

Genotypic characterisation of ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) pathogens from groundwater

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DECLARATION

I, Baimphetlhetse Belah Twala, declare that the dissertation entitled “Genotypic characterisation of ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter species*) pathogens from groundwater” hereby submitted for the degree of Masters of Science (Biology), has not previously been submitted to another University for a degree. I further declare that the work contained therein is my own in design and execution and that all the materials contained therein have been duly acknowledged.

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DEDICATION

This work is dedicated to the Lord Almighty who gave me strength, my husband, Thabani Twala who supported me throughout, my daughters, Siphesihle and Phumla Twala as well as my friend Kokeletso Mooketsi who never got tired of supporting me.

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DEFINITIONS OF TERMS

Acinetobacter: A genus of non-motile, non-fastidious, catalase-positive, oxidase oxidase-negative, aerobic, Gram Gram-negative bacillus bacteria.

Antibiotic resistance: Ability The ability of a microorganism to withstand the effects of an antibiotic.

Antibiotics: Compounds produced by microorganisms that can inhibit the growth or kill microorganisms.

Bacillus: A rod rod-shaped bacterium.

Bacteraemia: Presence of bacteria in the bloodstream.

Bactericidal: refers to the agent that kills bacteria.

Bacteriophage: is a type of virus that infects bacteria.

Bacteriostatic: an agent that prevents the growth of bacteria.

Biofilms: complex microbial communities living as a thin layer on biotic or abiotic surfaces, implanted in a matrix of extracellular polymeric substances.

Causative agent: a toxin or toxic chemical that causes illness.

Cocci: any bacterium or archaeon that has a spherical, ovoid, or generally round shape.

Enterobacteriaceae: A large family of Gram Gram-negative, non-spore spore-forming bacilli found in soil and water, as well as in plants and animals.

Enterococcus: The genus that comprises species that are characterized by single, paired or short-chained, Gram-positive, catalase-negative and facultative anaerobic cocci.

Epidemiology: The study of how often diseases occur in different groups of people and why.

Facultative anaerobe: an organism that metabolizes energy with or without oxygen.

Genotype: complete set of the genetic material of an organism.

Groundwater: Water that is located below the surface of the earth in soil pore spaces and in the fractures of rock formations.

Immunocompromised: Having an impaired immune system.

Klebsiella: species that are Gram Gram-negative non-motile, encapsulated, rod rod-shaped, lactose- fermenting and facultative anaerobes belonging to the family Enterobacteriaceae.

Metritis: Inflammation of the wall of the uterus.

Nosocomial: Originating or taking place in a hospital.

Outbreak: A sudden rise in the incidence of a disease.

Pathogenesis: This is the process by which a disease or disorder develops.

Pathogenic: Causing disease.

Pathogenicity: Ability of an organism to cause disease or harm to a host.

Pathogens: an organism that causes disease.

Phagocytosis: Process whereby living cells called phagocytes ingest or engulf other cells or particles.

Plasmid: A small, circular, double-stranded DNA molecule that is distinct from a cell's chromosomal DNA.

Pneumoniae: Infection of the air sacs of the lungs.

Pseudomonas: Gram-negative rod bacteria that are widespread, inhabiting soil, water, plants and animals.

Rhinoschleroma: A chronic specific disease of the nose and upper respiratory passages caused by *Klebsiella rhinoscleromatis* bacilli.

Septicemia: a life-threatening complication that can happen when bacteria from another infection enter the blood and spread throughout the body.

Staphylococcus: A genus of bacteria that are Gram-positive cocci that can be observed under a microscope as individual organisms in pairs and irregular grapelike clusters.

Therapeutic: relating to the healing of disease.

Ubiquitous: present, appearing, or found everywhere.

Urinary tract infection: An infection in any part of the urinary system such as the kidneys, bladder or urethra.

Virulence factor: Molecules produced by pathogens that contribute to the pathogenicity of the organism and enable them to achieve colonization of a niche in the host.

