatment.

1.4 Research Questions

1. Is the molecular technique suitable for the confirmation of *Klebsiella pneumoniae*?
2. Can PCR effectively detect specific resistance genes in clinical *K. pneumoniae* isolates?

1.5 Research Hypothesis

1.5.1 Null hypothesis (H_0)

PCR will not accurately detect *K. pneumoniae* in clinical isolates and its specific resistance genes associated with MDR-*Kp.*

1.5.2 Alternative hypothesis (H_A)

PCR will accurately detect *K. pneumoniae* in clinical isolates and its specific resistance genes associated with MDR-*Kp.*

1.6 Objectives

1.6.1 General objective

To evaluate Polymerase Chain Reaction (PCR) for the detection of multidrug resistance *K. pneumoniae* (MDR-*Kp*).

1.6.2 Specific objectives

1. To confirm the *K. pneumonia* using PCR.
2. To identify the resistance genes for multidrug-resistance *K. pneumoniae* (MDR-*Kp*) using PCR.

CHAPTER 2

LITERATURE REVIEW

2.1 History of *K. pneumoniae*

Carl Friedlander first described *K. pneumoniae* in 1882. He characterized it as an encapsulated bacillus following the isolation of the bacterium from the lungs of individuals who succumbed to pneumonia. Initially identified as Friedlander's bacillus, the bacterium acquired the name Klebsiella in 1886. *K. pneumoniae* is a gram-negative, encapsulated, non-motile bacterium present in the environment, linked to pneumonia in individuals with alcohol use disorder or diabetes mellitus (Jondle et al., 2018). The bacterium generally inhabits the mucosal surfaces of the oropharynx and gastrointestinal tract in humans. Upon entering the body, the bacterium may exhibit significant virulence and resistance to antibiotics (Aghamohammad et al., 2020).

2.2 Etiology of *K. pneumoniae*

K. pneumoniae is a member of the Enterobacteriaceae family and is characterized as a gram-negative, encapsulated, and non-motile bacterium. The polysaccharide capsule (CPSs) of the organism is the primary virulence factor, enabling the bacteria to evade opsonophagocytosis and serum-mediated killing by the host. As of now, 77 distinct capsular types have been examined, and Klebsiella species lacking a capsule generally exhibit reduced virulence. A secondary virulence factor is lipopolysaccharides (LPS) that envelop the external surface of gram-negative bacteria (Rønning et al., 2019). The detection of lipopolysaccharides triggers an inflammatory cascade in the host organism and is a primary contributor to the sequelae of sepsis and septic shock. Fimbriae, another virulence factor, enable the organism to adhere to host cells. Siderophores

represent an additional virulence factor essential for the organism's ability to induce infection in hosts. In 1929, Alexander Fleming first identified resistance to beta-lactam antibiotics in gram-negative organisms (Tsereteli et al., 2018). Since then, *K. pneumoniae* has been extensively researched and has demonstrated the production of a beta-lactamase that hydrolyzes the beta-lactam ring in antibiotics.

2.3 Prevalence of *K. pneumoniae*

Humans serve as the primary reservoir for *K. pneumoniae* (Figure 2.1). In the general community, 5% to 38% of individuals carry the organism in their stool and 1% to 6% in the nasopharynx. The main reservoirs of infection are the patient's gastrointestinal tract and the hands of hospital personnel (Esposito et al., 2018). It can lead to a nosocomial outbreak. However, higher rates of colonization have been reported in those of Chinese ethnicity (27%) and those who experience chronic alcoholism (Guo et al., 2017). In hospitalized patients, the carrier rate for *K. pneumoniae* is much higher than that in the community. In one study, carrier rates as high as 77% can be seen in the stool of those hospitalized and are related to the number of antibiotics given (Walter et al., 2018).

