Genotypic characterization of hypervirulent Enterobacterales isolates from Intensive Care Unit patients at the Kenyatta National Hospital

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(H56/34346/2019)

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December 2023

DECLARATION

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LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
CTX-M	Cefotaximase-Munich
DNA	Deoxyribonucleic acid
ICU	Intensive care unit
IMP	Imipenemase
KNH	Kenyatta National Hospital
KPC	*Klebsiella pneumoniae* carbapenemase
MDR	Multidrug resistance
NDM	New Delhi Metallo-β-lactamases
ONT	Oxford Nanopore Technology
OXA	Oxacillinase
RNA	Ribonucleic acid
SHV	Sulfhydryl variable
ST	Strain type
TEM	Temoniera
UoN	University of Nairobi
WGS	Whole genome sequencing
VIM	Veronaintegrin-encoded Metallo-β-lactamase

DEFINITION OF TERMS

Adapter trimming: cutting off the short adapter sequences from genomic sequences.

Antibiotic: A specific type of antimicrobial medication that inhibits bacterial growth, used to treat, and prevent bacterial infections.

Antibiotic susceptibility: the inability of bacteria to proliferate when an antibiotic is administered as per prescribed dosage.

Antimicrobial resistance: the ability of bacteria to survive and multiply in the presence of an antibiotic that they would be considered sensitive to.

Beta-lactam antibiotics: antibiotics with a beta-lactam ring in their molecular structure that prevent bacteria from synthesizing their cell walls.

De-multiplexing: processing of decoding barcode information after sequencing to know which sequences came from which samples.

Genome: the complete genetic information set that contains all the data needed for the organism to function.

Genes: the basic unit of heredity in a specific chromosome location that codes for a specific protein leading to a particular characteristic or function.

Hypervirulence: the ability of bacteria, viruses, or fungi to cause extremely severe illness.

Mutations: alterations in DNA sequences that may result in changes in the structure of a gene.

Multidrug- resistance: the ability of bacteria to acquire resistance to more than two classes of antimicrobial agents.

Molecular markers: specific regions in DNA that are used to express certain characteristics.

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ABSTRACT

Introduction: Intensive care units (ICUs) are hotspots for antibiotic resistance emergence, propagated by misuse and overuse of broad-spectrum antibiotics. The emergence of multidrug-resistant bacteria, including potentially dangerous hypervirulent strains result in severe and hard-to-treat infections, posing significant public health threats. The aim of this study was to establish the presence of hypervirulent Enterobacterales bacteria among isolates obtained from ICU patients at Kenya's Kenyatta National Hospital.

Methods: The study was a retrospective laboratory study that involved the analysis of 40 Extended Spectrum Beta-Lactamase (ESBL) and carbapenemase-producing Enterobacterales isolates from diverse clinical samples including tracheal aspirates, urine, blood and pus swabs. These isolates were subjected to culture, identification, and antibiotic susceptibility testing using VITEK®2. DNA was extracted and whole-genome sequencing was performed using the Oxford Nanopore MinION protocol. Bioinformatic tools ResFinder 2.1, Virulence Finder 2.0, Plasmid Finder databases 2.1, and the virulence factor database were used to analyze the bacterial genomes for AMR genes, virulence genes, and plasmids.

Results: Out of 33 viable isolates, 22 were successfully identified and sequenced, consisting of 15 *Escherichia coli* and 7 *Klebsiella pneumoniae* isolates. These isolates exhibited multidrug-resistant traits, with nearly complete resistance to beta-lactam antibiotics. Carbapenem resistance was rare, except in one *E. coli* isolate. No carbapenemase genes were found, however ESBL production was evident across the Enterobacterales isolates, with presence of genes encoding β-lactamases such as bla_{OXA-1} (13/22, 59%), $bla_{OXA-534}$ (3/22, 13%), $bla_{CTX-M-15}$ (18/22, 82%), bla_{EC} (13/22, 59%), bla_{TEM} (7/22, 32%), bla_{CMY} (1/22, 5%), bla_{SHV} (8/22, 36%), and ompK (6/22, 27%) encode ESBL production and consequently resistance to cephalosporins. Notably, key hypervirulent genes were identified among *E. coli* isolates, including attachment-fimH- (15/15, 100%), toxin production sat- (5/15, 33%), biofilm formation csgA- (14/15, 93%), and capsule formation kspE- (10/15, 67%). For *K. pneumoniae* isolates, notable efflux pump transporters were acrAB- (4/7, 57%), and mtrD- (1/7, 14%).

Conclusion: The existence of ESBL-producing Enterobacterales, associated with resistance to multiple classes of antibiotics and heightened virulence, presents a substantial healthcare risk for ICU patients. Therefore, health care workers should strictly observe infection prevention protocols and exercise judicious antibiotic usage to reduce the dissemination of AMR.

CHAPTER 1

1.1. INTRODUCTION

AMR poses a great threat to humanity, thus a growing worldwide concern. The effective measures implemented following the introduction of antibiotics to prevent and treat various infections in humans and animals are now under threat due to the emergence of AMR(1).

Infections due to drug resistant Enterobacterales are a leading cause of adverse health outcomes resulting in deaths, increased hospitalization duration, and high costs of treatment (1). In 2019 AMR resulted in 1.27 million deaths globally, surpassing deaths due to malaria (640,000) and HIV (960,000) (2). Reported deaths due to AMR in sub-Saharan Africa (SSA) were 4 times higher than in the high-income countries (2). Without efforts to mitigate AMR, it is projected that by the year 2050 one person could die every 3 seconds and up to 10 million people will die annually globally due to drug-resistant infections (3).

The increasing occurrence of multi-drug resistant microorganisms poses a safety concern for patients, healthcare professionals, and healthcare administrators at a global scale (4). Several investigations indicate a substantial surge in drug resistance rates within SSA. Notably, a study conducted on bloodstream infections at a major referral hospital in Ethipia revealed a rise in ESBL resistance, increasing from 0.7% to 30.3% among *E. coli* isolates and from 11.8% to 90.5% in *Klebsiella spp* (5). Kenya, like many other nations is grappling with this menace, and has reported presence of multi-drug resistant bacteria within hospitals and communities (4). A previous study on AMR within rural and urban hospitals and communities in Kenya showed a prevalence of 65% for ESBL-producing Enterobacterales colonization among hospitalized patients, along with 11% for carbapenem-producing Enterobacterales (6). This shows the burden of these resistant bacteria in hospital settings. Infection with these bacteria has been shown to result in severe and hard-to-treat infections, increased lengths of hospitalization and subsequently increased cost of treatment (7).

The global proliferation and dissemination of antibiotic-resistant bacteria is facilitated by repeated antibiotic exposure and irrational antibiotic use (8). Treatment of bacterial infections must be tailored to the causative pathogen (9). However, in low and middle-income countries, insufficient, unequipped and lack of adequate diagnostic facilities drives empirical treatment with broad spectrum antibiotics (10). Third-generation cephalosporins are also widely

available in developing countries, which has led to their increased and indiscriminate usage, increasing the danger of the emergence of multidrug-resistant bacteria (11). In hospital settings, up to 70% of patients in ICUs are treated with third line antibiotics, among which are last reserve antibiotics such carbapenems and colistin which are last reserve antibiotics (12). Due to the overuse of broad-spectrum antibiotics, ICUs are hotspots for the establishment of antibiotic resistance, putting ICU patients at an elevated risk of colonization and infection with MDR bacteria (13). These antibiotics are important as the last treatment options for severe bacterial infections that are not susceptible to other classes of antibiotics. Third-line antibiotics are now being abused and misused, causing selective antibacterial pressure and the rise of drug and multidrug resistant bacteria like ESBL, and carbapenem-producing Enterobacterales (CPE). Therefore, it is not surprising that there are reports of these multidrug resistant bacteria in clinical settings all over the world, and they are not limited by geographic borders (14,15).

Enterobacterales are particularly concerning given their quick ability to colonize and spread, as well as the limited treatments available for drug resistant Enterobacterales. There is evidence that Enterobacterales can quickly acquire resistance genes leading to acquisition of antibiotic resistance to multiple classes of antibiotics. Owing to this, infections due to these resistant bacteria are difficult to treat and have high fatality rates (16,17).

Hypervirulent Enterobacterales bacteria harbor multiple AMR and virulence genes that aid in evading the host immune system (18). These bacteria are linked to invasive infections with significantly higher rates of morbidity and mortality, a phenomenon observed in hospitals worldwide. These strains are responsible for hospital outbreaks, leading to difficult-to-treat infections, and can readily cause severe disease in immunocompromised individuals, hospitalized patients, as well as in healthy individuals within the community. Among the hypervirulent bacterial strains in Enterobacterales documented are *E. coli* ST131, ST15, *K. pneumoniae* ST23, ST258, and *Enterobacter* ST133 (19–21). AMR needs to be monitored to develop targeted interventions because the rise in hypervirulent Enterobacterales poses a threat with detrimental outcomes to both human and animal health.

1.2. JUSTIFICATION

A recent study conducted in Kenya has revealed that the occurrence of ESBL-producing Enterobacterales and carbapenemase-producing Enterobacterales is notably higher in hospital environments, at 65% and 11%, respectively, in contrast to lower percentages of 49% and 1%, respectively, observed in community settings (6). Further, up to 70% of critical care patients

receive empirical treatment with broad-spectrum antibiotics, which can consequently select for resistant and potentially hypervirulent strains of ESBL- and carbapenemase-producing bacteria (12). These infections have higher morbidity and mortality rates and increase the costs of treatment among hospitalized patients. With poor infection prevention control practices, these bacteria can be easily transmitted among ICU patients resulting in hospital outbreaks.

1.3. SIGNIFICANCE

The diversity and distribution of hypervirulent ESBL and carbapenemase-producing Enterobacterales in Kenyan hospitals is unknown. It remains unclear which strains circulate among key patient populations, e.g., ICU patients, and what the underlying genomic determinants are. WGS was used to characterize ESBL and carbapenemase genes among Enterobacterales strains isolated from ICU patients at the Kenyatta National Hospital (KNH) between January-June 2021 to detect presence of hypervirulent bacteria. The findings from this study will support Kenya's AMR surveillance goals, one of which is the early detection of emerging antibiotic-resistant bacterial strains and will further provide a basis for setting up effective targeted control measures in the ICUs within KNH.

1.4. STUDY QUESTIONS

i. What are the antibiotic susceptibility profiles of ESBL and carbapenemase-producing Enterobacterales isolates among ICU patients at KNH?
ii. What are the phenotypic and genotypic differences among hypervirulent ESBL and carbapenemase-producing Enterobacterales isolates?
iii. What is the relative abundance of hypervirulent genes among ESBL and carbapenemase-producing Enterobacterales isolates?

1.5. STUDY OBJECTIVES

1.5.1. Broad objective

To determine the presence and the genomic characteristics of hypervirulent bacteria among Enterobacterales bacteria isolated from ICU patients at the Kenyatta National Hospital between January and June 2021.

1.5.2. Specific objectives

1. To describe the antibiotic susceptibility profiles of the ESBL and carbapenemase-producing Enterobacterales isolates.
2. To describe the phenotypic and genotypic differences in the hypervirulent bacteria among ESBL and carbapenemase-producing Enterobacterales isolates.

3. To determine the relative abundance of hypervirulent genes in ESBL and carbapenemase-producing Enterobacterales isolates.

CHAPTER 2

2. LITERATURE REVIEW

2.1 AMR and infections in ICU

AMR poses a formidable global health threat, affecting individuals across diverse healthcare settings. The alarming escalation of resistant strains is underscored by the World Health Organization, noting its ubiquity in every country. Contributing factors include the misuse of antibiotics in human and animal health, coupled with suboptimal infection prevention practices. Globally, the impact of AMR is profound, resulting in heightened mortality rates, prolonged illnesses, and increased healthcare expenditures.

Within ICUs, the impact of AMR is particularly pronounced. The ICU environment, characterized by high antibiotic usage, invasive procedures, and close patient proximity, creates a breeding ground for the rapid transmission of resistant strains. ICU patients are at an increased risk of both colonization and acquisition of bacterial infections caused by multidrug-resistant pathogens (13). Critically ill patients are often subjected to empirical antibiotic therapy, intensifying the selection pressure for resistance (2). Additionally, risk of infection among these ICU patients is also due to use of respiratory ventilators, urinary catheterization, central venous catheterization increased lengths of hospitalization, high patient-staff contact, and their critical health states (3).

In Kenya, over 70% of patients in ICUs receive antibiotics daily during their hospitalization and studies have shown that 30% to 60% of antibiotics use in ICUs is unnecessary (3,22). The misuse and excessive usage of antibiotics is among factors attributed to the increased rise of bacteria strains that are resistant to antibiotics. These bacteria are frequently isolated in healthcare facilities, primarily in ICUs in which there is frequent and indiscriminate administration of large amounts and last-line antibiotics as a prophylactic measure due to the critical state of the patients (11,23). Drug-resistant Enterobacterales such as *E. coli* and *K. pneumoniae* are the most isolated bacteria in ICUs and strikingly hypervirulent strains of Enterobacterales are also being isolated from patients and hospital surfaces (24). The cross-transmission of resistant bacteria within ICUs poses a significant challenge, leading to outbreaks and limiting treatment options.

Hypervirulent Enterobacterales strains carry multiple AMR and virulence genes and are associated with hospital outbreaks, severe illness, and death (25). These include *E. coli* strain type (ST)131, ST15 (20), *K. pneumoniae* ST23, ST 258 (19), and Enterobacter ST133 (21).

These hypervirulent strains are implicated in invasive disease syndromes such as pneumonia, bacteremia, urinary tract infections, wound and soft tissue infections and liver abscess (19–21).

Asia and the Middle East have seen the emergence of carbapenem resistance due to the high prevalence of ESBL bacteria and the accompanying rise in carbapenem use (26). ESBL infections rates of up to 80% have been reported in Ethiopia and up to 60% with carbapenemase producing bacteria among patients in ICU(18). Research on AMR in Kenyan hospitals and communities reported ESBL-producing Enterobacterales prevalence of 65% and carbapenem-producing Enterobacterales 11% colonization among hospitalized patients (6).

2.2 Treatment of severe ESBL and carbapenemase-producing bacterial infections

ESBL and carbapenemase-producing bacteria, commonly encountered in Enterobacterales such as *E. Coli* and *K. pneumoniae*, exhibit resistance to beta-lactam antibiotics. The ESBL enzymes, often plasmid-mediated, confer resistance by modifying the antibiotic's beta-lactam ring (27). Apart from beta-lactams, ESBL producers may also display co-resistance to other antibiotic classes, complicating treatment options. Resistance to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole is frequently observed, limiting the available therapeutic options. In ICUs, antibiotics are crucial for treating severe ESBL and carbapenemase-producing bacterial infections. Empirical therapy frequently involves the use of carbapenem antibiotics like meropenem or combination therapy with a beta-lactam/beta-lactamase inhibitor (e.g., piperacillin-tazobactam) in cases of suspected ESBL-producing bacteria. For infections caused by carbapenemase-producing bacteria, treatment options become more limited due to the high level of resistance, however colistin is recommended (28). Other options may include fluoroquinolones, folates, aminoglycosides, or combinations of these agents. (29).