Virulence gene: A gene whose presence or activity in an organism's genome is responsible for the pathogenicity of an infective agent.

LIST OF ABBREVIATIONS

(MSCRAMMs)	Microbial surface components recognizing adhesive matrix molecules
ABSSSI:	acute bacterial skin and soft tissue structure infections
algD:	alginate
AMPs:	Antimicrobial peptides
aPDI:	Antimicrobial photodynamic inactivation
AS:	Aggregation substance
BEA:	Bile Esculin Agar
BEA:	Bile Esculin Agar
CRE:	Carbapenem-resistant Enterobacteriaceae
DNA:	Deoxyribonucleic acid
EMBA:	Eosin Methylene Blue Agar
ESBLs:	Extended -spectrum beta lactamase
ESKAPE:	<i>Enterococcus faecium</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> , <i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i> and <i>Enterobacter</i> species.
Esp:	Extracellular surface protein
FDA:	Food and Drug Administration
FIB:	faecal indicator bacteria
FimH:	Adhesin
HAI:	Hospital-acquired infection
HLR:	high-level resistance
KPC:	<i>Klebsiella pneumoniae</i> carbapenamase

LAB:	lactic acid bacteria
LasB:	Elastase
LPS:	Lipopolysaccharide
MDR:	Multi-drug resistance
MRSA	Methicillin Resistant <i>Staphylococcus Aureus</i>
MSA:	Mannitol Salt Agar
PBPs:	Penicillin-binding proteins
PCR:	Polymerase chain reaction
PFTs:	Pore-forming toxins
PLA:	phospholipase A
PLC:	phospholipase C
PLD:	phospholipase D
PPL:	priority pathogen list
PS:	photosensitizer
rpoS:	RNA polymerase, sigma
rRNA:	ribosomal ribonucleic acid
Sea:	Staphylococcal enterotoxins A
Sec:	Staphylococcal enterotoxins C
SFP:	staphylococcal food poisoning
Spp:	Species
TTSS:	Type III secretion systems
UTI:	Urinary tract infection
VRE:	vancomycin-resistant enterococci;
WHO:	World Health Organization
Ycfm:	outer membrane lipoprotein

ABSTRACT

This study aimed to investigate the presence, antibiotic susceptibility profiles, and virulence genes determinants of ESKAPE pathogens isolated from water obtained from different groundwater sources of some villages of Zeerust and Mahikeng in the North-West Province, South Africa. To achieve this, 60 samples of water were obtained randomly from taps and boreholes in Zeerust and Mahikeng between December 2020 and October 2021. ESKAPE pathogens were isolated and positively identified using morphological (Gram-staining), biochemical (oxidase and catalase) tests and molecular (species-specific PCR) methods. The antimicrobial resistance profiles of the isolates were determined using the Kirby-Bauer disc diffusion method. Virulence genes were also screened using PCR. Among all the samples examined, a total number of 5, 16, 18 and 32 isolates were amplified for the species-specific genes of *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *E. cloacae* respectively. None of the isolates amplified for the *E. faecium* and *A. baumannii* species-specific genes. A large proportion (60%–100%) of the isolates were resistant to piperacillin, ampicillin, amoxicillin, cefuroxime and ceftazidime. On the other hand, low-level resistance (0%–33%) was observed for gentamicin and kanamycin. In addition, out of five *S. aureus* isolates, 2 (40%) amplified for the *sec* gene while 3 (60%) amplified for the *sea* gene. The *fimH-1* gene was detected in 38% of the *K. pneumoniae* isolates while the *ycfM* gene was detected in 63% of the isolates. One of the major virulence factors of *Pseudomonas aeruginosa*, the enzyme elastase (LasB) was detected in 61% of the isolates while *algD* was detected in 50% of the isolates. For the *E. cloacae* isolates, 94% and 22% amplified for the *fliI* and *rpoS* genes respectively. The findings of this study indicate that groundwater from some areas of Zeerust and Mahikeng is contaminated and this can cause serious

health problems in the communities, especially for immunocompromised individuals. Therefore, there is a need for routine testing and surveillance of groundwater and implementation of basic water treatment methods in the communities such as boiling and usage of sodium hypochlorite solutions.