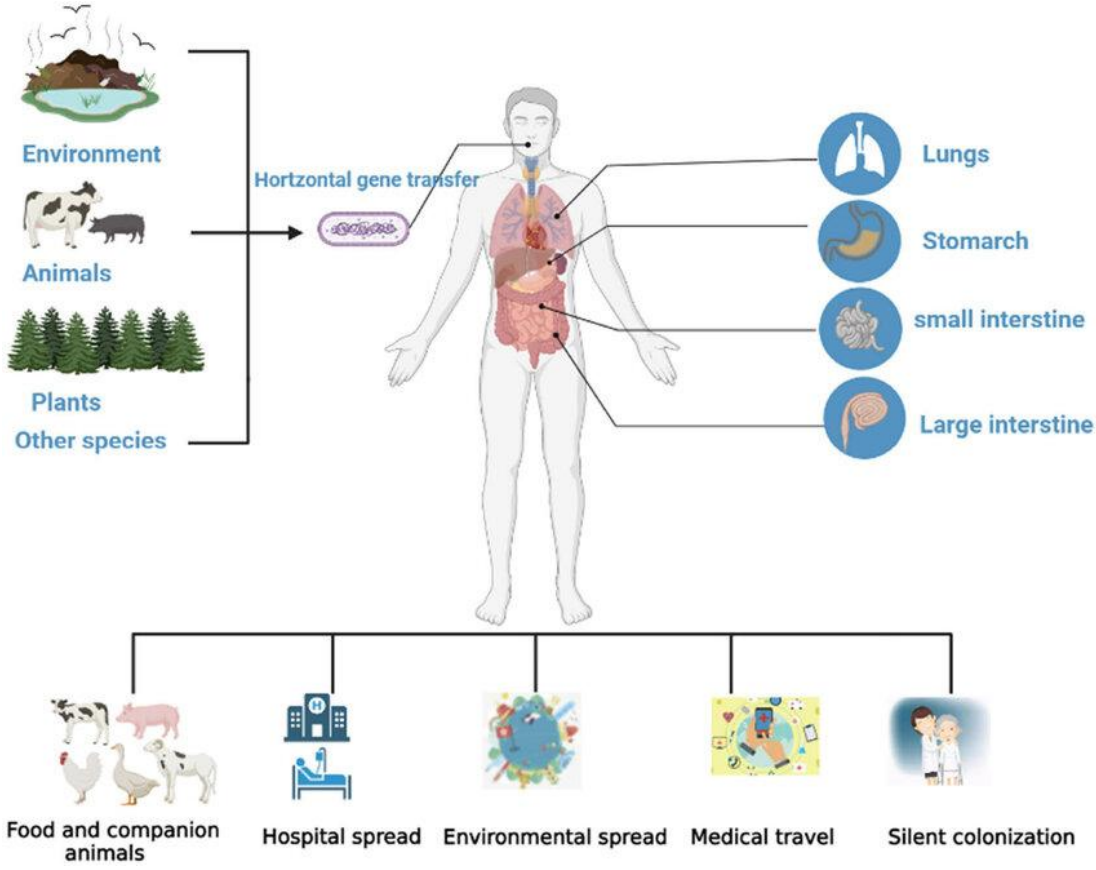

Figure 2.1: Transmission of *K. pneumoniae*. Adapted from Li et al. (2023).

Pneumonia caused by *K. pneumoniae* can be categorized as either community-acquired or hospital-acquired pneumonia. In Western culture, it is estimated that around 3% to 5% of community-acquired pneumonia cases are attributable to *K. pneumoniae* infections, whereas in developing countries like Africa, this figure may reach approximately 15% of all pneumonia cases. *K. pneumoniae* constitutes roughly 11.8% of global hospital-acquired pneumonia cases. Among individuals who acquire pneumonia while on a ventilator, 8% to 12% of cases are attributed to *K. pneumoniae*, whereas only 7% occur in non-ventilated patients. Mortality rates in patients with alcoholism and septicemia vary from 50% to 100% (Ashurst & Dawson, 2018). Figure 2.2 depicts the prevalence of *Kp.* globally. Regional studies in Asia and Africa indicate prevalence rates of ESBL-producing Enterobacteriaceae at 40% (95% CI, 34.0–47.0)

in Pakistan and 42% (95% CI, 34.0–50.0) in East Africa. Conversely, Europe and North America exhibited reduced rates, with ESBL prevalence at 5% (95% CI, 2.0–8.0) and 3% (95% CI, 1.0–5.0), respectively. Nonetheless, the rates of MDR *K. pneumoniae* in South America (72.4%; 95% CI, 53.8–85.6) and certain European regions surpassed previous estimates, influenced by endemic ESBL-producing strains and elevated transmission within healthcare environments (Abbas et al., 2023).

Figure 2.2: Global prevalence of *K. pneumonia*. Severity is differentiated by color. Severe cases have endemic spread of *Kp*, while moderate severity and low-severity regions have moderate case studies of infection and hospital-reported cases of *Kp* infection, respectively. Adapted from Abbas et al. (2023).

2.4 Pathophysiology of *K. pneumoniae*

Host defense against bacterial invasion primarily relies on two factors: polymorphonuclear granulocytes, which phagocytize bacteria, and serum complement proteins, which exhibit bactericidal properties. The alternative pathway of the activation of complement is more active during *K. pneumoniae* infection. Neutrophil myeloperoxidase along with lipopolysaccharide-binding protein aid in the defense against *K. pneumoniae* infection. It possesses a polysaccharide capsule composed of

intricate acidic polysaccharides that influence their pathogenicity. The capsule safeguards bacteria against phagocytosis and serum bactericidal proteins. It attaches to host cells via numerous fimbrial and non-fimbrial adhesions, which is essential to the infectious process (Li et al., 2014).