2.2.1 Monobactams

Monobactams differ from other beta-lactam antibiotics like penicillins and cephalosporins by having a distinct single beta-lactam ring structure. Monobactams like aztreonam interact with penicillin-binding proteins (PBPs), affecting the development of the bacterial cell wall. Notably, they are less susceptible to degradation by beta-lactamases in comparison to other beta-lactam antibiotics, rendering them efficacious against certain ESBL producing bacteria (30).

2.2.2 Beta-Lactams

Beta-lactams, including cephalosporins and carbapenems, are the cornerstone of ICU antibiotic therapy. They disrupt bacterial cell wall synthesis by binding to PBPs. Beta-lactam antibiotics are a good option for empirical therapy because of their wide spectrum of activity and effectiveness against both gram-positive and gram-negative bacteria (31).

2.2.3 Carbapenems

Carbapenems, like meropenem and ertapenem, are reserved beta-lactam antibiotics. They are effective against both gram-positive and gram-negative bacteria. Their mechanism of action involves PBP binding and thus disrupting bacterial cell wall synthesis and resulting in cell death (31).

2.2.4 Colistin

Colistin is considered a last-resort antibiotic when no other effective treatment options are available. It is often used in cases of severe infections brought on by bacteria that are MDR or extensively drug resistant (XDR), such as carbapenem-resistant Enterobacterales. It compromises the integrity of the bacterial cell membrane by creating pores, leading to cell death. Colistin is effective against a wide range of bacteria, including those resistant to other classes antibiotics (32).

2.2.5 Combination Therapy

Combination therapy involves use of two or more antibiotics in severe bacterial infections or when dealing with ESBL bacteria and carbapenem-resistant bacteria. Combination therapies include a beta-lactam antibiotic paired with a beta-lactamase inhibitor to enhance their effectiveness against ESBL and some carbapenemase producers. Examples include Ampicillin/sulbactam, Amoxicillin/clavulanic acid, and Piperacillin/tazobactam (31).

2.2.6 Folates

Folate antibiotics such as trimethoprim-sulfamethoxazole target bacterial dihydrofolate reductase (DHFR), the key enzyme in the folate metabolic pathway. By inhibiting DHFR, these antibiotics interfere with the synthesis of tetrahydrofolate (THF), an essential cofactor in the biosynthesis of nucleic acids (DNA and RNA). This disruption ultimately impairs bacterial DNA replication and formation of proteins. Their spectrum of activity includes both gram-negative and gram-positive bacteria (33).

2.2.7 Aminoglycosides

Aminoglycosides, such as gentamicin, amikacin, and kanamycin act binding to the bacterial ribosome (30S subunit), interfering with protein synthesis, and leading to the production of faulty, nonfunctional proteins and ultimately results in bacterial cell death. Aminoglycosides have a broad-spectrum of activity on gram-negative bacteria and some gram-positive bacteria (34).

2.2.8 Fluoroquinolones

Fluoroquinolones, like ciprofloxacin, obstruct DNA replication and repair by inhibiting bacterial DNA gyrase and topoisomerase IV and consequently resulting in cell death. They also possess a broad-spectrum activity (35).

2.3 Antibiotic resistance in Hypervirulent Enterobacterales

Hypervirulent bacteria have acquired various antibiotic resistance mechanisms such as the generation of beta-lactamase enzymes, efflux pump systems, modifications to antibiotic target sites, and the presence of plasmid-borne resistance genes. The primary resistance mechanism observed in Enterobacterales involves the production of enzymes that render antibiotics inactive, along with various non-enzymatic processes (36). Resistance may be increased intrinsically due to chromosomal gene mutations that mediate the expression of efflux pumps, reduce cellular permeability, modify drug-target active sites, or are acquired through other mechanisms. It might also occur due to the cross-transfer of DNA transposable elements carrying drug resistance genes, such as plasmids that encode ESBL and carbapenemase enzymes, aminoglycosides inactivating enzymes, or non-enzymatic mechanisms like the generation of quinolone resistance genes (37).

2.3.1 Extended-spectrum beta-lactamases (ESBL)

ESBL enzymes in bacteria act against beta-lactam antibiotics such as penicillin and cephalosporins. The enzymes are site-specific β-lactamase inhibitors that render these antibiotics ineffective. Worldwide reports of these drug resistant enzymes being found among humans, animals, and in the environment, all point to a One health issue. Most ESBL-producing bacteria are in the Enterobacterales order, with *E. coli* being among bacteria ESBL producing bacteria (38). These organisms have also been reported in communities and healthcare facilities in Africa (39), but a high incidence of ESBL-producing Enterobacterales

has been identified in healthcare facilities, notably in ICUs (40). This is linked to increased antibiotic consumption and prolonged hospitalization periods, which foster the spread of these antibiotic-resistant bacteria. Due to the high cost of treatment and potential threat to patient lives, the presence of ESBL-producing Enterobacterales in hospitals can burden healthcare systems (1).

ESBLs can be stratified into two classes, class A and class D, depending on their active sites. Serine in the active site of Class A enzymes preferentially hydrolyzes penicillin. Penicillinase, TEM-1, and SHV-1 are examples of class A -lactamases, whereas OXA is a class D enzyme (41). Class A ESBLs and their variants are produced by the majority of Enterobacterales. These enzymes are majorly plasmid encoded facilitating their high transferability within clinical settings. Nosocomial outbreaks in ICU associated with ESBLs producing Enterobacterales have also been reported, presenting a public health risk (42).

2.3.1.1 TEM

TEM accounts for approximately 90% of ampicillin resistance in *E. coli* and *K. pneumoniae* and is the most frequently identified ESBL gene (41). Additionally, it has been discovered in *Salmonella* spp., *Proteus mirabilis*, *Proteus rettgeri*, *Pseudomonas aeruginosa*, and *Morganella morganii*. It majorly hydrolyses penicillins and first generation cephalosporins. Several variants of TEM are emerging, and this is because of selective pressure on use of various β-lactams and not a single β-lactam. TEM producing clinical isolates such as *E. coli* are reportedly resistant to inhibitors hence a challenge in clinal settings (41).

2.3.1.2 SHV

SHV is plasmid encoded and predominantly seen in *K. pneumoniae*, while *Citrobacter diversus*, *E. coli*, and *P. aeruginosa* have also been shown to harbor it. Although SHV comes in a variety of forms, they are not comparable with TEMs. The variations occur from a serine for glycine exchange at position 238 (which facilitates the hydrolysis of ceftazidime) or a lysine for glutamate exchange at position 240 (which facilitates the hydrolysis of cefotaxime) (41).

2.3.1.3 CTX-M

This enzyme has 40% similarity to TEM and SHV (43). They are plasmid encoded and mostly hydrolyze cefotaxime (41). They are primarily seen in strains of *E. coli* and *Salmonella enterica* serovar *Typhimurium*, among other Enterobacteriaceae.

2.3.2 Carbapenemase

Carbapenemase enzymes are stratified into three different classes of β -Lactamase enzymes namely class A, B, and D. Both class A and class D are similar due to the presence a serine residue at their active sites that helps in the β -Lactam ring opening during antibiotic inactivation, thus are called serine β-lactamases (SBLs), whereas class B is distinguishable by the presence of metallo-β-lactamases (MBLs) whose active sites utilizes zinc ion for bond hydrolysis (44).

2.3.2.1 Class A carbapenemases

These enzymes are either plasmid or chromosomally encoded, and include *K. pneumoniae* carbapenemases (KPCs), imipenem-hydrolyzing β-lactamase (IMI), Guiana extended spectrum carbapenemase (GES), *Serratia fonticola* carbapenemase, *Serratia marcescens* enzyme and non-metallo-carbapenemase-A. The most prevalent of these is KPC, and many variants of it have been identified (45). *Bla*KPC genes are plasmid-encoded and can easily be transferred across different species of bacteria through horizontal gene transfer (44). Strains carrying this gene are normally multidrug-resistant (MDR) as they have acquired resistance to almost all β -lactams antibiotics as well as other groups of antibiotics such as aminoglycosides, fluoroquinolones, and trimethoprim/sulfamethoxazole (44). IMI strains are usually sensitive to extended spectrum cephalosporins, however show resistance to Imipenem, and since they are chromosomally embedded, they are of less clinical relevance (44). GES are plasmid encoded and contain point mutations which drives its activity in conferring resistance to carbapenem. Clavulanic acid partially inhibits Class A carbapenemases (45).

2.3.2.2 Class B carbapenemases

This group primarily consists of metallo-lactamases (MBLs), which includes the Verona integron-encoded metallo-lactamase (VIM), imipenem-resistant pseudomonas-type carbapenemases (IMP types), and most recently discovered, the New Delhi metallo-lactamase-1 (NDM-1) type (46). The first case of MBL was described in 1966 in Bacillus cereus, which was chromosomally encoded (47). However, since then, there has been an increase in plasmid encoded MBLs strains in clinical samples that are associated with life-threatening infections, particularly among ICU patients. Except for monobactams, most β-lactam antibiotics are ineffective against bacteria that produce MBLs (47).

VIM was initially discovered in Verona, Italy, in 1999 in *P. aeruginosa* and this has been detected in most countries including Kenya (46) mainly implicated in nosocomial infections and especially in hospitalized patients with wound infections. Except for aztreonam, enteric

bacteria including *K. pneumoniae* and *E. coli* that harbor the New Delhi MBL (NDM) gene are frequently resistant β-lactams. This AMR gene is of great concern due to its rapid global spread and resistance to several classes of antibiotics. Class B Carbapenemases are inhibited by EDTA but not clavulanic acid (45).

2.3.2.3 Class D carbapenemases

This comprises Oxacillinase (OXA) enzymes that hydrolyze Oxacillin. For penicillins, the precursor enzyme OXA-2 exhibits significant hydrolysis activity, whereas for carbapenems, it exhibits weak hydrolysis activity. In Kenya, most investigations have documented the existence of microorganisms that produce these enzymes (44). Since β-lactamase inhibitors do not block these enzymes and there are currently no known inhibitors for them, this poses a danger to human health with potentially fatal outcomes. They are either chromosomally or plasmid encoded.

2.3.3 Alteration of target sites

Alteration of target sites involves genetic mutations that change the structure of target sites for antibiotic action, reducing or eliminating antibiotic binding. Bacteria can modify various target sites, including ribosomal subunits, enzymes, and proteins, to decrease antibiotic binding affinity. Alterations in bacterial ribosomal subunits results in resistance to antibiotics like macrolides, tetracyclines, and aminoglycosides. Mutations in bacterial DNA gyrase and topoisomerase IV, which are the targets of fluoroquinolone antibiotics rendering them ineffective. Modifications of PBPs result in resistance to beta-lactam antibiotics (48,49).

2.3.4 Efflux pumps

Efflux pump systems are specialized membrane proteins that actively remove antibiotics from bacterial cells, diminishing the amount of drug inside the cell. These pumps can provide resistance against a broad spectrum of antibiotics, irrespective of their specific mechanisms of action. They frequently play a role in MDR, where the bacteria develop resistance to multiple antibiotic classes, and MDR efflux pumps can simultaneously expel various antibiotics (49).

2.3.5 Biofilm formation

Biofilms are organized bacterial colonies coated in an extracellular polymeric substance (EPS) protective matrix. A biofilm is a multicellular, three-dimensional structure formed when bacteria cling to surfaces and one another. They secrete EPS, which creates a protective shield that can limit the penetration of antibiotics (49,50).

2.4 Virulence genes

Hypervirulent Enterobacterales strains are characterized by the presence of various virulence factors that enable them to evade host immune responses, invade tissues, and cause rapid disease progression (51). Among the most prominent virulence genes are those encoding for adhesins (e.g., fimH, fyuA), toxins (e.g., sat, hlyE), iron acquisition systems (e.g., iutA), biofilm formation (e.g., csgA, mrkC), and capsule formation (e.g., rmpA) (20,25). These genes contribute to bacterial survival and virulence, allowing hypervirulent strains to outcompete commensal strains and establish infections. These genes can be found on mobile genetic elements such as plasmids or genomic islands, and they often encode virulence factors such as enzymes, iron chelation, adhesins, toxins, and secretion systems that enable the bacteria to attach to host cells, evade the immune system, and damage host tissues. Additionally, certain virulence genes in Enterobacterales, such as enzymes encoding ESBLs and carbapenemases may also confer resistance to antibiotics, resulting in difficult-to-treat infections (18).

2.5 Plasmids in Enterobacterales

Plasmids are crucial genetic elements in bacteria that play a significant role in horizontal gene transfer, including the transmission of antibiotic resistance genes (52). Incompatibility (Inc) plasmids, a widely studied subgroup, are known for their diverse genetic cargo, including resistance determinants, virulence factors, and other adaptive traits. Inc plasmids are widespread in Enterobacterales, comprising various incompatibility groups such as IncF, IncI, IncP, IncN, IncH, and IncA/C (53). These plasmids often carry a combination of resistance genes, which can confer multidrug resistance. Their ability to host diverse genetic elements makes them essential players in bacterial adaptation. Horizontal gene transfer accelerates the spread of antibiotic resistance genes, facilitating the development of multidrug-resistant strains. The ease with which Inc plasmids can move between bacteria contributes to the rapid dissemination of resistance, posing a significant challenge for antimicrobial therapy (53).

2.6 Detection of AMR and virulence genes

Bacterial drug resistance can be identified either phenotypically (through culture and susceptibility) or genotypically (by characterization of AMR genes). Antimicrobial susceptibility tests (ASTs) using several techniques, such as the antibiotic disc diffusion test, the minimum inhibitory concentration in broth or agar media, the E-test, and the automated VITEK® 2 system, are used to detect resistant phenotypes (54).

Genomic identification of the AMR and virulence genes needs molecular techniques, which may include amplification of target genes, whole-genome sequencing (WGS), metagenomics, random amplified polymorphic DNA (RAPD), PCR-RFLP, RAPD paired with restriction

fragment length polymorphism. While more expensive than conventional procedures, molecular techniques allow for quick detection of slow-growing organisms and genomic identification of bacterial AMR and virulence genes in non-cultivable organisms (55).

2.7 Bacterial Whole-genome sequencing using Oxford nanopore technology.

Whole genome sequencing (WGS) has been shown to be valuable as a method for routine infection control and, for bacterial and viral pathogens identification, and as the main diagnostic tool to identify antibiotic resistance. Bacterial whole genome sequencing has significantly improved the knowledge of the genetics of various bacterial infections and led to the identification of novel-new target sites for antibiotics (56). Given the present trend of developing antibiotic resistance, particularly multidrug resistance in bacteria, the importance of this application cannot be overlooked.

Information needed to identify, speciate, and characterize AMR genes, virulence genes and mobile genetic elements is present in the genomic data obtained from bacterial WGS. Bacterial WGS is now possible even in developing nations due to the decreased cost of sequencing brought on by technological improvements (57). Among the third-generation sequencing techniques using nanopore technology for nucleic acid analysis is the Oxford Nanopore Technology (ONT) MinION (57). At its core, the technology uses a nanopore protein in a polymer membrane that is electrically resistant. DNA strand complexes are applied to the membrane-binding nanopore for sequencing. The movement of individual DNA molecules within the nanopore causes some electrical current disruptions. Single nucleotide bases are calculated by calculating the difference in the current flowing through the membrane pore. The Oxford Nanopore portable pocket-size MinION has demonstrated the potential of fully sequencing genomes in a single assay (58).