Key words: ESKAPE pathogens, characterisation, groundwater, genotypic, virulence genes.

CHAPTER ONE

INTRODUCTION AND PROBLEM STATEMENT

1.1. General introduction public health significance of ESKAPE pathogens

ESKAPE is an acronym for a group of bacteria consisting of both Gram-positive and Gram-negative species, namely, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species (Santajit & Indrawattana, 2016). ESKAPE pathogens are receiving substantial attention recently due to their growing multidrug resistance and virulence potential. Multidrug resistance is among the top three threats to global public health thus multidrug resistant ESKAPE isolates present a great challenge in clinical practice (Santajit & Indrawattana, 2016). These organisms are responsible for nosocomial and community-acquired infections such as ventilator-associated pneumoniae and *S. aureus*-induced diabetic foot infection (Lin et al., 2018). The acronym ESKAPE was first used in 2008 by Rice to set apart a group of pathogens that were responsible for nosocomial infections and also resistant to currently marketed antimicrobial drugs (Karlowsky et al., 2017a). Moreover, ESKAPE pathogens belong to "Priority 1: Critical" and "Priority 2: High" pathogen groups on the WHO's priority pathogens list of multidrug-resistant organisms that contribute significantly to the burden of disease in developed and developing nations (Navidinia et al., 2017). ESKAPE pathogens are of great public health significance, not only because they contribute a large proportion of nosocomial infections but also because they represent new paradigms in the pathogenesis, and transmission of resistance determinants globally (Rice, 2008). Improved understanding of the virulence, resistance, transmission and pathogenicity of these pathogens may lead to innovative strategies for the development of new effective anti-infective agents to combat the complications they cause (Pendleton et al., 2013).

The natural hosts of ESKAPE pathogens are humans and animals thus poor hygiene and improper farm management techniques provide opportunities for contamination of environmental sources (Nyenje et al., 2012; Hrenovic et al., 2017; Amarasiri et al., 2020). Potential sources of contamination include sewage spills, improper deposition of hospital waste, anthropogenic activities, and improper disposal of agricultural waste (Nyenje *et al.*, 2012; Hrenovic et al., 2017). ESKAPE pathogens are a leading cause of nosocomial infections especially in humans (Boucher et al., 2009; Santajit and Indrawattana, 2016). The potential of these pathogens to harbour and express antibiotic-resistant determinants enhances their public health significance (Boucher et al., 2009; Santajit and Indrawattana, 2016). Moreover, their ability to adapt to altered environmental conditions by exchanging genetic material with other microorganisms through horizontal gene transfer makes them a source for the spread of virulent and resistance determinants (Huddlestone, 2014). The potential to acquire antibiotic resistance determinants by foodborne pathogens including ESKAPE pathogens is a serious public health threat presenting severe concerns for regulatory bodies, clinicians and the general society (WHO, 2003). The presence of antimicrobial resistance genes in ESKAPE pathogens reduces treatment options for serious infections, increases the burden of disease, and increased death rates due to treatment failure and requires a coordinated global response for antimicrobial resistance surveillance. Given that nosocomial ESKAPE bacteria represent paradigms of resistance, pathogenesis, and disease transmission (Santajit & Indrawattana, 2016) knowledge of the virulence and drug-resistant profiles of ESKAPE pathogens in circulation is imperative.