2.5 Virulence Factors

Lipopolysaccharide (LPS) and capsular polysaccharide (CPS) are two critical virulence factors of *K. pneumoniae* in the etiology of sepsis. LPS comprises lipid A, core, and O-polysaccharide antigen to evade complement-mediated lysis. The CPS is the external layer of the pathogen composed of polymorphonuclear cells, which confers resistance to phagocytosis. CPS diminished bacterial cell interaction by decreasing the quantity of C3 attached to the bacteria and by serving as a barrier to impede contact between macrophage receptors and their ligands on the bacterial surface. CPS is essential and also regulates the interaction between surfactant protein D (SP-D) and *K. pneumoniae*. SP-D facilitates aggregation and enhances phagocytic clearance by human alveolar macrophages. In the presence of CPS, C3 and SP-D are unable to bind, leading to the clearance of microorganisms from the lower respiratory tract, which results in pneumonia (Podschun et al., 1998). Figure 2.3 presents different virulence factors associated with *K. pneumoniae*.

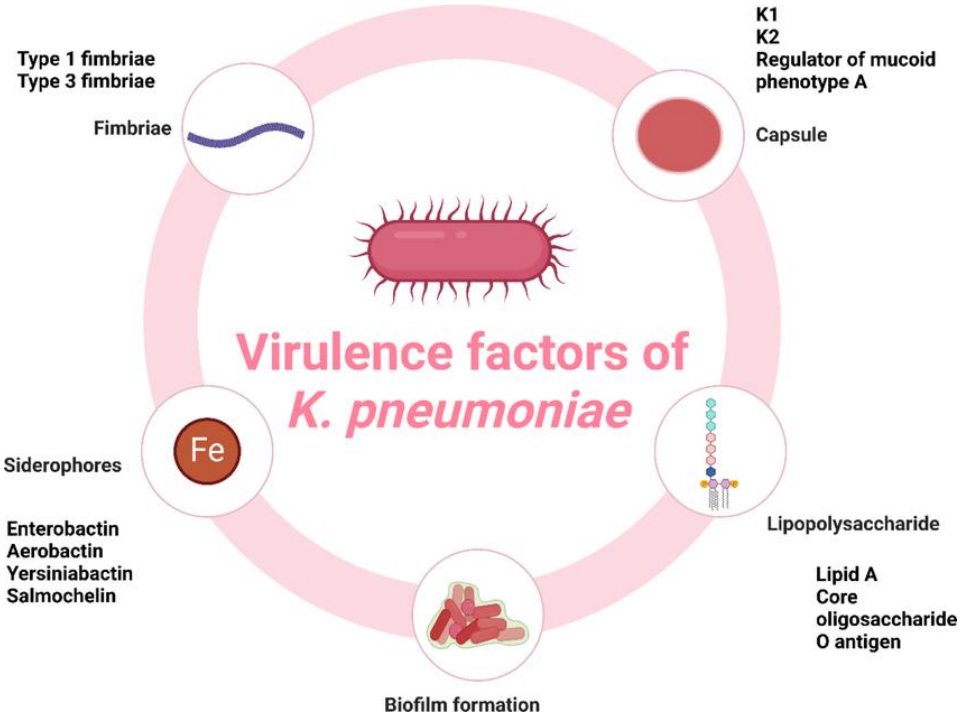

Figure 2.3: Virulence factors of *K. pneumoniae*. Adapted from Selim et al. (2024).

2.6 Clinical Symptoms

K. pneumoniae is a recognized etiological agent of community-acquired pneumonia. Symptoms linked to community-acquired *K. pneumoniae* encompass abrupt onset, elevated fever, and a gelatinous sputum. Dyspnea and coughing are prevalent symptoms as well. If introduced into the bloodstream, *K. pneumoniae* can cause meningitis, which affects the central nervous system. Urinary tract infections and bacteraemia are primary outcomes of Klebsiella infections. Hospital patients with prolonged stays in Intensive Care Units, receiving extended courses of broad-spectrum antibiotics or possessing comorbidities, are at risk of acquiring a nosocomial infection. A significant number of hospital patients and faculty members infected with *K. pneumoniae* will experience pneumonia, bacteraemia, and urinary tract infections (Yang et al., 2019).