WGS emerges as a pivotal future direction for AMR surveillance, providing unparalleled precision in characterizing resistance profiles and enabling real-time monitoring of resistance dynamics. With its ability to uncover comprehensive genomic insights, WGS holds the promise of transforming our understanding of AMR evolution, facilitating targeted interventions, and bolstering global efforts to combat AMR.(59). In Kenya, studies have applied the technique in identification of AMR genes including carbapenemase genes and in understanding the genomic transmission pathways of drug-resistant bacteria (15,60).

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Study design

Retrospective laboratory study.

3.2 Study site

KNH hospital is a 1,800-bed referral and tertiary-care hospital which is also the Teaching University Hospital. The ICU capacity has a total of 20 beds. The study used archived bacterial Enterobacterales isolates obtained from patients admitted to ICUs in KNH. Isolates were obtained from various samples including tracheal aspirates, blood samples, pus swabs and urine samples. The isolates were analyzed in the bacteriology lab at the University of Nairobi Institute of Tropical and Infectious Diseases building, within the KNH complex.

3.3 Study population

These included ESBL and carbapenemase-producing Enterobacterales isolates. For this study, hypervirulent isolates were classified by the presence of multiple AMR genes resulting in multidrug- resistance and presence of key virulence genes for invasion, iron chelation, toxin production, biofilm formation and immune evasion (20,21).

3.3.1 Inclusion criteria

- All ESBL and carbapenemase-producing Enterobacterales isolates.

3.3.2 Exclusion criteria

- Enterobacterales isolates which on revival did not grow.
- Enterobacterales isolates which on sequencing had low quality reads.

3.4 Sample size

For this study, sample size referred to all ESBL and carbapenemase-producing Enterobacterales isolated from the ICU between January and June 2021. All viable bacterial isolates that met the inclusion criteria above were characterized; these were 40 in total.

This sample size was determined based on the number of ESBL and carbapenemase-producing Enterobacterales during the specified time frame. The frequency of these isolates was 40 and thus all available isolates within the identified period were utilized in the study.

3.5 Sampling technique

Convenience sampling was employed to select ESBL and carbapenemase-producing Enterobacterales isolates that were included in the study, and thus all, 40 isolates were included.

3.6 Variables

3.6.1 Independent variables

- Patient factors: Gender and age (abstracted from main study)
- Sample type (abstracted from main study)

3.6.2 Dependent variables

- Enterobacterales bacteria isolate types.
- Antibiotic susceptibility profile.
- AMR and virulence genes associated with hypervirulence.

3.7 Study materials

These included the bacterial isolates obtained from the main study, lab supplies for bacterial culture and DNA extraction, MinION flow cell for WGS, and patient data including age, gender and location of admission that were abstracted from existing data in the main study.

3.8 Laboratory procedures

3.8.1 Revival of Enterobacterales isolates.

Isolates archived in skimmed milk-tryptone-glucose-glycerol broth and stored at -80 ^{o}C were thawed at ambient temperature 24 – 25 °C for 15 minutes. The isolates were then plated on MacConkey agar and then kept at 37 °C for 18–24 hours of incubation. Viable isolates were sub-cultured on Tryptic soy agar (TSA) and kept at 37 °C for 18–24 hours of incubation. Purified colonies from TSA were further sub-cultured on Tryptic soy broth at 37 °C for 18– 24 hours (Appendix 1).

3.8.2 Identification confirmation and antibiotic susceptibility testing.

Isolate identification and AST were performed using the automated VITEK®2 system. The isolates were emulsified in 0.5% saline to create bacterial suspensions, and a densitometer was used to standardize the turbidity to 0.5 McFarland's. VITEK®2 GN ID and GN 83 AST cards were dipped into the suspension and loaded onto the VITEK®2. All isolates were tested for antibiotic susceptibility against a panel of 17 antibiotics including amikacin, ampicillin, ampicillin/sulbactam, amoxicillin/clavulanate, aztreonam, ceftazidime, ceftriaxone, cefotaxime, cefuroxime, cefuroxime/axetil, cefazolin, cefepime, ciprofloxacin, gentamicin,

meropenem, piperacillin/tazobactam, and trimethoprim/ sulfamethoxazole. The AST results were interpreted according to the 2020 Clinical and Laboratory Standards Institute guidelines (61). Isolates were grouped as ESBL-producing if susceptible carbapenems tested but non-susceptible (intermediate or resistant) to ceftriaxone and/or ceftazidime. Isolates were classified as carbapenemase-producing if resistant to the carbapenem (meropenem) tested.

3.8.3 DNA extraction

DNA extraction for the isolates was done by utilizing the Qiagen DNeasy UltraClean Microbial kit according to the instructions provided by the manufacturer. Quantification of the extracted DNA was done using the Qubit 4 fluorometer. DNA concentration was normalized to ≥ 50 ng/µL by adding nuclease free water for downstream molecular analysis. The full procedure for genomic bacterial DNA extraction and quantification, (Appendix 2 and 3).

3.8.4 Genomic sequencing of Enterobacterales isolates

The extracted DNA was subjected to repair and end-prep processing using the NEBNext End repair module (E7546, New England Biolabs, Ipswich, MA, USA) and NEBNext FFPE DNA Repair Mix (M6630, NEB), End-repair DNA purification was carried out using Agencourt AMPure-XP beads (A63880, Beckman Coulter). DNA libraries were prepared using the Oxford Nanopore genomic sequencing kit, SQK-LSK109 (ONT, UK) and native barcoding expansion kits (EXP-NBD104, EXP-NBD114) according to the guidelines provided by the manufacturer (Appendix 4).

To ligate prepared genomic DNA to native barcode adapters, Blunt/TA Ligase Master Mix (M0367S, NEB) were utilized. The barcoded DNA was further purified using the AMPure XP beads. Quick T4 DNA ligase was used to join adaptors to the pooled DNA (E6056). After priming the MinION flow cell using the flush buffer and flush tether, the barcoded pooled DNA was loaded onto the primed MinION flow cell for whole genome sequencing (WGS).

3.9 Data analysis

The data was entered, coded, and cleaned into a Microsoft Excel spreadsheet. The study investigator reviewed data for completeness, checked for missing information and removed duplicate data. Data analysis was done using bioinformatics software and descriptive statistics done using Microsoft Excel.

Objective 1: To describe the antibiotic susceptibility profiles of the ESBL and carbapenemase-producing Enterobacterales.

Hypervirulent bacteria have demonstrated the presence of numerous AMR genes that could potentially provide resistance against various antibiotic classes. To assess the phenotypic susceptibility of these bacteria to antibiotics, their response to a range of antibiotics was analyzed using the VITEK®2 GN 83AST cards.

Objective 2: To describe the phenotypic and genotypic differences in the hypervirulent strains among ESBL and carbapenemase-producing Enterobacterales.

Whole genome sequence data analysis: Raw sequence data was analyzed using the MinKNOW program (Oxford Nanopore technologies, UK, Oxford Science Park) and summarized using the NanoPlot data plotting tool (https://github.com/wdecoster/NanoPlot). Base-calling was done using guppy base caller (https://github.com/nanoporetech). De-multiplexing and adapter trimming were done using Porechop (https://github.com/rrwick/Porechop), a tool for demultiplexing reads from FASTQ files. De-novo genomes were assembled and aligned using the Canu software (https://github.com/marbl/canu). Assembly characteristics were checked using quast (http://bioinf.spbau.ru/quast). Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/) was used to comprehensively identify and analyze AMR genes in addition to screening the sequences using ResFinder version 2.1, Virulence Finder version 2.0, Plasmid Finder databases version 2.1 and virulence factor database.

Isolates were considered potentially hypervirulent based on AMR and select virulence genes. These included bacteria that had multiple AMR genes and thus exhibited MDR phenotypic characteristics and presence of key virulence genes for invasion (fyuA), iron chelation (iutA), attachment (fimH), toxin production (sat), efflux transporters (acr, ibeA/B, waa) and immune evasion (traT). The proportion of isolates with these genes was divided by the number of Enterobacterales isolates (n/N).

Co-occurrences of antibiotic resistance genes, virulence, and plasmids in the bacteria were determined by screening the sequences using ResFinder 2.1, Virulence Finder 2.0, Plasmid Finder databases 2.1, and virulence factor database databases. Identified AMR and virulence genes in the isolates have been described using heatmaps.

Objective 3: To determine the relative abundance of hypervirulent genes ESBL and carbapenemase-producing Enterobacterales isolates.

Relative abundance of the hypervirulent genes was determined by calculating (x/N*100), where 'x' represented the count of identified hypervirulent bacteria, and 'N' was the total number of successfully sequenced isolates. Tables have been used to illustrate the descriptive statistics of the hypervirulent genes found among the Enterobacterales isolates.

3.10 Quality control/ quality assurance

American Type Culture Collection (ATCC) BAA-196 *E. coli* and 700603 *K. pneumoniae* strains organisms were used as the positive control whereas DNA/RNA free water was used as the negative control in the molecular analysis. Molecular analyses were performed by a trained molecular biologist and microbiologist. Approved standard operating procedures were followed. Materials and reagents were prepared following manufacturer's instructions and prescribed SOPs. All equipment were calibrated and validated prior to running the tests.

3.11 Ethical Considerations and approval

Authorization to conduct the study was obtained from KNH-UoN Ethics and Research Committee, approval number P88/02/2023 and National commission for science, technology, and innovation (NACOSTI) License number NACOSTI/P/23/28006 (Appendix 5). The research study used archived samples from the primary study approved the KNH-UoN ERC (P632/11/2020). All the laboratory procedures adhered to the basic principles of good clinical laboratory practice. The data was kept in a password-protected computer that was only available to the study investigator and did not include any patient identifiers.

3.12 Data storage and security

Data variables were collected on Microsoft Excel database for data cleaning, storing, monitoring, and manipulation. For confidentiality, laboratory isolated IDs were used instead of direct patient identifiers such as names. The computer used for the data storage was password protected and was only accessible to the study principal investigator and the data scientist. Data was backed up weekly onto an external hard drive, which was stored securely.

CHAPTER 4

4 RESULTS

4.1 Enterobacterales isolates

A total of 40 isolates were available for analysis and were all included in the revival process. The Enterobacterales isolates archived at -80 °C in skimmed milk were thawed and revived on MacConkey agar plates. Only 33 isolates were viable after culture. After incubation at 37 °C for 24 hours, the isolates were presumptively identified based on their colonial morphology, i.e., large, pink colonies for *E. coli* (Figure 1A) and large pink and mucoid colonies for *Klebsiella* spp. (Figure 1B)**.**

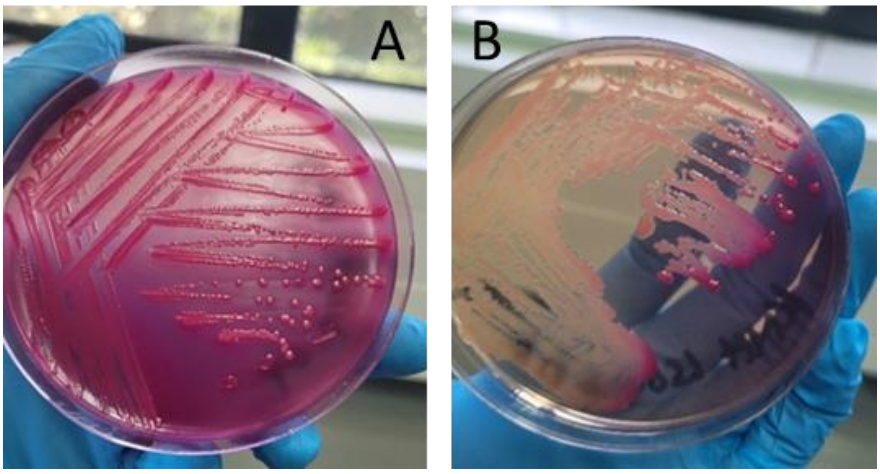


Figure 1: Growth of the presumptive *E. coli* and *Klebsiella* spp. on MacConkey agar.

4.2 Phenotypic analysis of AMR of the clinical isolates

VITEK®2 confirmatory ID of the presumptive Enterobacterales on culture plates identified 2 types of bacterial organisms *E. coli* (n = 15) and *K pneumoniae* (n = 7). All the 22 isolates had MDR phenotypes with resistance to more than three antibiotics in different classes. One Enterobacterales isolate E. coli_KNH_13 was resistant to all the antibiotics tested. Resistance of more than 80% among the isolates was observed with amikacin, aztreonam, ceftazidime, ciprofloxacin, ceftriaxone, cefotaxime, cefuroxime, cefuroxime/axetil, cefazolin, ampicillin/sulbactam, sulfamethoxazole/trimethoprim (Figure 2 and Figure 3).

Objective 1: Antibiotic susceptibility profiles of the isolates

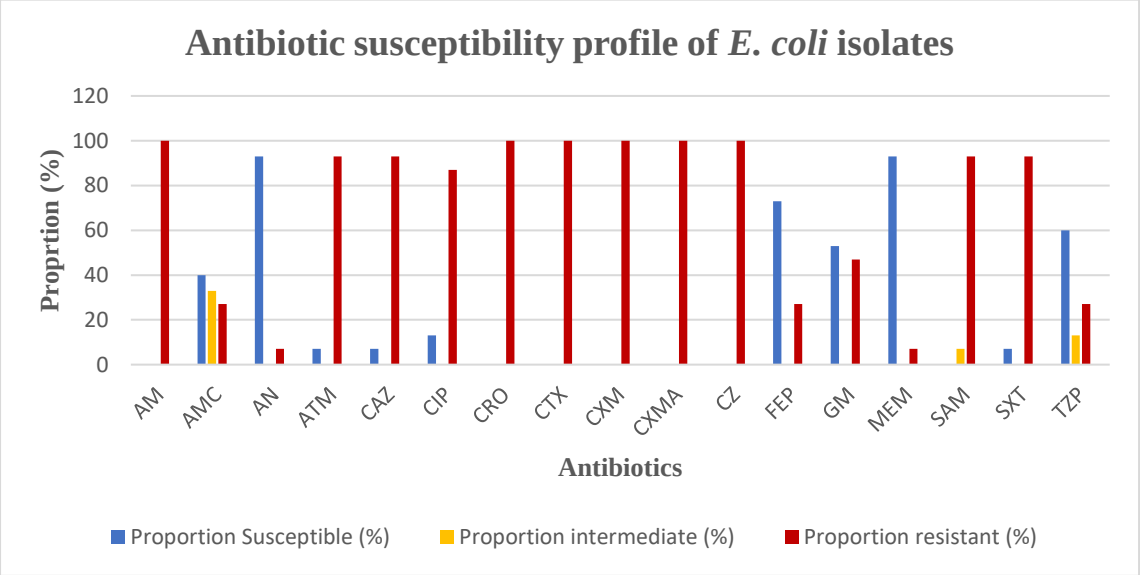


Figure 2: Antibiotic susceptibility profile of the *E. coli* isolates

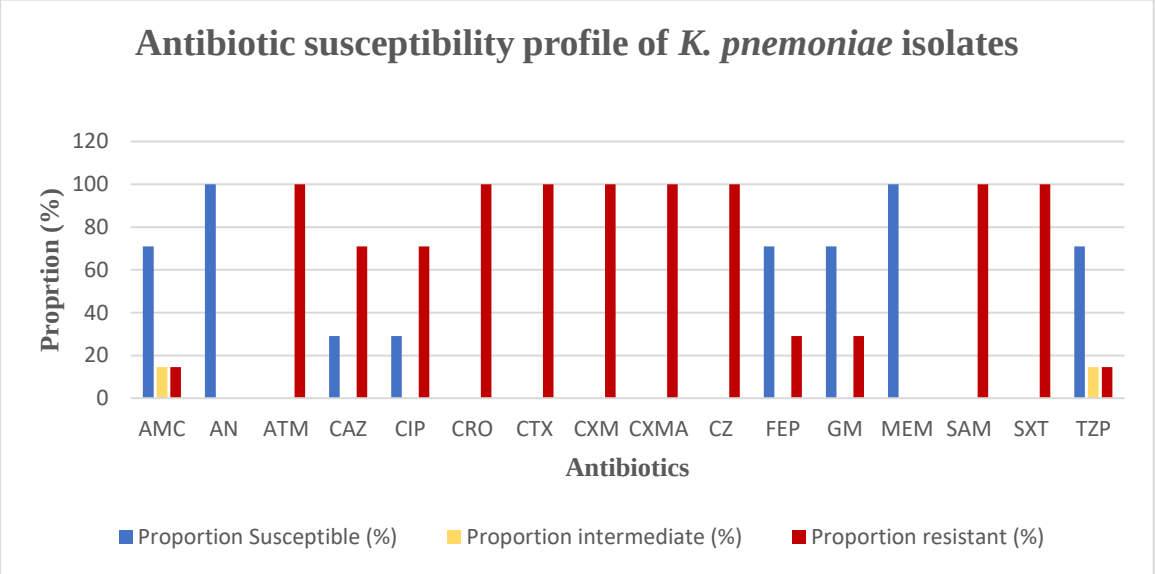


Figure 3: Antibiotic susceptibility profile of the *K. pneumoniae* isolates

Antibiotic: AM, ampicillin; AMC, amoxicillin/clavulanate, AN, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; CXMA, Cefuroxime/Axetil; CZ, cefazolin; FEP, cefepime; GM, gentamicin; MEM, meropenem; SAM, ampicillin/sulbactam; SXT, sulfamethoxazole/trimethoprim, TZP, piperacillin/tazobactam.