1.1.1. *Enterococcus faecium*

Enterococci are part of the normal flora of the intestinal tract of humans and animals (Moneoang and Bezuidenhout, 2009). *Enterococcus* species are characterized by single, paired or short-chained, Gram-positive, catalase-negative and facultative anaerobic cocci (Castillo-Rojas et al., 2013). The public health significance of *Enterococcus* species is enhanced by their potential to exhibit both intrinsic or acquired resistance to several antibiotic agents such as glycopeptides, β -lactams, and fluoroquinolones. Moreover, their ability to exhibit high-level aminoglycosides resistance to antibiotics gentamicin and streptomycin results in reduced therapeutic options for patients infected with enterococci (Arias et al., 2010). In humans, *E. faecium* and *E. faecalis* are the most frequently identified species in food and clinical samples (Martino et al., 2018) and these species contribute significantly to diseases that may present more life-threatening complications, especially to infants, the elderly as well as immunocompromised individuals (Martino et al., 2018). Enterococcus species also harbour virulence gene determinants including *asa1* (aggregation substances), *gelE* (gelatinase), *cylA* (cytolysin), *esp* (enterococcal surface protein), *hly* (hyaluronidase), *efaA* (endocarditis antigen), and *ace* (adhesion of collagen) (Wang et al., 2020).

1.1.2. *Staphylococcus aureus*

Bacteria belonging to the genus *Staphylococcus* are Gram-positive cocci whose cells occur either in pairs or as irregular grapelike clusters (Baorto and Boarto, 2016). *Staphylococcus aureus* is found in the environment and also forms part of the human normal flora thus most often detected on the skin and mucous membranes of most healthy individuals (Ateba et al., 2010). Although these organisms occur as normal flora

in their hosts, some staphylococci have been associated with diseases in humans and other mammals (Taylor & Unakal, 2017). The organism does not normally cause infections in healthy individuals but has the potential to cause a variety of serious infections such as bacteraemia, pneumonia, deep abscesses, osteomyelitis, endocarditis, phlebitis, meningitis, and urinary tract infections in susceptible patients (Taylor & Unakal, 2017). In humans, these complications are often associated with hospitalized patients. Staphylococci are divided into two groups based on their ability to clot blood plasma (the coagulase reaction). The coagulase-positive staphylococci (CPS) such as *S. aureus* constitute the most pathogenic species while coagulase-negative staphylococci (CNS) comprising over 30 species were previously considered to be non-pathogenic (Foster, 1996). The ability of *S. aureus* to harbour multiple antibiotic resistance mechanisms, especially to Methicillin, coupled with the expression of various virulence factors (surface proteins, capsule, immunoglobulin binding protein A, and numerous toxins) accounts for their link with outbreaks worldwide.

1.1.3. *Klebsiella pneumoniae*

Klebsiella species are Gram-negative non-motile, encapsulated, rod-shaped, lactose fermenting and facultative anaerobes belonging to the family Enterobacteriaceae (Younis et al., 2016; Asha et al., 2017). *Klebsiella* spp. are ubiquitous as they are widely distributed in water, soil, plants and sewage water and also exist as normal flora in the gastrointestinal tract of animals and humans (Namratha et al., 2015). These species are opportunistic pathogens that are responsible for nosocomial infections (Chakraborty et al., 2016). *Klebsiella pneumoniae* is the most frequently isolated species that accounts for a large proportion of *Klebsiella* infections (Gundestrup et al., 2014). Infections caused by *K. pneumoniae* include septicaemia, pneumoniae, urinary tract infections and

chronic lung disorders (Vasaikar et al., 2017). These infections have severe public health implications for patients especially in developing countries (Rice, 2008).

1.1.4. *Acinetobacter baumannii*

Members of the genus *Acinetobacter* are non-motile, non-fastidious, catalase-positive, oxidase-negative, aerobic, Gram-negative bacilli (Lee et al., 2017). *Acinetobacter* species are ubiquitous and can be found in a variety of environmental sources such as sewage, dump sites, vegetables, animals and humans (Al Atrouni et al., 2016). Although the genus *Acinetobacter* has more than 50 species, *Acinetobacter baumannii* is the species with great clinical significance and has therefore been associated with several hospital-acquired infections of the skin, bloodstream, urinary tract and other soft tissues (Lee et al., 2017).