2.7 Diagnosis

K. pneumoniae can be isolated from blood, urine, pleural fluid, and wounds. A gram stain of a sputum sample from a patient may facilitate the diagnosis of *K. pneumoniae*. Cultures must be collected from locations including open wounds, peripheral or central intravenous access sites, urinary catheters, and respiratory apparatus. Chest radiography can facilitate the diagnosis of a *K. pneumoniae* infection. The organism typically inhabits one of the upper lobes of the lungs but may also affect the lower regions. The lobe will exhibit edema and may, in numerous instances, generate abscesses (Para et al., 2018).

2.8 Treatment

2.8.1 Antibiotic therapy

K. pneumoniae exhibits resistance to several antibiotics, resulting in severely restricted treatment options. The selection of antibiotic therapy for *K. pneumoniae* is contingent upon the affected organ system. Antibiotics exhibiting significant intrinsic activity against *K. pneumoniae* comprise cephalosporins, carbapenems, aminoglycosides, and quinolones. These treatments are initially employed as monotherapy or in combination. Current protocols for community-acquired *K. pneumoniae* pneumonia entail a 14-day course of treatment with either a third or fourth-generation cephalosporin as monotherapy, a respiratory quinolone as monotherapy, or a combination of the aforementioned regimens with an aminoglycoside. If the patient is allergic to penicillin, a regimen of aztreonam or a respiratory fluoroquinolone should be administered. Carbapenem can be utilized as monotherapy for nosocomial infections until sensitivity results are available (Liu et al., 2018; Mitharwal et al., 2016).

2.8.2 Resistance

Upon diagnosing ESBL, carbapenem therapy should be commenced due to its global sensitivity profile. Upon diagnosis of carbapenem-resistant Enterobacteriaceae (CRE), it is imperative to seek infectious disease consultation to inform treatment strategies. Various antibiotic alternatives for treating CRE encompass polymyxins, tigecycline, Fosfomycin, aminoglycosides, or dual therapy with carbapenems. The combination therapy of two or more agents, as previously stated, may reduce mortality in comparison to monotherapy alone (Arnold et al., 2011).

2.9 Multidrug Resistance in *K. pneumoniae*

MDR in *K. pneumoniae* constitutes a major public health issue, predominantly fueled by the organism's capacity to acquire and propagate resistance genes. Multiple mechanisms facilitate the emergence of MDR in *K. pneumoniae*, encompassing the synthesis of beta-lactamases, the presence of efflux pumps, and modifications in target sites. The main mechanism is the synthesis of beta-lactamases, enzymes that hydrolyze beta-lactam antibiotics, thereby rendering them ineffective. ESBLs and carbapenemases are especially alarming. ESBLs provide resistance to penicillins, cephalosporins, and aztreonam, whereas carbapenemases, including Klebsiella pneumoniae carbapenemase (KPC), inactivate carbapenems, frequently utilized as last-resort antibiotics (Yang et al., 2023).

A notable mechanism is the existence of efflux pumps, which actively extrude antibiotics from the bacterial cell, thereby diminishing their intracellular concentration and efficacy (Figure 2.4). Efflux pumps can provide resistance to various classes of antibiotics, including tetracyclines, fluoroquinolones, and aminoglycosides (Padilla et

al.,2010).

Moreover, mutations in target sites may result in antibiotic resistance. Modifications in penicillin-binding proteins (PBPs) can diminish the binding affinity of beta-lactam antibiotics, whereas mutations in DNA gyrase and topoisomerase IV can impart resistance to fluoroquinolones (Huy, 2024).