K. pneumoniae bacteria has been established to have intrinsic resistance to ampicillin and thus data on ampicillin resistance omitted (62).

Table 1: Antibiotic classes and observed resistance in the isolates.

Antibiotic classification	Antibiotic	Resistant *E. coli* (n=15)	Resistant *K. pneumoniae* (n=7)
Monobactam	Aztreonam	14 (93%)	7 (100%)
Aminopenicillin	Ampicillin	15 (100%)	7 (100%)
Aminopenicillin + β-lactamase inhibitor	Ampicillin/sulbactam	14 (93%)	7 (100%)
Penicillin+ β-lactamase inhibitor	Amoxicillin/clavulanic acid	4 (27%)	1 (14%)
Ureidopenicillin β-lactamase inhibitor	Piperacillin/tazobactam	4 (27%)	1 (14%)
First generation cephalosporins	Cefazolin	15 (100%)	7 (100%)
Second generation cephalosporins	Cefuroxime	15 (100%)	7 (100%)
	Cefuroxime/axetil	15 (100)	7 (100%)
Third generation cephalosporins	Ceftazidime	14 (93%)	5 (71%)
	Ceftriaxone	15 (100%)	7 (100%)
	Cefotaxime	15 (100%)	7 (100%)
Fourth generation cephalosporins	Cefepime	4 (27%)	2 (29%)
Carbapenems	Meropenem	1 (7%)	0 (0%)
Folate pathway inhibitor	Trimethoprim/sulfamethoxazole	14 (93%)	7 (100%)
Aminoglycosides	Amikacin	1 (7%)	0 (0%)
	Gentamicin	7 (47%)	2 (29%)
Fluoroquinolones	Ciprofloxacin	13 (87%)	5 (71%)

Table 1 displays the antibiotic resistance patterns observed in the *E. coli* and *K. pneumoniae* isolates. Notably, the isolates exhibited substantial resistance to aztreonam, a monobactam, with (14/15, 93%) resistance in *E. coli* and complete resistance (7/7, 100%) in all *K. pneumoniae* isolates. Furthermore, all the 22 isolates, comprising both *E. coli* and *K. pneumoniae*, displayed a 100% resistance rate to various antibiotics, including ampicillin from the penicillin group, cefazolin (a first-generation cephalosporin), cefuroxime/axetil (a second-generation cephalosporin), and third generation cephalosporins encompassing cefuroxime, cefotaxime/axetil, and ceftriaxone.

For the β-lactam antibiotics with beta-lactamase inhibitors such highest resistance was observed in ampicillin/sulbactam, with all but one of the *E. coli* isolates being resistant (14/15, 93%) and all the *K. pneumoniae* isolates being resistant (7/7, 100%). Amoxicillin/clavulanic acid combination showed negligible resistance with (4/15, 27%) and (1/7, 14%) and similarly piperacillin/tazobactam (4/15, 27%) and (1/7, 14%) among *E. coli* and *K. pneumoniae* isolates respectively.

The least resistance was observed with meropenem, a carbapenem antibiotic with only one isolate, *E. coli* (1/15, 7%). There was 7/7, 100% susceptibility observed among the *K. pneumoniae* isolates to meropenem.

Among the aminoglycoside antibiotics almost 50% resistance was observed with gentamicin with (7/15, 47%) and (2/7, 29%) among *E. coli* and *K. pneumoniae*, respectively. All *K. pneumoniae* isolates exhibited complete susceptibility (7/7, 100%) to amikacin, while among the *E. coli* isolates, resistance was detected in only 1 case (1/15, 7%). High and similar rates of resistance to fluoroquinolones were observed among the isolates, with percentages of (6/7, 86%) and (13/15, 87%) for *K. pneumoniae* and *E. coli*, respectively. On the other hand, minimal resistance was observed for nitrofurantoin, with (2/15, 13%) and (2/7, 29%) among *E. coli* and *K. pneumoniae* isolates, respectively.

Table 2: Combination of resistance phenotype among *E. coli* Isolates.

Resistance phenotype	*E. coli* (N=15)
	n (%)
ATM-AM- AMC- CRO- CTX- CXM- CXMA- CZ- SAM- SXT- TZP- CIP	2 (13)
	2 (13)
ATM- AM- AMC- CRO- CTX- CXM- CXMA- CZ- SAM- SXT- CIP	1 (7)
ATM- AM- AMC- CAZ- CRO- CTX- CXM- CXMA- CZ- SAM- TZP	1 (7)
ATM-AM- CRO- CTX- CXM- CXMA- CZ- SAM- SXT- CIP	1 (7)
ATM- AM- AMC- CRO- CTX- CXM- CXMA- CZ- SAM- SXT- GM- CIP	1 (7)
ATM- AM- CRO- CTX- CXM- CXMA- CZ- FEP- SXT- CIP	1 (7)
ATM-AM- CRO- CTX-CXM- CXMA- CZ- SAM- SXT- CIP	1 (7)
ATM- AM- CRO- CTX- CXM- CXMA- CZ- SAM- SXT- GM	1 (7)
AM-CRO- CTX- CXM- CXMA- CZ- SAM- SXT- CIP	1 (7)
ATM- AM- AMC- CAZ- CRO- CTX- CXM- CXMA- CZ- FEP- SAM- SXT- TZP- GM- CIP	2 (13)
ATM- AM- AMC- CAZ- CRO- CTX- CXM- CXMA- CZ- FEP- MEM- SAM- SXT- TZP- AN- GM- CIP	1 (7)

Antibiotic: AM, ampicillin; AMC, amoxicillin/clavulanate, AN, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; CXMA, Cefuroxime/Axetil; CZ, cefazolin; FEP, cefepime; GM, gentamicin; MEM, meropenem; SAM, ampicillin/sulbactam; SXT, sulfamethoxazole/trimethoprim, TZP, piperacillin/tazobactam. **Phenotype:** S, susceptible; I, intermediate; R, resistant.

Three combination phenotypes resistance were observed in 2 *E. coli* isolates each, these were "ATM-AM- AMC- CRO- CTX- CXM- CXMA- CZ- SAM- SXT- TZP- CIP", "ATM-AM-AMC- CRO- CTX- CXM- CXMA- CZ- SAM- SXT- TZP- CIP", and "ATM- AM- AMC-CAZ- CRO- CTX- CXM- CXMA- CZ- FEP- SAM- SXT- TZP- GM- CIP". The other combination phenotypes resistance were unique across the other isolates.

Table 3: Combination of resistance phenotype among *K. pneumoniae* Isolates.

Resistance phenotype	*K. pneumoniae* (N=7)
	n (%)
ATM- CRO- CTX- CXM- CXMA- CZ- SAM- SXT	1 (14)
ATM- CAZ- CRO- CTX- CXM- CXM- CZ- SAM- SXT	1 (14)
ATM- CRO- CTX- CXM- CXMA- CZ- SAM- SXT- CIP	1 (14)
ATM- CAZ- CRO- CTX- CXM- CXMA- CZ- SAM- SXT- CIP	1 (14)
ATM- AMC- CRO- CTX- CXM- CXMA- CZ- SAM- SXT- GM- CIP	1 (14)
ATM- AMC- CAZ- CRO- CTX- CXM- CXMA- CZ- FEP- SAM- SXT- TZP- GM- CIP	1 (14)
ATM- CAZ- CRO- CTX- CXM- CXMA- CZ- FEP- SAM- SXT- TZP- GM- CIP	1 (14)

Antibiotic:AMC, amoxicillin/clavulanate, AN, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; CXMA, Cefuroxime/Axetil; CZ, cefazolin; FEP, cefepime; GM, gentamicin; MEM, meropenem; SAM, ampicillin/sulbactam; SXT, sulfamethoxazole/trimethoprim, TZP, piperacillin/tazobactam. **Phenotype:** S, susceptible; I, intermediate; R, resistant.

The *K. pneumoniae* isolates showed unique combination of phenotypic resistance across all the isolates.

Table 4: Proportion of Enterobacterales isolates among ICU patients.

Variable	Counts	Proportion (%)	*P*-value
Age (years)			0.9733
< 18	3	14	
18 - 40	12	55	
41 - 70	6	27	
> 70	1	5	
Sex			0.609
Male	16	73	
Female	6	27	
Sample type			0.999
Tracheal aspirate	11	50	
Urine	7	32	
Blood	2	9	
Pus	3	9	
Bacterial isolates			
E. coli	15	65	
K. pneumoniae	7	30	

Table 4 shows the proportion of Enterobacterales among the ICU patients. The isolates were more prevalent among the patients between 18-40 years (12/22, 55%). There was no statistical significance in the distribution of the isolates according to age group, p= 0.9733 and this can be attributed to small sample size. Proportion of the isolates based on gender showed higher prevalence among male patients (16/22, 73%) than the female patients (6/22, 27%). This difference in distribution was also not statistically significant, p= 0.609. Fifty percent (11/22, 50%) of the isolates were obtained from tracheal aspirates. However, the difference in distribution of isolates based on sample types was also not statically significant, p= 0.999.

4.3 Whole genome sequencing

A total of 22 out of the 33 Enterobacterales isolates were successfully sequenced after DNA quantification threshold of 1000 ng and quality threshold score (Q score) of >6 was applied on reads eliminating 5 and 6 isolates, respectively. In total, 108,000 reads were recorded, corresponding to 900,320,000 bases. The average read length was 16890 bases (Figure 2). MinION run results of the sequencing is depicted below.

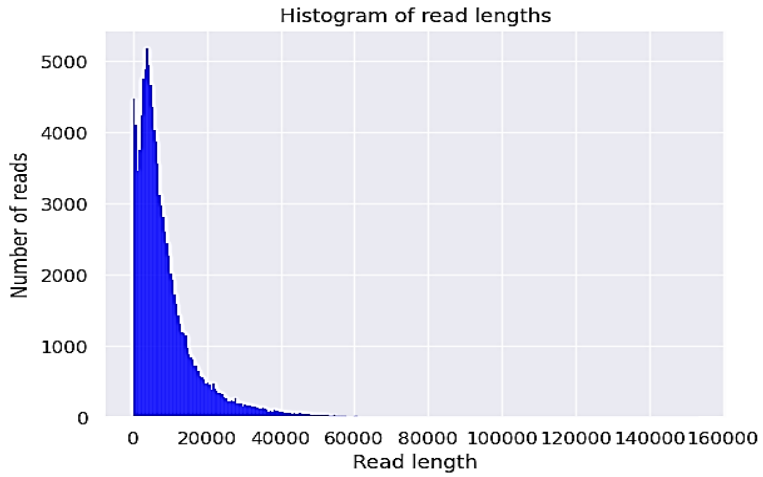


Figure 4: Read lengths against the number of reads.

4.4 Sequence assembly

Assemblies were compared and meticulously chosen according to specific criteria: minimal count of mismatches, misassemblies, contigs, GC (%) content, N50 length, L50, and genome coverage, as evaluated using the quality assessment tool QUAST (http://bioinf.spbau.ru/quast). The highest N50 among the *E. coli* assemblies measured 5,128,195, coinciding with the greatest contig length, and the L50 value was 1 (Table 17). The shortest length of the contig was 362814 bases. Among the *K. pneumoniae* isolates, the largest N50 of the assemblies was 4244064, which was corresponding to the largest contig length and the L50 was 1. The average GC content for *E. coli* isolates was 50.79% which is within the range of 50.4% and 50.8% for

reference *E. coli* (63), while that of *K. pneumoniae* was 57.06% (reference range: 57.22 - 57.46%) (64).

Objective 2: To describe the phenotypic and genotypic differences in the hypervirulent bacteria

4.5 AMR genes

In total, 38 AMR genes conferring resistance to eight antimicrobial classes were identified in the 22 isolates. These included genes that confer resistance to aminoglycosides (*aaa*(3)-IId, *aac*(6')-Ib-cr, *aad*A5, *aph*(3")-Ib), *aph*(6'), beta-lactams (bla_{OXA-1}, $bla_{OXA-534}$, $bla_{CTX-M-15}$, bla_{EC}, bla_{TEM}, bla_{CMY}, bla_{SHV}, *ompK*), quinolones (*gyrA, oqxA, oqxB*), tetracyclines (*tetA, tetB, tetD* , sulfonamides (*sul1*, *sul2*), phenicols (*catA, catB*), fosfomycins (*fosA*), rifamycins (*rpoB*), and macrolides (*ermB*), Figure 3 above. The genes bla_{OXA-1} (13/22, 59%), $bla_{OXA-534}$ (3/22, 13%), $bla_{CTX-M-15}$ (18/22, 82%), bla_{EC} (13/22, 59%), bla_{TEM} (7/22, 32%), bla_{CMY} (1/22, 5%), blaSHV (8/22, 36%) and ompK (6/22, 27%) encode ESBL production and consequently resistance to cephalosporins. Concordance between AMR genes and the observed resistance phenotype was evident for beta-lactam antibiotics, fluoroquinolones, aminoglycosides, and inhibitors of the folate pathway.