1.1.5. *Pseudomonas Aeruginosa*

Pseudomonas aeruginosa is a Gram-negative rod bacterium that is widespread, inhabiting soil, water, plants and animals (Lee et al., 2011). *P. aeruginosa* is an opportunistic pathogen responsible for nosocomial infections in people who have weak immune systems, especially those suffering from cystic fibrosis (Stover et al., 2000). This organism is also associated with a wide range of infections such as urinary tract infections, wound infections, septicaemia and neonatal infections (Trautmann et al., 2008).

1.1.6. *Enterobacter species*

The genus *Enterobacter* consists of a Gram-negative, facultatively anaerobic, rod-shaped, non-spore-forming bacteria belonging to the family Enterobacteriaceae (Trivedi

et al., 2015). These organisms are also ubiquitous and widely distributed in soil and sewage and also form part of the enteric microflora of the human gastrointestinal tract (Mezzatesta et al., 2012). The genus *Enterobacter* consists of six species with *Enterobacter cloacae* emerging as the most frequently isolated species in patients. The public health significance of this pathogen globally amplifies the need to conduct surveillance studies given that it can cause nosocomial infections such as urinary tract infections, osteomyelitis and neonatal meningitis in humans (Mezzatesta et al., 2012; Ren et al., 2010).

ESKAPE pathogens are a leading cause of hospital-acquired infections (HAIs) worldwide (Tabah et al., 2012), and this is worsened by the increasing levels of antimicrobial resistance that is now a significant cause of morbidity and mortality. It is therefore obvious that understanding the transmission dynamics of these pathogens, as well as their transfer between patients, healthcare workers, and the environment is critical to evaluate potential intervention methods, and identify possible environmental reservoirs for the pathogens as well as pathogen-specific transmission behaviours.

1.2. Problem statement

Water is a natural resource that plays a very crucial role in sustaining life, economic development, food security and poverty reduction worldwide (Odonkor & Ampofo, 2013). Despite this, about 1,1 billion individuals worldwide lack access to potable water supplies, leaving them with no option but to rely on untreated surface and groundwater for their daily needs (Sobsey et al., 2008). Human and anthropogenic activities interfere with the natural water cycle thereby decreasing the microbial quality of ground and surface water. There are a variety of man-made pollutants that contaminate water

sources thus presenting severe challenges to consumers (Sasakova et al., 2018). In South Africa, it is estimated that about two million people residing in rural communities do not have access to basic water services and depend on wells, springs and boreholes for their day-to-day water needs (Nkuna et al., 2014b).

The Northwest Province of South Africa is one of nine provinces. The province is bordered by Botswana to the North, the Kalahari desert to the West, Gauteng to the East, and the Free State to the South. Although the principal minerals that are mined in the province are gold, uranium, platinum and diamonds, the province is termed the "Platinum Province", due to its wealth from the mining of the metal. The province has a population of 3,2 million inhabitants with Mahikeng serving as the provincial capital city. The Northwest Province has been faced with increasing challenges relating to the provision of water in certain communities (South African Government, 2021). While the government through its municipal structures recognizes that this challenge infringes on the basic right of access to water as stipulated in the Constitution of the Republic of South Africa, residents in some rural communities obtain water directly from unprotected sources that have not undergone any formal treatment (Nkwe et al., 2015a). Water from these sources is unsafe for drinking and serves as a major contributing factor to the emergence and spread of waterborne infections thus enhancing the global burden of infectious and enteric diseases such as diarrhoea in humans (Sobsey et al., 2008). Waterborne diseases have been associated with high mortality in humans (Sobsey et al., 2008). It is estimated that more than 5 million people die from water-related diarrheal diseases every year in developing countries, of which 84% of them are children aged between 0 and 14 years (Eneh, 2011; Sobsey et al., 2008).