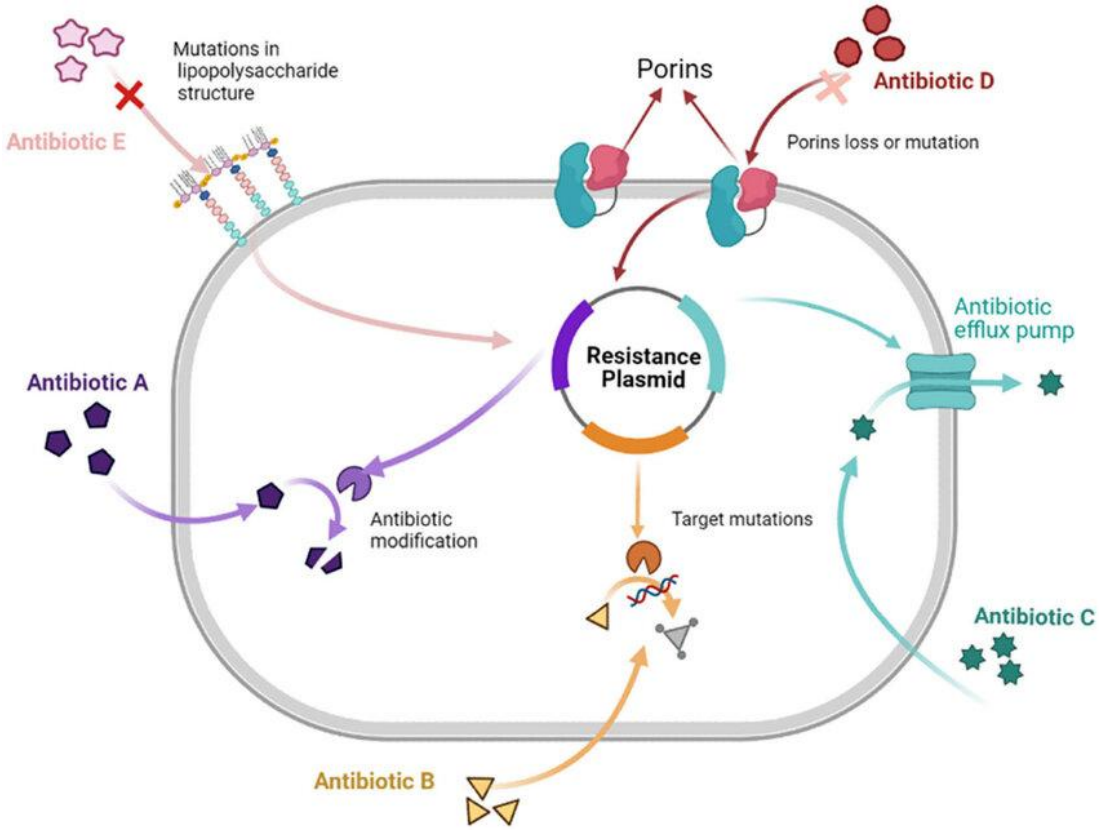

Figure 2.4: Mechanisms conferring antibiotic resistance to *K. pneumoniae*. Adapted from Li et al. (2023).

Horizontal gene transfer (HGT) is essential for the dissemination of resistance genes among *K. pneumoniae* and other bacterial species. HGT) transpires via mechanisms including conjugation, transformation, and transduction, enabling the swift spread of resistance determinants among bacterial populations. Environmental factors, including the excessive and improper use of antibiotics in medical and agricultural contexts, intensify the issue by generating selective pressure that promotes the survival and

expansion of resistant strains. The emergence of hypervirulent strains that integrate virulence and resistance characteristics presents an additional challenge, as these strains can induce severe infections in otherwise healthy individuals (Yang et al., 2023). Comprehending the mechanisms driving MDR in *K. pneumoniae* is crucial for formulating effective strategies to address this threat. Continuous research and surveillance are essential for monitoring resistance trends and informing the development of novel therapeutic options.

2.10 Genes in MDR in *K. pneumoniae*

2.10.1 β-Lactamase Resistance Genes

β-Lactamase generated by *K. pneumoniae* hydrolyzes the β-lactam ring in antibiotics, leading to resistance against β-lactam antibiotics. *K. pneumoniae* exhibits inherent resistance to various β-lactamase genes due to the widespread presence of the SHV β-lactamase in its genomic sequence, with ampicillin resistance being a characteristic feature of the organism (Table 2.1).

2.10.2 Extended-spectrum beta-lactamases (ESBLs)

ESBLs are plasmid-mediated antibiotic resistance mechanisms found in the accessory genome. The broad-spectrum activity of ESBL genes against β-lactams, including third-generation carbapenems, is obstructed by clavulanic acid. *K. pneumoniae* that produces ESBL has emerged as a common pathogen in hospital infection outbreaks. CTX-M has progressively replaced TEM and SHV as the predominant genotype of ESBLs due to the availability of plasmids and transposons that produce blaCTX-M-type ESBLs (Li et al., 2001). Additional ESBL genotypes were also conveyed to *K. pneumoniae* through horizontal gene transfer, encompassing blaOXA type ESBLs and

the rare genes bla GES, bla SFO, bla PER, bla TLA, bla VEB, and bla KLUC-5. *K. pneumoniae* that produces ESBL is increasingly prevalent globally, with endemic rates reaching as high as 50% in certain regions. Carbapenems have historically been the preferred therapy for managing ESBL-producing bacterial infections (Li et al., 2018).