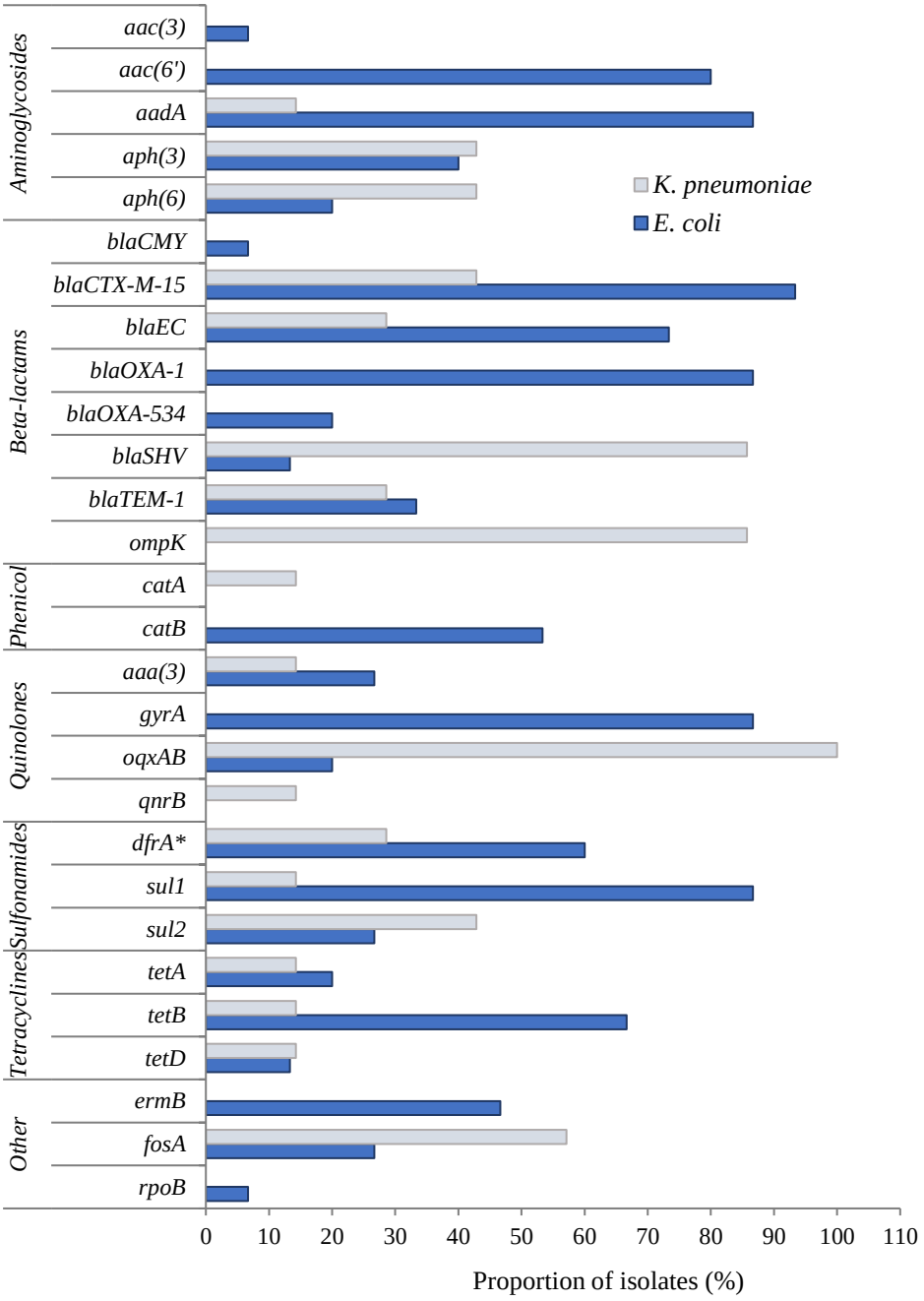


Figure 5: AMR genes identified among *E. coli* and *K. pneumoniae* isolates.

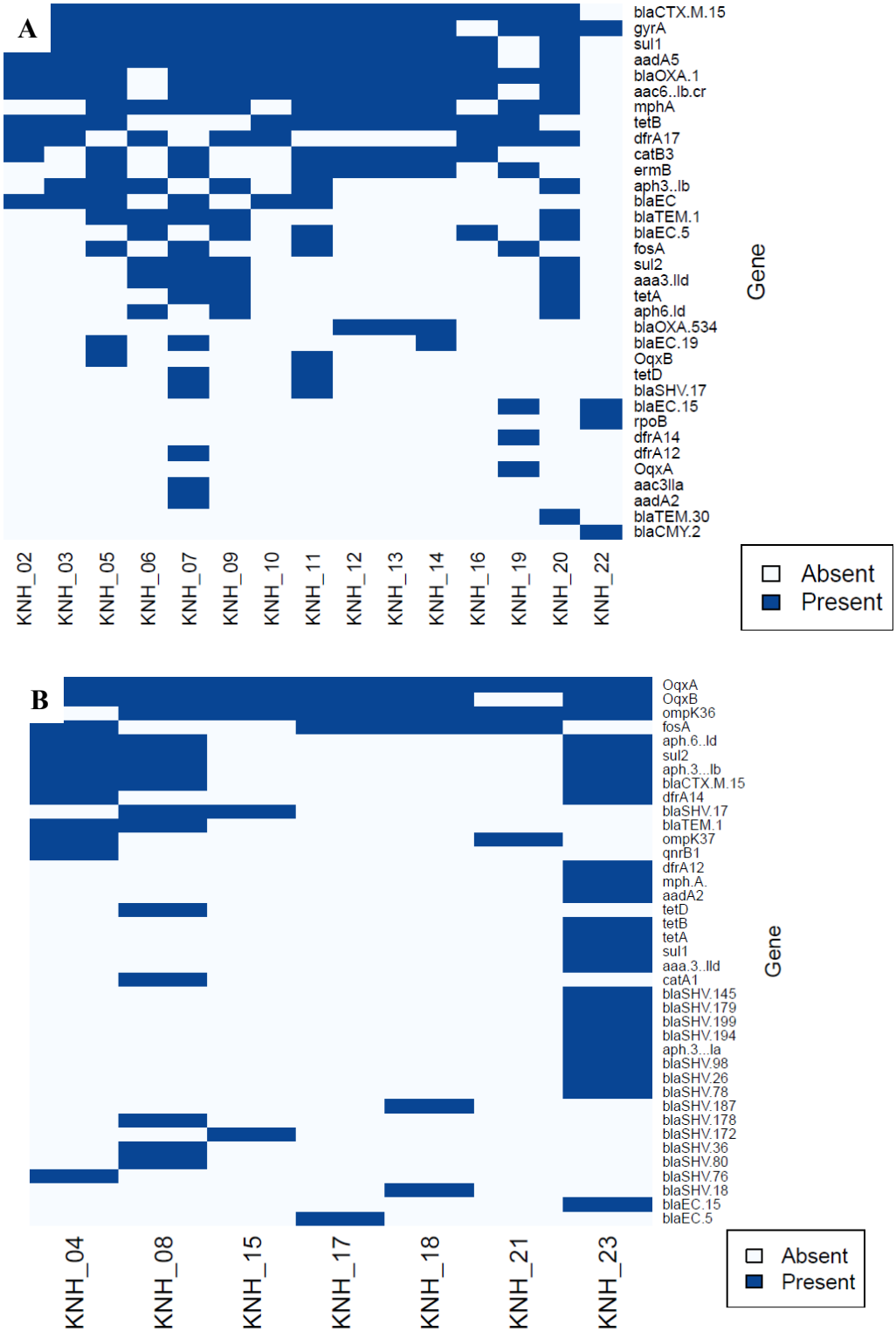


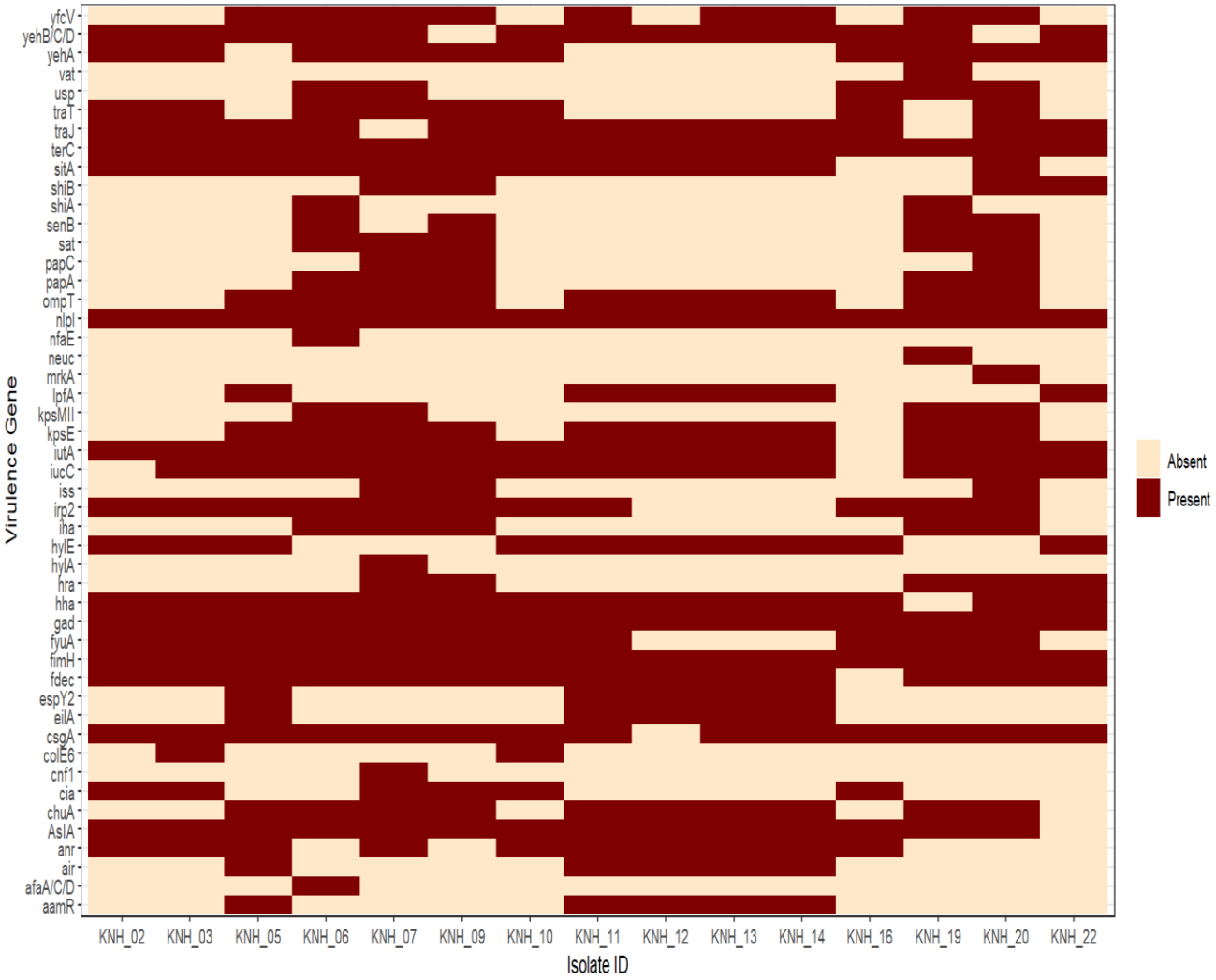
Figure 6: AMR genes identified in each *E. coli* (A) and *K. pneumoniae* (B) isolate.

4.6 Virulence genes

A total of 27 virulence genes were detected among the *E. coli* isolates. At least 5 virulence genes were detected in all 15 *E. coli* isolates (100%) evaluated. These were gad, fimH, nlpl, terC and yehD. The main virulence genes identified were fimH (15/15100%), fyuA (11/15,

73%), traT (8/15, 53%), and sat (5/15, 33%) and traJ (13/15, 87%). Other key virulence genes identified included: attachment (afa 1/15, 7%), cia (6/15, 40%), ipfA (6 /15, 40%)) biofilm formation (csgA 14/15, 93%), iss 3/15, 20%)) type III secretion system (espY2 5/15, 33%)), iron chelation (iucC 13/15, 87%), senB 4/15, 27%)), immune evasion (neuc 1/15, 7%) isolate, ompT 10/15, 67%)) toxin production (sat 5/15, 33%), hlyA 1/15, 7%) and cnf1 1/15, 7%)) and capsule genes (kpsE 10/15, 67%), kpsMII 3/15, 20%)), Figure 5A.

A



B

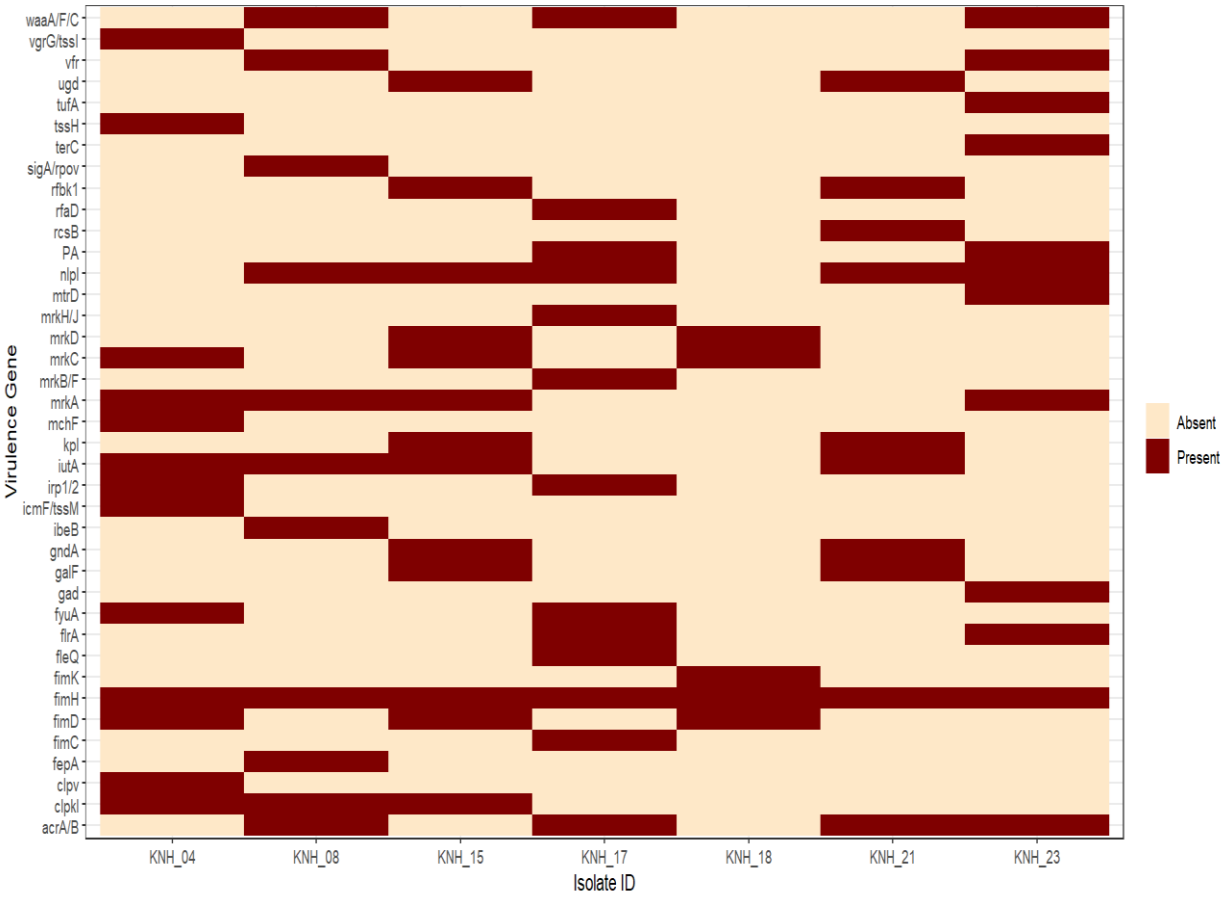


Figure 7: Virulence genes identified in each *E. coli* (A) and *K. pneumoniae* (B) isolate.