Table 2.1: MDR-related genes in *K. pneumoniae*

Characteristic	Gene Name	Gene Functions
β-Lactam	blaSHV, blaTEM, blaCTX	ESBLs
	blaGES, blaSFO, blaPER, blaTLA, blaVEB, blaKLUC-5	Lateral gene transfer
	bla KPC Bla NDM, bla VIM, bla IMP, and bla OXA	Carbapenemase
	blaCMY, blaDHA, blaFOX, blaMOX	AmpC plasmids

2.10.3 Carbapenem resistance genes

The usage of carbapenems has risen markedly due to the multidrug-resistant phenotypic traits of ESBL-producing *K. pneumoniae* strains. Plasmid-regulated carbapenem enzymes remain the primary focus of multidrug resistance pathways. KPC is a serine-based class β-lactamase, recognized as the predominant and most detrimental carbapenemase in K. pneumoniae. Bla NDM, blaVIM, blaIMP, and blaOXA are several carbapenemase genes identified in *K. pneumoniae*. NDM-1 can

render numerous carbapenemase-producing Enterobacteriaceae (CPE) resistant to various frequently utilized clinical antibiotics, leading to challenging clinical management of CPE and elevated mortality rates (Papp-Wallace et al., 2010). In the absence of the carbapenemase gene, *K. pneumoniae* may develop carbapenem resistance due to porin loss, enhanced efflux pump activity, and overproduction of β-lactamases, including ESBL and AmpC. Clinically, CRKP infection presents a significant challenge in medical practice (Cristina et al., 2018).

2.11 Role of TEM, CTXM-1, NDM genes

Effective treatment of infections resulting from *K. pneumoniae* depends on the accurate detection of resistance genes. The existence of these genes can substantially influence antibiotic selection. The TEM and CTX-M-1 genes encode ESBLs, which impart resistance to various beta-lactam antibiotics, such as penicillins and cephalosporins (Dehshiri et al., 2018). Failure to identify these genes may result in clinicians prescribing ineffective antibiotics, consequently causing treatment failure and extended infection duration. The NDM gene encodes New Delhi Metallo-beta-lactamase, which confers resistance to carbapenems, frequently regarded as last-resort antibiotics (Ghenea et al., 2022). Recognizing NDM is essential to prevent the administration of carbapenems and to choose alternative therapies that are more likely to be efficacious.

Furthermore, the identification of these resistance genes significantly improves infection control measures. Understanding the precise resistance mechanisms enables healthcare institutions to execute targeted infection control measures. Patients carrying bacteria with NDM genes may necessitate isolation to avert the transmission of these highly resistant organisms to other susceptible patients. Furthermore, identifying these

genes facilitates the monitoring of resistant strain dissemination within healthcare environments, allowing for prompt interventions to mitigate outbreaks (Castanheira et al., 2021).

Thirdly, from a public health standpoint, tracking the prevalence and distribution of resistance genes such as TEM, CTX-M-1, and NDM is crucial for epidemiological surveillance. This information aids public health authorities in comprehending the dynamics of antibiotic resistance within community and healthcare environments. It also guides the formulation of guidelines and policies designed to mitigate the proliferation of resistant bacteria (Ghenea et al., 2022). Data regarding the prevalence of these genes can inform antibiotic stewardship programs, encouraging the prudent use of antibiotics to reduce the development of resistance (Castanheira et al., 2021).

Moreover, the identification of these resistance genes aids in research and development initiatives focused on the discovery of novel antimicrobial agents and diagnostic tools. Comprehending the genetic foundation of resistance may facilitate the creation of innovative pharmaceuticals that can circumvent or suppress the resistance mechanisms. It also assists in the development of rapid diagnostic tests that can swiftly identify resistant strains, enabling timely and suitable treatment (Ghenea et al., 2022).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Flow Chart