A total of 23 virulence genes were detected among the *K. pneumoniae* isolates. Attachment gene fimH was detected in all the isolates, while the others were distributed across the isolates in different frequencies. Genes associated with hypervirulence multidrug efflux transporter (mtrD 1/7, 14%), acrAB 4/7, 57%)), endotoxin (galF 2/7, 29%)), efflux pumps (waa 2/7, 29%), acr 2/7, 29%), ibeB 1/7, 14%)), Type III secretion system (mrk 1/7, 14%)), iron chelation (irp 2/7, 29%), iutA 4/7, 57%)) and attachment (fim 7/7, 100%), tufA 1/7, 14%)), Figure 5B.

Objective 3: To describe the relative abundance of hypervirulent genes

Table 5: Relative abundance of hypervirulent genes among Enterobacterales isolates.

	E. coli		*K. pneumoniae*	
Virulence factor encoded	Gene	n (%)	Gene	n (%)
Adhesins/Attachment	fimH	15 (100%)	fimH	7 (100%)
	fyuA	11 (73%)	fyuA	2 (29%)
Iron chelation	iutA	14 (93%)	iutA	4 (57%)
	fyuA	11 (73%)	fyuA	2 (29%)
	iucC	13 (87%)	irp	2 (29%)
	sitA	12 (80%)		
Biofilm formation	csgA	14 (93%)	mrkC	3 (43%)
	iss	3 (20%)		
Efflux pumps			acrAB	4 (57%)
			ibeB	1 (14%)
			waa	2 (29%)
			mtrD	1 (14%)
Capsule formation	kpsE	10 (67%)	vfr	2 (29%)
Toxin production	cnf1	1 (7%)	ugd	2 (29%)
	sat	5 (33%)	galF	2 (29%)

4.7 Presence of plasmid replicons

WGS data analysis identified 8 plasmid replicons in 15 *E. coli* isolates. All 15 isolates harbored multiple plasmid replicons, except for one isolate which had only one replicon. Six different types of Inc with different frequencies were found including IncFIA, IncFIB (AP001918), IncFII, Incl1-1(Alpha), IncFIB (H89-Phage Plasmid) *and* IncY. Additionally, two types of col replicon plasmids were also identified. These were col156 which several isolates had and col (BS512) which was found in only one isolate (Table 4).

Table 6: Plasmids identified among *E. coli* isolates.

Plasmid	E 02	E 03	E 05	E 06	E 07	E 09	E 10	E 11	E 12	E 13	E 14	E 16	E 19	E 20	E 22
IncFIA	+	+	+		+		+	+	+	+	+		+		+
IncFIB	+	+	+	+	+	+	+	+	+	+	+		+	+	
IncFII	+	+	+	+	+	+	+	+	+	+	+	+		+	
Incl1-1(Alpha)	+	+		+	+	+	+					+	+		
col156		+		+		+	+							+	
col (BS512)								+							
IncFIB				+											
IncY				+											
Total plasmids	4	5	3	6	4	4	5	4	3	3	3	2	3	3	1

Among the *K. pneumoniae* isolates, 5 plasmid replicons were identified in 5 of the 7 isolates. Four isolates harbored single plasmid replicons, except for one that had 3 plasmid replicons. The 5 types of replicon plasmids identified were IncFIB (K), IncFIA (PBK30683), Incl1-1 (Alpha), IncHI1B (pNDM-MAR) and IncR (Table 5).

Table 7: Plasmids identified among *K. pneumoniae* isolates.

Plasmid	K04	K08	K15	K17	K18	K21	K23
IncFIB (K)	+	+					+
IncFIA (PBK30683)		+					
Incl1-1 (Alpha)					+		
IncHI1B (pNDM-MAR)		+					
IncR			+				
Total plasmids	1	3	1	0	1	0	1

CHAPTER 5

5 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The goal of this study was to determine the presence and the genomic characteristics of hypervirulent bacteria among Enterobacterales strains isolated from ICUs patients at the Kenyatta National Hospital. The isolates were characterized using several approaches, including the identification of antibiotics susceptibility profiles, resistance and virulence and the detection of phylogenetic groups, all of which are crucial for AMR surveillance, managing patient treatment and outbreaks within hospitals.

The study findings reveal presence of MDR bacteria among ICU patients. *E. coli* and *K. pneumoniae* were the Enterobacterales isolates that were identified, and these two Enterobacterales have been consistently identified as frequently encountered in previous research studies as well (12,65,66). Significant resistance was observed in β-lactam antibiotics with high resistance of up to up to 100% resistance to second and third generation cephalosporins among the isolates. These findings are in line with related studies that depict that antibiotic-resistant Enterobacterales is a critical pathogen in the fight against antimicrobial resistance as evidenced by rapidly rising levels of resistance to several classes of antibiotics including beta-lactams (6,67,68). Similarly, AMR genes associated with beta-lactam resistance including $bla_{CTX\text{-}M\text{-}15}$ (14/15, 93%), $bla_{OXA\text{-}1}$ (13/15, 87%), $bla_{OXA\text{-}534}$ (3/15, 20%), bla_{EC} (11/15, 73%) and $bla_{TEM\text{-}1}$ (5/15, 33%) in *E. coli* and bla_{SHV} (6/7, 86%), ompK (6/7, 86%), $bla_{CTX\text{-}M}$ (3/7, 43%) among the *K. pneumoniae isolates* and thus concordance phenotypic and genotypic resistance.

The study revealed the presence of AMR genes across other classes of antibiotics including aminoglycosides, fluoroquinolones, and sulfonamides within the bacterial isolates, underscoring the concerning prospect of multidrug resistance, and emphasizing the urgent need for targeted interventions. Among the *E. coli* isolates, gryA (13/15, 87%) was the most common fluoroquinolone resistance gene whereas quinolone resistance gene was oqxAB (7/7, 100%) *K. pneumoniae* isolates. Consequently, high phenotypic resistance was observed with ciprofloxacin (13/15, 87%) and (5/7, 71%) among the *E. coli* and *K. pneumoniae* respectively. Similar findings from a tertiary hospital in Nigeria have been described (69).

The identification of hypervirulent bacteria in this study, characterized by the coexistence of multiple virulence genes, signifies a significant potential for severe illnesses in affected individuals. The presence of virulence-associated genes, including adhesins, iron chelation, biofilm formation, capsule formation, and toxin production, within both *E. coli* and *K. pneumoniae* isolates suggests an enhanced capacity to evade host defenses, establish infections, and contribute to disease severity. These findings were also observed from a study in China that sought to characterize hypervirulent *K.pneumoniae* and pathogenic *E. coli* (70,71). It is noteworthy that drug efflux pump genes were observed in the *K. pneumoniae* isolates.

Plasmids are the main vehicle for AMR genes transmission, which can be transferred horizontally between bacteria. In this study, we found *IncF* plasmids were the predominant plasmids observed in both the *E. coli* isolates and *K. pneumoniae* isolates. These were IncFIA (11/15, 85%), INCFIB (AP001918) (13/15, 87%) and IncFII (13/15, 87%). This is in accordance with previous studies where IncF plasmids were found predominantly (72,73). Among the *K. pneumoniae* isolates, the IncFIB-K was common (3/7, 43%), like a study in Kenya (19).

There are a few limitations to this study. First, the small sample size could not establish any statical significance to the differences in distribution of the Enterobacterales isolates by age or gender. Second, the genetic relatedness of the isolates, which would have been crucial for determining potential patient-to-patient and intra-ward transmission of these bacteria, was not established. Third, only a few antibiotics were included in the VITEK®2 antibiotic susceptibility testing cards, which were not confirmed with other phenotypic methods. Lastly, no in-vivo tests were performed to confirm the virulence genes activities, hence the results that some of these bacteria could be hypervirulent are only predictions. However, some of these bacteria exhibited concordance in the genotypic and phenotypic antibiotic resistance patterns.

5.2 Conclusion

1. This study reveals the presence of MDR strains among ESBL-producing Enterobacterales isolates within ICUs.
2. The study elucidates both phenotypic and genotypic differences among ESBL-producing Enterobacterales isolates, revealing the coexistence of antimicrobial resistance genes and hypervirulent genes.
3. There is a shared relative abundance of hypervirulent genes between *E. coli* and *K. pneumoniae* bacteria, with efflux pumps predominantly found in the *K. pneumoniae* isolates.

5.3 Recommendations

1. These findings highlight the need for increased surveillance to keep track of AMR within hospital settings.
2. The presence of AMR genes with phenotypic and genotypic concordance highlights the importance of targeted therapeutic strategies.
3. More studies adopting genomic methods are essential for effective AMR surveillance.

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APPENDICES

1. Isolate handling procedures

This method was used to confirm the viability and purity of the isolates.

1.1. Isolate revival

i. The isolates stored in Trypticase soy broth glycerol were identified, removed from the freezer -80°C, and allowed to thaw.

ii. 2 Clean the outside of the cryogenic vials using a disinfectant wipe soaked in 70% alcohol.

iii. 3 Subculture onto MacConkey to make it easier to detect any contaminants that may have been introduced as the cryogenic vials were opened.

iv. 4 Incubate at 37°C for 18 - 24 hours. Extended incubation period than normal is required as a proportion of the bacteria may be sub-lethally injured due to the preservation process and need time to recover on the nutrient-rich medium.

v. 5 After the growth is observed in the appropriate medium, parameters of purity check shall be done and recorded and colonial morphology.

vi. 6 The pure, viable culture colonies were used for inoculum preparation VITEK®2 Identification and Antimicrobial Susceptibility Testing System.

vii. VITEK®2 was used for the identification of the organism and determine the antimicrobial susceptibility.

1.2. Suspension preparation

i. Suspension was prepared by transferring enough colonies of pure culture using a sterile swab in 3.0 ml sterile saline (aqueous 0.45% to 0.50% NaCl, pH4.5 to7.0) in a plastic polystyrene test tube.

ii. The turbidity was adjusted to 0.50 -0.60 McFarland turbidity range measured using a turbidity meter called DensiChekTM.

1.3. Inoculation

i. Identification cards GN ID and GN AST-83 cards were inoculated with micro-organism suspensions test and controls using an integrated vacuum apparatus.

ii. The test tube containing the micro-organism were placed into a unique rack (cassette), and the ID cards were placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube.

iii. The filled cassette was then transported automatically into a vacuum chamber station for test well filling.

1.4. Incubation

i. 1 Automatically, inoculated cards were passed and loaded into the carousel incubator.

ii. 2 The cards were incubated at 35.5 + 1.0oC.

iii. 3 Every card was removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator until the next read time.

iv. 4 Data was collected at 15-minute intervals during the entire incubation period.

1.5. Antimicrobial Susceptibility testing (AST)

i. Antimicrobial susceptibility cards (AST GN-83) were inoculated with identified micro-organism suspension and controlled using the integrated vacuum apparatus.

ii. The test tube containing the micro-organism was placed into a unique rack (cassette), and an antimicrobial susceptibility card was placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube.

iii. It was bar-coded for data entry.

iv. The filled cassette will then be transported automatically into a vacuum chamber station for test well filling.

v. Data was collected at 15-30-minute intervals during the entire incubation period.

1.6. Results

i. Identification: The ID-GN database interpreted the results, and the results were obtained automatically.

ii. AST: The Minimum Inhibitory Concentration (MIC) of the microbial susceptibility was determined and identified as susceptible, intermediate, or

resistant according to CLSI 2020 standards. The results were extracted automatically from the VITEK®2 system.

2. DNA extraction procedure

DNeasy® UltraClean® SOP For the isolation of high-quality DNA from microbial cultures

1. Add 1.8 ml of microbial (bacteria, yeast) culture to a 2 ml Collection Tube (provided) and centrifuge at 10,000 x g for 30 s. Decant the supernatant and spin the tubes again at 10,000 x g for 30 s. Completely remove the supernatant with a pipette tip.

   Note: Depending on the type of microbial culture, it may be necessary to centrifuge longer than 30 s. This step concentrates and pellets the microbial cells. It is important to pellet the cells completely and remove all the culture media in this step.

2. Resuspend the cell pellet in 300 µL of PowerBead Solution and gently vortex to mix. Transfer resuspended cells to a PowerBead Tube.

   Note: The PowerBead Solution contains salts and a buffer that stabilizes and homogeneously disperses the microbial cells prior to lysis.

3. Add 50 µL of Solution SL to the PowerBead Tube.

   Note: To increase yields, to minimize DNA shearing or for difficult cells. Solution SL contains SDS, and other disruption agents required for cell lysis. In addition to aiding in cell lysis, SDS also breaks down fatty acids and lipids associated with the cell membrane of several organisms. SDS may precipitate when cold but heating at 55°C will dissolve the SDS. Solution SL can be used while it is still warm.

4. Secure PowerBead Tubes horizontally using the Vortex Adapter.
5. Vortex at maximum speed for 10 min.

   Note: This step creates the combined chemical/mechanical lysis conditions required to release desired nucleic acids from microbial cells. Many cell types will not lyse without this chemically enhanced bead beating process. The vortex action is typically all that is required; however, more robust bead beaters may also be used. In most cases bead beating times may be shorter with other devices, but you run the risk of increased DNA shearing. This process is compatible with fast prep machines.

6. Make sure the 2 ml PowerBead Tubes rotate freely in the centrifuge without rubbing.
7. Centrifuge the tubes at a maximum of 10,000 x g for 30 s.

   Note: The cell debris is sent to the bottom of the tube while DNA remains in the supernatant.

8. Transfer the supernatant to a clean 2 ml Collection Tube (provided)

   Note: Expect 300–350 µL of supernatant. Volume will vary depending on the size of the cell pellet in Step 1.

9. Add 100 µL of Solution IRS to the supernatant and vortex for 5 s. Incubate at 4°C for 5 min.
10. Centrifuge the tubes at 10,000 x g for 1 min.

    Note: Solution IRS contains a reagent to precipitate non-DNA organic and inorganic material, including cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

11. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2 ml Collection Tube (provided).

    Note: Expect 450 µL of supernatant. The pellet at this point contains non-DNA organic and inorganic materials, including cell debris and proteins. For the best DNA quality and yield, avoid transferring any of the pellet.

12. Add 900 µL of Solution SB to the supernatant and vortex for 5 s.

    Note: Solution SB is a highly concentrated salt solution. It sets up the high-salt condition necessary to bind DNA to the MB Spin Column membrane in the following step.

13. Load about 700 µL into a MB Spin Column and centrifuge at 10,000 x g for 30 s. Discard the flow-through, add the remaining supernatant to the MB Spin Column, and centrifuge again at 10,000 x g for 30 s. Note: Each sample processed will require 2–3 loads. Discard all flow-through. DNA is selectively bound to the MB Spin Column silica membrane. Contaminants pass-through the filter membrane, leaving only the DNA bound.
14. Add 300 µL of Solution CB and centrifuge at 10,000 x g for 30 s.

    Note: Solution CB is an ethanol-based wash solution used to further clean the DNA bound to the MB Spin Column silica filter membrane. This wash solution removes residues of salt and other contaminants but allows the DNA to stay bound to the silica membrane.

15. Discard the flow-through. Centrifuge at 10,000 x g for 1 min.

Note: The flow-through is waste, containing ethanol wash solution and contaminants that did not bind to the MB Spin Column membrane. This step removes any residual Solution CB (ethanol wash solution). It is critical to remove all traces of Solution CB because it can interfere with downstream DNA applications.

16. Place the MB Spin Column in a new 2 ml Collection Tube (provided). Note: Be careful not to splash any of the liquid on the MB Spin Column.
17. Add 50 µL of Solution EB to the center of the white filter membrane.

    Note: Placing the Solution EB (elution buffer) in the center of the small white membrane will make sure the entire membrane is wet. This will result in more efficient release of bound DNA.

18. Centrifuge at 10,000 x g for 30 s.

    Note: As Solution EB passes through the silica membrane, DNA is released and flows through the membrane and into the Collection Tube. The DNA is released because it can only bind to the MB Spin Column membrane in the presence of salt. Solution EB is 10 mM Tris pH 8 and does not contain salt.

19. Discard the MB Spin Column. The DNA is now ready for downstream applications.

    Note: Store DNA frozen (–30 °C to –15°C/–90°C to –65°C) as Solution EB does not contain EDTA.

3. DNA quantification procedure

1. Set up two assay tubes for the standards (three for the protein or RNA IQ assay) and one assay tube for each sample.
2. Prepare the Qubit™ working solution by diluting the Qubit™ reagent 1:200 in Qubit™ buffer. Prepare 200 µL of working solution for each standard and sample.
3. Prepare the assay tubes according to Table 8.

Table 8: DNA quantification assay mix

	Standard assay tubes	User sample assay tubes
Working solution from step 2	190 µL	180–199 µL
Standard solution (from kit)	10 µL	_
User sample	_	1–20 µL
Total volume in each assay tube	200 µL	200 µL

4. Vortex all tubes for 2–3 seconds.
5. Incubate the tubes for 2 minutes at room temperature (15 minutes for the Qubit™ protein assay).
6. Insert the tubes in the Qubit™ Fluorometer and take readings. For detailed instructions refer to the Qubit™ Fluorometer manual.

4. Oxford MinION Sequencing Protocol

4.1. Checking the flow cell

Flow cells, when shipped, always contain a QC DNA molecule present in a buffer. This molecule produces a distinctive nanopore signal. The MinKNOW software uses the signal to check the nanopore array's integrity before the flow cell is used by giving the number of simultaneously available channels for the experiment. A minimum of 800 nanopores is required to conduct a successive sequencing run.

4.2. DNA repair and end-prep

DNA repair is achieved using NEBNext FFPE DNA repair mix (M6630). NEBNext Ultra II End Repair/dA-Tailing Module was also utilized in the end-prep step to prepare 1000ng of sheared gDNA (E7546). In a 1.5 ml Eppendorf DNA LoBind tube, the following were combined.

1. Thaw the AMPure XP Beads (AXP) and DNA Control Sample (DCS) at room temperature and mix by vortexing. Keep the beads at room temperature and store the DNA Control Sample (DCS) on ice.
2. Prepare the NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice.
3. In a 0.2 ml thin-walled PCR tube, add in the reagents in Table 8.

Table 9: DNA repair and end-prep mastermix

Reagent	Volume
DNA sample	11 µL
Diluted DNA Control sample (DCS)	1 µL
NEBNext FFPE DNA Repair Buffer	0.875 µL
Ultra II End-prep Reaction Buffer	0.875 µL
Ultra II End-prep Enzyme Mix	0.75 µL
NEBNext FFPE DNA Repair Mix	0.5 µL
Total	15 µL

4. Ensure the components are thoroughly mixed by pipetting. Close the tubes (or seal the plate) and spin down in a centrifuge.
5. Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.
6. Transfer each sample into a clean 1.5 ml Eppendorf DNA LoBind tube.
7. Resuspend the AMPure XP beads (AXP) by vortexing.

8. Add 15 µL of resuspended AMPure XP Beads (AXP) to each end-prep reaction and mix by flicking the tube.
9. Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
10. Prepare 500 µL of fresh 70% ethanol in nuclease-free water.
11. Spin down the samples and pellet the beads on a magnet until the eluate is clear and colorless. Keep the tubes on the magnet and pipette off the supernatant.
12. Keep the tube on the magnet and wash the beads with 200 µL of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. If the pellet was disturbed, wait for beads to pellet again before removing the ethanol.
13. Repeat the previous step.
14. Briefly spin down and place the tubes back on the magnet for the beads to pellet. Pipette off any residual ethanol. Allow to dry for 30 seconds, but do not dry the pellets to the point of cracking.
15. Remove the tubes from the magnetic rack and resuspend the pellet in 25 µL nuclease-free water. Spin down and incubate for 2 minutes at room temperature.
16. Pellet the beads on a magnet until the eluate is clear and colourless.
17. Remove and retain 25 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.
18. Dispose of the pelleted beads.
19. Quantify 1 µL of each eluted sample using a Qubit fluorometer.

4.3. Native barcode ligation

Prepare the NEB Blunt/TA Ligase Master Mix according to the manufacturer's instructions and place it on ice.

1. Thaw reagents at room temperature.
2. Spin down the reagent tubes for 5 seconds. Ensure the reagents are fully mixed by performing 10 full volume pipette mixes.
3. Thaw the Native Barcodes (NB01-24) required for your number of samples at room temperature. Individually mix the barcodes by pipetting, spin down, and place on ice.
4. Select a unique barcode for each sample to be run together on the same flow cell. Up to 24 samples can be barcoded and combined in one experiment.
   Note: Only use one barcode per sample.
5. In clean 0.2 ml PCR-tubes or a 96-well plate, add reagents as ordered in Table 9.

Table 10*:* Native barcode ligation mastermix

Reagent	Volume
500 ng end-prepped DNA	22.5 µL
Native Barcode (NB01-24)	2.5 µL
Blunt/TA Ligase Master mix	25 µL
Total	50 µL

6. Between each addition, pipette mix 10 - 20 times. Ensure the components are thoroughly mixed by gently pipetting and spin down briefly.
7. Incubate for 20 minutes at room temperature.
8. Pool all the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.
9. Resuspend the AMPure XP Beads (AXP) by vortexing.
10. Add AXP to the pooled reaction and mix by pipetting for a 0.4X clean.
11. Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.
12. Prepare 2 ml of fresh 70% ethanol in nuclease-free water.
13. Spin down the sample and pellet on a magnet for 5 minutes. Keep the tube on the magnetic rack until the eluate is clear and colourless, and pipette off the supernatant.
14. Keep the tube on the magnetic rack and wash the beads with 700 µL of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard. If the pellet was disturbed, wait for beads to pellet again before removing the ethanol.
15. Repeat the previous step.
16. Spin down and place the tube back on the magnetic rack. Pipette off any residual ethanol. Allow the pellet to dry for ~30 seconds, but do not dry the pellet to the point of cracking.
17. Remove the tube from the magnetic rack and resuspend the pellet in 35 µL nuclease-free water by gently flicking.
18. Incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.
19. Pellet the beads on a magnetic rack until the eluate is clear and colourless.
20. Remove and retain 35 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

4.4. Adapter ligation and clean-up

1. Prepare the NEBNext Quick Ligation Reaction Module according to the manufacturer's instructions, and place on ice.

2. Thaw the reagents at room temperature.
3. Spin down the reagent tubes for 5 seconds. Ensure the reagents are fully mixed by performing 10 full volume pipette mixes.
4. Spin down the Native Adapter (NA) and Quick T4 DNA Ligase, pipette mix and place on ice.
5. Thaw the Elution Buffer (EB) at room temperature, mix by vortexing, spin down and place on ice.
6. Thaw either Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB) at room temperature, mix by vortexing,
7. Spin down and place on ice.
8. In a 1.5 ml Eppendorf LoBind tube, mix in the order shown in Table 10.

Table 11: Adapter ligation and clean-up reaction mix

Reagent	Volume
Pooled barcoded sample	30 µL
Native Adapter (NA)	5 µL
NEBNext Quick Ligation Reaction Buffer (5X)	10 µL
Quick T4 DNA Ligase	10 µL
Total	55 µL

9. Ensure the components are thoroughly mixed by gently pipetting and spin down briefly.
10. Incubate the reaction for 20 minutes at room temperature.
11. Resuspend the AMPure XP Beads (AXP) by vortexing.
12. Add 20 µL of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting.
13. Incubate on a Hula mixer (rotator mixer) for 10 minutes at room temperature.
14. Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant.
15. Wash the beads by adding either 125 µL Long Fragment Buffer (LFB) or Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.
16. Repeat the previous step.
17. Spin down and place the tube back on the magnet. Pipette off any residual supernatant.
18. Remove the tube from the magnetic rack and resuspend the pellet in 15 µL Elution Buffer.

19. Spin down and incubate for 10 minutes at 37°C. Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.
20. Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.
21. Remove and retain 15 µL of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

4.5. Priming and loading the SpotON flow cell

1. Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FLB) at room temperature before placing the tubes on ice as soon as thawing is complete.
2. Mix the Sequencing Buffer (SQB) and Flush Buffer (FLB) tubes by vortexing, spin down and return to ice.
3. Spin down the Flush Tether (FLT) tube, mix by pipetting and return yo ice.
4. Open the lid of the nanopore sequencing device and slide the flow cell's priming port cover so that the priming port is visible.
5. After opening the priming port, check for small bubbles under the cover. Draw back a small volume to remove any bubble.
6. Prepare the flow cell priming mix: add 30 µL of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FLB) and mix by pipetting up and down.
7. Load 800 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of bubbles. Wait for 5 minutes.
8. Thoroughly mix the contents of the Loading Beads (LB) by pipetting.
9. In a new tube prepare the library for loading as shown in Table 11.

Table 12: Priming and loading reaction mix

Reagent	Volume
Sequencing Buffer (SB)	37.5 µL
Loading Beads (LB), mixed immediately before use	25.5 µL
DNA library	12 µL
Total	75 µL

10. Mix the prepared library gently by pipetting up and down just prior to loading.
11. Add 75 µL of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

12. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.

4.6. Starting a sequencing run

1. Double-click the MinKNOW icon located on the desktop to open the MiKNOW GUI.
2. Choose the flow cell type from the selector box. Then mark the flow cell as “selected”.
3. Click the “New Experiment” button at the bottom left of the GUI.
4. On the New Experiment popup screen, select the running parameters for your experiment from the individual tabs.
5. Allow the script to run to completion.

5. Sample types and bacteria isolated from ICU patients.

Table 13: Patient particulars and bacteria isolated from each sample.

Isolate ID	Gender	Age	Ward	Sample Type	Bacterial isolate
E. coli_KNH_02	Male	38	CCU	Urine	*E. coli*
E. coli_KNH_03	Male	21	CCU	Tracheal aspirate	*E. coli*
E. coli_KNH_05	Male	32	CCU	Pus swab	*E. coli*
E. coli_KNH_06	Male	43	CCU	Tracheal aspirate	*E. coli*
E. coli_KNH_07	Male	43	CCU	Tracheal aspirate	*E. coli*
E. coli_KNH_09	Female	36	CCU	Urine	*E. coli*
E. coli_KNH_10	Male	42	ICU	Urine	*E. coli*
E. coli_KNH_11	Male	27	ICU	Urine	*E. coli*
E. coli_KNH_12	Female	38	CCU	Blood	*E. coli*
E. coli_KNH_13	Female	28	ICU	Urine	*E. coli*
E. coli_KNH_14	Male	34	CCU	Tracheal aspirate	*E. coli*
E. coli_KNH_16	Male	82	CCU	Tracheal aspirate	*E. coli*
E. coli_KNH_19	Male	48	ICU	Tracheal aspirate	*E. coli*
E. coli_KNH_20	Female	48	ICU	Tracheal aspirate	*E. coli*
E. coli_KNH_22	Male	2	ICU	Tracheal aspirate	*E. coli*
Klebsiella_KNH_04	Male	34	ICU	Tracheal aspirate	*K. pneumoniae*
Klebsiella_KNH_08	Male	40	ICU	Urine	*K. pneumoniae*
Klebsiella_KNH_15	Female	54	CCU	Blood	*K. pneumoniae*
Klebsiella_KNH_17	Female	36	CCU	Urine	*K. pneumoniae*
Klebsiella_KNH_18	Male	12	CCU	Tracheal aspirate	*K. pneumoniae*
Klebsiella_KNH_21	Male	38	CCU	Tracheal aspirate	*K. pneumoniae*
Klebsiella_KNH_23	Male	17	CCU	Pus swab	*K. pneumoniae*

6. Sample types and bacteria isolated from ICU patients.

Table 14: Patient particulars and bacteria isolated from each sample.

Isolate ID	Gender	Age	Ward	Sample Type	Bacterial isolate
E. coli_KNH_02	Male	38	CCU	Urine	*E. coli*
E. coli_KNH_03	Male	21	CCU	Tracheal aspirate	*E. coli*
E. coli_KNH_05	Male	32	CCU	Pus swab	*E. coli*
E. coli_KNH_06	Male	43	CCU	Tracheal aspirate	*E. coli*
E. coli_KNH_07	Male	43	CCU	Tracheal aspirate	*E. coli*
E. coli_KNH_09	Female	36	CCU	Urine	*E. coli*
E. coli_KNH_10	Male	42	ICU	Urine	*E. coli*
E. coli_KNH_11	Male	27	ICU	Urine	*E. coli*
E. coli_KNH_12	Female	38	CCU	Blood	*E. coli*
E. coli_KNH_13	Female	28	ICU	Urine	*E. coli*
E. coli_KNH_14	Male	34	CCU	Tracheal aspirate	*E. coli*
E. coli_KNH_16	Male	82	CCU	Tracheal aspirate	*E. coli*
E. coli_KNH_19	Male	48	ICU	Tracheal aspirate	*E. coli*
E. coli_KNH_20	Female	48	ICU	Tracheal aspirate	*E. coli*
E. coli_KNH_22	Male	2	ICU	Tracheal aspirate	*E. coli*
Klebsiella_KNH_04	Male	34	ICU	Tracheal aspirate	*K. pneumoniae*
Klebsiella_KNH_08	Male	40	ICU	Urine	*K. pneumoniae*
Klebsiella_KNH_15	Female	54	CCU	Blood	*K. pneumoniae*
Klebsiella_KNH_17	Female	36	CCU	Urine	*K. pneumoniae*
Klebsiella_KNH_18	Male	12	CCU	Tracheal aspirate	*K. pneumoniae*
Klebsiella_KNH_21	Male	38	CCU	Tracheal aspirate	*K. pneumoniae*
Klebsiella_KNH_23	Male	17	CCU	Pus swab	*K. pneumoniae*

7. Minimum Inhibitory Concentration (MIC) values and interpretation.

Table 15: MIC values from VITEK®2 AST data and their interpretation.