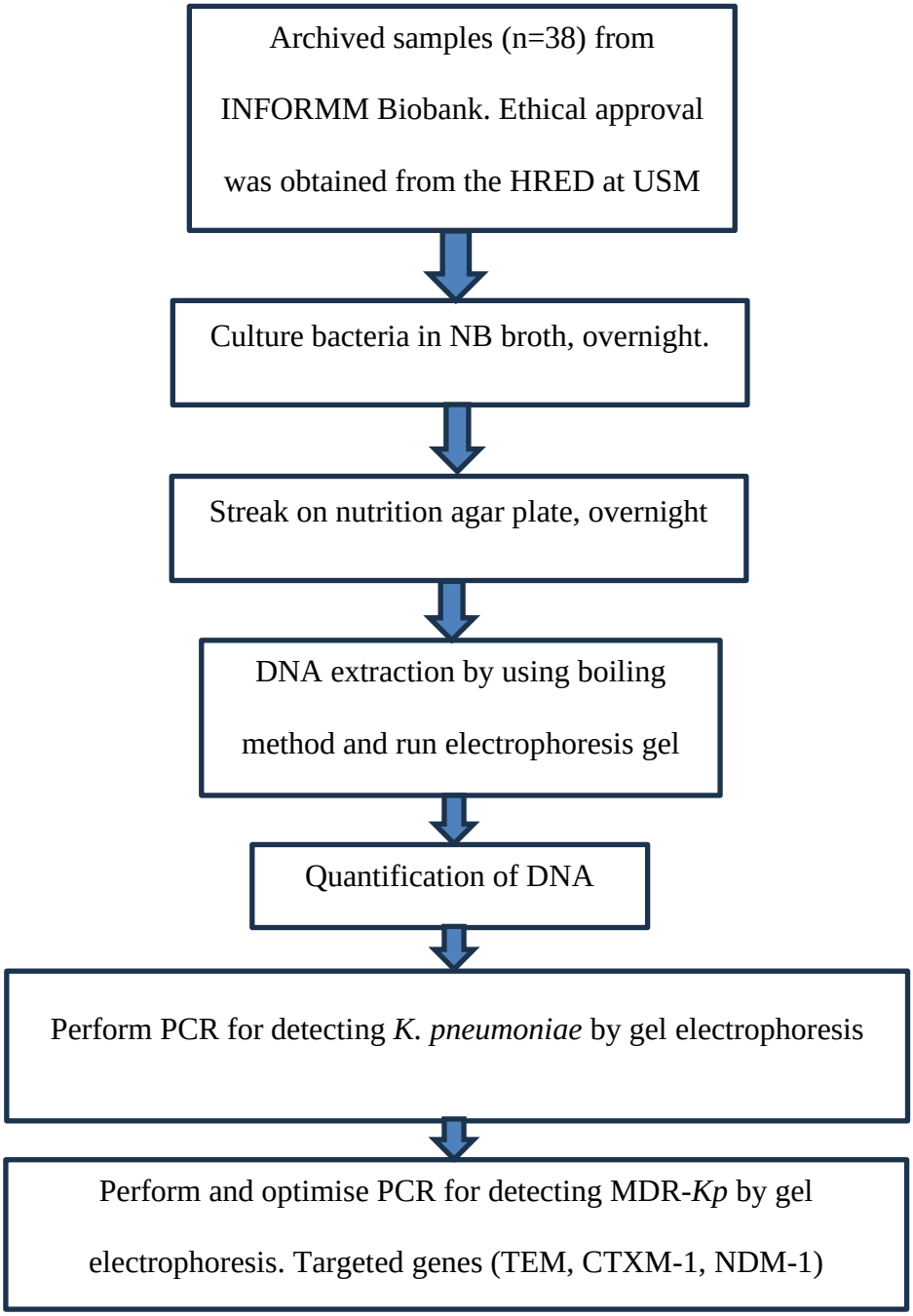

Figure 3.0: Study flowchart.

3.2 Materials

3.2.1 Chemicals and Reagents

The chemicals and reagents used in this study are listed in Table 3.1.

Table 3.1. List of chemicals and reagent

Chemicals/Reagents	**Supplier/Brand**
100 bp DNA Ladder	Thermo Fisher Scientific, USA
70% Ethanol	HmbG Chemicals, Malaysia
5x GoTaq(®)Flexi Buffer	Promega, USA
50X Tris-Acetate-EDTA (TAE) Buffer, pH 8.0	1st BASE, Singapore
Agarose powder	Vivantis, USA
dNTPs mix (10 mM each)	Promega, USA
FloroSafe DNA Stain	1st BASE, Singapore
Go Taq DNA polymerase	Promega, USA
$MgCl_2$ solution (10 mM)	Promega, USA
Nuclease free water	New England Biolabs, USA
Normal saline	Merck, Germany
Nutrient agar	OXOID, England
Nutrient broth	OXOID, England

3.2.2 Apparatus and equipment

The laboratory apparatus and equipment used in this study are listed in Table 3.2

Table 3.2: List of laboratory apparatus and equipment

Consumables	Supplier, country
Agarose apparatus	Bio-rad, Germany
Beaker 100 mL	SCHOTT, Germany
Centrifuge machine	Hettich Zentrifugen, Germany
Centrifuge tube 50 mL	Biologix, USA
Culture plate	BIOMEDIA
Electric balance	Shimadzu, Japan
Erlenmeyer flask 100 mL	SCHOTT, Germany
GeneFlash	Syngene,UK
Incubator machine	Thermo Fisher Scientific, USA
Incubator shaker	Thermo Fisher Scientific, USA
Mass balance	Goettingen, Germany
Measuring cylinder 100 mL	SCHOTT, Germany
Microcentrifuge tube 0.65 mL	Golden Gate Bioscience, USA
Microcentrifuge tube 1.7 mL	Golden Gate Bioscience, USA
Micropipette p10	Eppendorf, Germany
Micropipette p100	Eppendorf, Germany
Micropipette p1000	Eppendorf, Germany
Micropipette tips (blue)	Gaia Science (M) Sdn. Bhd., Malaysia
Micropipette tips (clear)	Axygen, USA
Micropipette tips (yellow)	Axygen, USA
Microwave Oven	Samsung, South Korea
Purifier Class II Biosafety Cabinet	Labconco, USA
Refrigerator	Panasonic, Japan
Schott bottle 1000 mL	SCHOTT, Germany