	AM		AMC		AN		ATM		CAZ		CIP		CRO		CTX		CXM		CXMA		CZ		FEP		GM		MEM		SAM		SXT		TZP	
Isolate Number	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**	**MIC**	**Result**
E. coli_KNH_02	>= 32	R	16	I	8	S	>=64	R	16	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	<=2	S	<=1	S	<=0.25	S	>=32	R	>=320	R	64	I
E. coli_KNH_03	>= 32	R	>=32	R	8	S	>=64	R	>=64	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	>=16	R	<=0.25	S	>=32	R	>=320	R	128	R
E. coli_KNH_05	>= 32	R	16	I	16	S	32	R	16	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	8	S	<=1	S	<=0.25	S	>=32	R	>=320	R	8	S
E. coli_KNH_06	>= 32	R	8	S	<=2	S	16	R	16	R	<=0.25	S	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	4	S	>=16	R	<=0.25	S	>=32	R	>=320	R	<=4	S
E. coli_KNH_07	>= 32	R	16	I	16	S	>=64	R	16	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	8	S	>=16	R	<=0.25	S	>=32	R	>=320	R	8	S
E. coli_KNH_09	>= 32	R	16	I	16	S	16	R	16	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	8	S	>=16	R	<=0.25	S	>=32	R	>=320	R	8	S
E. coli_KNH_10	>= 32	R	16	I	8	S	16	R	16	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	<=2	S	<=1	S	<=0.25	S	>=32	R	>=320	R	64	I
E. coli_KNH_11	>= 32	R	8	S	<=2	S	>=64	R	16	R	1	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	<=2	S	<=1	S	<=0.25	S	>=32	R	>=320	R	4	S
E. coli_KNH_12	>= 32	R	>=32	R	<=2	S	>=64	R	>=64	R	<=0.25	S	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	<=1	S	<=1	S	<=0.25	S	>=32	R	20	S	>=128	R
E. coli_KNH_13	>= 32	R	>=32	R	>=64	R	>=64	R	>=64	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	>=16	R	>=16	R	>=32	R	>=320	R	>=128	R
E. coli_KNH_14	>= 32	R	8	S	<=2	S	16	R	16	R	1	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	<=2	S	<=1	S	<=0.25	S	>=32	R	>=320	R	<=4	S
E. coli_KNH_16	>= 32	R	8	S	<=2	S	4	S	4	S	>=4	R	32	R	>=64	R	>=64	R	>=64	R	>=64	R	<=2	S	<=1	S	<=0.25	S	>=32	R	>=320	R	<=4	S
E. coli_KNH_19	>= 32	R	4	S	<=2	S	>=64	R	16	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	<=1	S	<=0.25	S	16	I	>=320	R	<=4	S
E. coli_KNH_20	>= 32	R	16	I	16	S	16	R	16	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	8	S	>=16	R	<=0.25	S	>=32	R	>=320	R	8	S
E. coli_KNH_22	>= 32	R	>=32	R	8	S	>=64	R	>=64	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	>=16	R	<=0.25	S	>=32	R	>=320	R	>=128	R
Klebsiella_KNH_04	>= 32	R	4	S	<=2	S	16	R	8	S	1	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	<=2	S	<=1	S	<=0.25	S	>=32	R	>=320	R	8	S
Klebsiella_KNH_08	>= 32	R	8	S	<=2	S	16	R	8	S	1	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	<=2	S	<=1	S	<=0.25	S	>=32	R	>=320	R	<=4	S
Klebsiella_KNH_15	>= 32	R	8	S	<=2	S	>=64	R	16	R	<=0.25	S	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	8	S	>=16	R	<=0.25	S	>=32	R	>=320	R	<=4	S
Klebsiella_KNH_17	>= 32	R	>=32	R	4	S	>=64	R	>=64	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	>=16	R	<=0.25	S	>=32	R	>=320	R	>=128	R
Klebsiella_KNH_18	>= 32	R	16	I	<=2	S	>=64	R	>=64	R	<=0.25	S	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	<=1	S	<=0.25	S	>=32	R	>=320	R	8	S
Klebsiella_KNH_21	>= 32	R	8	S	<=2	S	>=64	R	>=64	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	4	S	<=1	S	0.3	S	>=32	R	>=320	R	16	S
Klebsiella_KNH_23	>= 32	R	8	S	<=2	S	>=64	R	>=64	R	>=4	R	>=64	R	>=64	R	>=64	R	>=64	R	>=64	R	8	S	<=1	S	<=0.25	S	>=32	R	>=320	R	64	I

Antibiotic: AM, ampicillin; AMC, amoxicillin/clavulanate, AN, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; CXMA, Cefuroxime/Axetil; CZ, cefazolin; FEP, cefepime; GM, gentamicin; MEM, meropenem; SAM, ampicillin/sulbactam; SXT, sulfamethoxazole/trimethoprim, TZP, piperacillin/tazobactam. **Phenotype:** S, susceptible; I, intermediate; R, resistant.

8. Resistance phenotypes of the of the isolates.

Table 16: Resistance phenotypes identified among each isolate.

Isolate ID	Resistance phenotype
E. coli_KNH_02	ATM, AM, AMC, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, TZP, CIP
E. coli_KNH_03	ATM, AM, AMC, CAZ, CRO, CTX, CXM, CXMA, CZ, FEP, SAM, SXT, TZP, GM, CIP
E. coli_KNH_05	ATM, AM, AMC, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, CIP
E. coli_KNH_06	ATM, AM, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, GM,
E. coli_KNH_07	ATM, AM, AMC, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, GM, CIP
E. coli_KNH_09	ATM, AM, AMC, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, GM, CIP
E. coli_KNH_10	ATM, AM, AMC, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, TZP, CIP
E. coli_KNH_11	ATM, AM, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, CIP
E. coli_KNH_12	ATM, AM, AMC, CAZ, CRO, CTX, CXM, CXMA, CZ, SAM, TZP
E. coli_KNH_13	ATM, AM, AMC, CAZ, CRO, CTX, CXM, CXMA, CZ, FEP, MEM, SAM, SXT, TZP, AN, GM, CIP
E. coli_KNH_14	ATM, AM, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, CIP
E. coli_KNH_16	AM, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, CIP
E. coli_KNH_19	ATM, AM, CRO, CTX, CXM, CXMA, CZ, FEP, SXT, CIP
E. coli_KNH_20	ATM, AM, AMC, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, GM, CIP
E. coli_KNH_22	ATM, AM, AMC, CAZ, CRO, CTX, CXM, CXMA, CZ, FEP, FOX, SAM, SXT, TZP, GM, CIP
Klebsiella_KNH_04	ATM, AM, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, CIP
Klebsiella_KNH_08	ATM, AM, AMC, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, GM, CIP
Klebsiella_KNH_15	ATM, AM, CRO, CTX, CXM, CXMA, CZ, SAM, SXT
Klebsiella_KNH_17	ATM, AM, AMC, CAZ, CRO, CTX, CXM, CXMA, CZ, FEP, SAM, SXT, TZP, GM, CIP
Klebsiella_KNH_18	ATM, AM, CAZ, CRO, CTX, CXM, CXMA, CZ, SAM, SXT
Klebsiella_KNH_21	ATM, AM, CAZ, CRO, CTX, CXM, CXMA, CZ, SAM, SXT, CIP
Klebsiella_KNH_23	ATM, AM, CAZ, CRO, CTX, CXM, CXMA, CZ, FEP, SAM, SXT, TZP, GM, CIP

Antibiotic: AM, ampicillin; AMC, amoxicillin/clavulanate, AN, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; CXMA, Cefuroxime/Axetil; CZ, cefazolin; FEP, cefepime; GM, gentamicin; MEM, meropenem; SAM, ampicillin/sulbactam; SXT, sulfamethoxazole/trimethoprim, TZP, piperacillin/tazobactam.

9. AMR genes grouped by antibiotic classification.

Table 17: Numbers and proportions (%) of isolates with AMR genes.

Antibiotic class	AMR genes	*E. coli, n (%)*	*K. pneumoniae, n (%)*
Beta-lactams	*blaOXA*	13 (87)	0 (0)
	blaCTX-M	14 (93)	3 (43)
	blaEC	11 (73)	2 (29)
	blaCMY	1 (7)	0 (0)
	blaTEM	5 (33)	2 (29)
	blaSHV	2 (13)	6 (86)
	OmpK	0 (0)	6 (86)
Aminoglycosides	*aph(3)*	6 (40)	3 (43)
	aac(3)	1 (7)	0 (0)
	aadA	13 (87)	1 (14)
	aph(6)	3 (20)	3 (43)
	aac(6')	12 (80)	0 (0)
Fluroquionolones	*gyrA*	13 (87)	0 (0)
	qnrB	0 (0)	1 (14)
	aaa(3)	4 (27)	1 (14)
Sulfa/Trimeth	*sul1*	13 (87)	1 (14)
	sul2	4 (27)	3 (43)
	dfrA	9 (60)	2 (29)
Phenicol	*catA*	0 (0)	1 (14)
	catB	8 (53)	0 (0)
Quinolones	*OqxAB*	3 (20)	7 (100)
	qnrB	0 (0)	1 (14)
Tetracyclines	*tetA*	3 (20)	1 (14)
	tetB	10 (67)	1 (14)
	tetD	2 (13)	1 (14)
Macrolides	*ermB*	7 (47)	0 (0)
Fosfomycin	*fosA*	4 (27)	4 (57)
Rifampin	*rpoB*	1 (7)	0 (0)

10. Virulence genes identified among Enterobacterales isolates.

Table 18: Proportional distribution of virulence genes among isolated bacteria.

	E. coli		*K. pneumoniae*	
Virulence factor encoded	Gene	n (%)	Gene	n (%)
Colonization factors	fimH	15 (100%)	fimH/D	7 (100%)
	lpfA	6 (40%)	tufA	1 (14%)
	pap	5 (33%)		
	yeh	15 (100%)		
	afa	1 (7%)		
Immune evasion	ompT	10 (67%)		
	neuc	1 (7%)		
Invasins	cia	6 (40%)	mrkH	1 (14%)
	hyl	11 (73%)	ibeB	1 (14%)
	iha	5 (33%)	flrA	2 (29%)
Homeostasis/Survival	AsIA	14 (93%)	irp	2 (29%)
	chuA	10 (67%)	clpv	1 (14%)
	gad	15 (100%)	sigA/rpov	1 (14%)
Secretion system	espY2	5 (33%)	icmF/tssM	1 (14%)
	terC	15 (100%)	tssH	1 (14%)
			mrk	1 (14%)
			terC	1 (14%)
Iron chelation	iutA	14 (93%)	iutA	4 (57%)
	fyuA	11 (73%)	fyuA	2 (29%)
	iucC	13 (87%)	irp	2 (29%)
	sitA	12 (80%)		
Biofilm formation	csgA	14 (93%)	mrkC	3 (43%)
	iss	3 (20%)		
Effux pumps			acr	3 (43%)
			ibeB	1 (14%)
			waa	2 (29%)
			mtrD	1 (14%)
Capsule	kpsE	10 (67%)	vfr	2 (29%)
Toxin	cnf1	1 (7%)	ugd	2 (29%)
	sat	5 (33%)	galF	2 (29%)
Conjugation	traJ	13 (87%)		

11. Quality assessment tool (QUAST) assembly statistics.

Table 19: Quality assessment tool (QUAST) assembly statistics

Isolate ID	Contigs	Largest contig	Total length	GC%	N50	L50
E. coli_KNH_02	18	1201859	5145239	50.91	822361	3
E. coli_KNH_03	13	3481314	5163745	50.87	3481314	1
E. coli_KNH_05	3	5128195	5305016	50.53	5128195	1
E. coli_KNH_06	9	5022444	5567494	50.68	5022444	1
E. coli_KNH_07	11	2232766	5598465	50.68	978482	2
E. coli_KNH_09	38	5105422	5578956	51.00	5105422	1
E. coli_KNH_10	14	1321730	5108385	50.89	699663	3
E. coli_KNH_11	11	1257921	5232304	50.56	923511	3
E. coli_KNH_12	26	751536	4781818	50.63	237831	6
E. coli_KNH_13	26	751536	4781818	50.63	237831	6
E. coli_KNH_14	26	751536	4781818	50.63	237831	6
E. coli_KNH_16	70	362814	6797296	50.48	127062	15
E. coli_KNH_19	10	2360078	5230247	50.61	1534297	2
E. coli_KNH_20	27	5111592	6544414	52.17	5111592	1
E. coli_KNH_22	4	4244064	4770413	50.65	4244064	1
Klebsiella_KNH_04	4	4193458	5614787	57.02	4193458	1
Klebsiella_KNH_08	22	1476282	5607363	57.01	556518	4
Klebsiella_KNH_15	23	1714740	5597548	56.99	723753	3
Klebsiella_KNH_17	36	382217	3327415	57.43	108853	10
Klebsiella_KNH_18	31	1332335	5606300	56.44	379192	4
Klebsiella_KNH_21	30	566395	4280941	57.51	272311	6
Klebsiella_KNH_23	24	738245	4716196	57.04	279261	6

Contigs: long stretches of DNA sequence assembled from shorter DNA fragments; GC content: percentage of nucleotides in the genome that are either guanine (G) or cytosine (C); N50 length: length of the shortest contig such that 50% of the genome is contained in contigs of that length or longer; L50 is the number of contigs required to cover 50% of the genome.

12. ETHICS APPROVAL

12.1 KNH-UoN ERC Approval

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Ref: KNH-ERC/A/201

16th May, 2023

Beatrice Atieno Oduor
Reg No. H56/34346/2019
Dept. of Medical Microbiology
Faculty of Health Sciences
University of Nairobi

KENYATTA NATIONAL HOSPITAL APPROVED 16 MAY 2023 P.O. Box 20723 - 00202 NAIROBI KNH/UoN-ERC

Dear Beatrice,

ETHICAL APPROVAL-RESEARCH PROPOSAL: GENOTYPIC CHARACTERIZATION OF HYPERVIRULENT ENTEROBACTERALES ISOLATES FROM INTENSIVE CARE UNIT PATIENTS AT THE KENYATTA NATIONAL HOSPITAL (P88/02/2023)

This is to inform you that KNH-UoN ERC has reviewed and approved your above research proposal. Your application approval number is **P88/02/2023.** The approval period is 16th May 2023 – 15th May 2024.

This approval is subject to compliance with the following requirements;

i. Only approved documents including (informed consents, study instruments, MTA) will be used.
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by KNH-UoN ERC.
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to KNH-UoN ERC 72 hours of notification.
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH-UoN ERC within 72 hours.
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to KNH-UoN ERC.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://research-portal.nacosti.go.ke and also obtain other clearances needed.

Yours sincerely,

DR. BEATRICE K.M. AMUGUNE
SECRETARY, KNH- UoN ERC

c.c. The Dean, Faculty of Health Sciences, UoN
The Senior Director, CS, KNH
The Chairperson, KNH- UoN ERC
The Assistant Director, Health Information Dept., KNH
The Chair, Dept. of Medical Microbiology, UoN
Supervisors: Dr. Ann Njeri Maina, Dept. of Medical Microbiology UoN
Dr. Sylvia Omulo, Paul G. Allen School of Global Health, Washington State University, USA

12.2 NACOSTI License

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

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Date of Issue: 27/July/2023

RESEARCH LICENSE

This is to Certify that Ms.. Beatrice Oduor of University of Nairobi, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Nairobi on the topic: Genotypic characterization of hypervirulent Enterobacterales isolates from Intensive Care Unit patients at the Kenyatta National Hospital for the period ending : 27/July/2024.

License No: **NACOSTI/P/23/28006**

228457

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See overleaf for conditions

13. PLAGIARISM REPORT

Genotypic characterization of hypervirulent Enterobacterales isolates from Intensive Care Unit patients at the Kenyatta National Hospital

Plagiarism report checked on 08/12/2023 by Dr. Anne Maina

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