Schott bottle 500 mL	SCHOTT, Germany
Spectrophotometer Nano-Drop 1000	Thermo Fisher Scientific, USA
Thermal Cycler (PCR machine)	Applied Biosystems, Malaysia
Thin-walled 0.5ul PCR tube	Axygen, USA
Water bath	Memmert, Germany

3.3 Bacterial Culture

3.3.1 Preparation of nutrient agar

To prepare the nutrient agar (NA), 14 gm of nutrient agar powder were weighed and dissolved in 500 mL of distilled water. The mixture was heated until it reached boiling, with constant stirring to ensure that the agar was completely dissolved resulting in a clear, light-yellow solution. The prepared solution was subsequently divided into 14 mL MacCartney bottles, with 5 mL dispensed into each one. Care was taken to ensure that all bottles had consistent volume. Following the dividing process, the bottles were sterilized in an autoclave at 121 °C for 15 minutes. After autoclaving, the bottles were cooled and positioned at an angle to allow the agar to solidify. These nutritional agars were appropriately stored till further use.

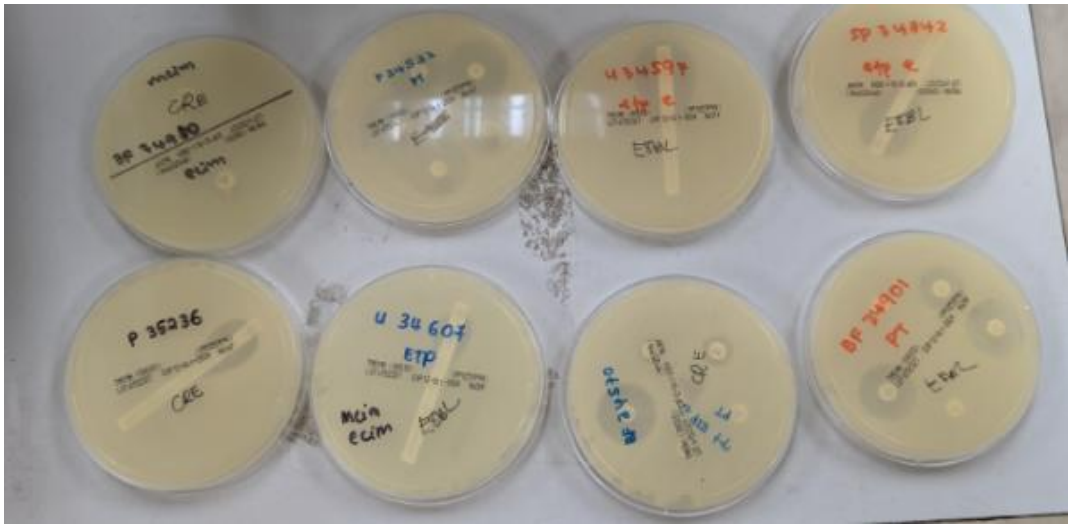

Figure 3.1: Collected clinical sample isolates.

3.3.2 Preparation of nutrient broth

To prepare the nutrient broth, 13 grams of nutrient broth powder consisting of Lab-Lemco powder (1 gm), yeast extract (2 gm), peptone (5 gm), and sodium chloride (5 gm) were weighed and dissolved in 900 mL of distilled water. The mixture was stirred continuously until all components were completely dissolved. The pH of the solution was then adjusted to 7.4 to ensure optimal growth conditions. After confirming the pH, the volume was made up to 1000 mL by adding additional distilled water. The prepared solution was then sterilized in an autoclave at 121 °C for 15 minutes to ensure it was free from contamination.

3.3.3 Preparation of nutrient saline

To prepare normal saline, 9 gm of sodium chloride (NaCl) were weighed and dissolved in 900 mL of distilled water. The solution was stirred thoroughly until the NaCl was completely dissolved. The pH of the solution was adjusted to 7.4 for maximum compatibility. Once the pH adjustment was completed, additional distilled water was