Large proportions of waterborne diseases result from the consumption or contact with water that is contaminated with pathogens. Although ESKAPE pathogens are notoriously responsible for some of the deadliest hospital-acquired infections (HAIs), their presence in water that is intended for human consumption or recreational purposes has been documented (Pendleton, 2013). These organisms are increasingly associated with multiple antibiotic resistance which presents serious public health concerns as well as therapeutic challenges (Karlowsky et al., 2017). This situation is aggravated by the development of resistance to almost all the clinically relevant antibiotics including vancomycin, methicillin, broad-spectrum β -lactams, carbapenems, fluoroquinolones, and aminoglycosides by ESKAPE pathogens (Santajit & Indrawattana, 2016). Multidrug-resistant (MDR) ESKAPE pathogens pose serious threats to immunocompromised individuals (Deslouches et al., 2015) thus their presence in water is a cause of concern in a country such as South Africa. There is a limited study on the occurrence of drug-resistant ESKAPE pathogens in South Africa (Ramsamy *et al.*, 2018), despite evidence of the presence of these organisms in untreated and treated wastewater (Pendleton et al., 2018). This fails to address concerns regarding the placement of all ESKAPE pathogens on the WHO priority list of antibiotic-resistant and public health significant pathogens (Ramsamy *et al.*, 2018). Moreover, evidence from the review of available literature reveals that there is currently no data for studies that collectively assessed the occurrence of ESKAPE pathogens despite the isolated report documenting them individually. In addition, previous studies in the North West Province have focused most often on enterococci and staphylococci (Ateba & Maribeng, 2011). Knowledge of the occurrence and resistance profiles of these pathogens is pivotal for

the development of effective antimicrobial agents or other alternative tools to eradicate these public health threats (Santajit & Indrawattana, 2016).

1.3 RESEARCH AIM AND OBJECTIVES

1.3.1. Research aim

This study aims to determine the genotypic characteristics of ESKAPE pathogens (from groundwater sources).

1.3.2. OBJECTIVES

- To isolate and determine the presumptive identities of ESKAPE pathogens.
- To confirm the identities of ESKAPE pathogens using molecular (PCR) techniques.
- To determine the antibiotic susceptibility profiles of ESKAPE pathogens and also assess the phenotypic similarities through clustering of antibiotic inhibition zone diameter data.
- To determine the pathogenic potentials of ESKAPE isolates through PCR amplification of selected virulence genes.

CHAPTER TWO

LITERATURE REVIEW

2.1. Antimicrobial resistance

Antimicrobial resistance refers to the ability of a microorganism to grow or survive in the presence of a concentration of an antimicrobial agent that is usually sufficient to inhibit or kill organisms of the same species (Sabtu et al., 2015). The genetic mutations in bacteria as well as the direct transfer of genes encoding resistance mechanisms are the primary causes of antimicrobial resistance (Sabtu et al., 2015; Odonkor, 2014). The usage of antimicrobial drugs irrationally and inappropriately provides favourable conditions for resistant microorganisms to emerge, spread and persist (Odonkor, 2014).

2.2. Antimicrobial resistance with reference to the ESKAPE pathogens

ESKAPE pathogens are among the most common opportunistic pathogens in nosocomial infections (Singh, 2018). The acronym ESKAPE reflects the ability of these organisms to “escape” the biocidal ability of antimicrobial agents and defy eradication by conventional therapies, which results in extensive morbidity and mortality for patients and increased resource utilization in healthcare (Navidinia, 2016; Ma et al., 2020).

2.3. Types of antibiotic resistance

There are two types of antibiotic resistance which include intrinsic or innate resistance as well as acquired resistance (Odonkor, 2014). Intrinsic or innate resistance refers to a natural insensitivity in certain bacterial strains that have never been susceptible to a particular antibiotic. For example, *P. aeruginosa* is intrinsically resistant to many classes of antibiotics because of the presence of a few porins in its outer membrane, therefore many antibiotics cannot penetrate the interior of these bacteria cells (Ma et al., 2020). Acquired resistance is the phenomenon whereby certain bacteria belonging to a

susceptible species are not inhibited or killed anymore by concentrations of an antibiotic that would normally do so, due to either chromosomal mutation or acquisition of exogenous genes (Odonkor, 2014).

2.4. Classification of antibiotics

Antibiotics are divided into two groups based on how they act, namely bacteriostatic and bactericidal. Bacteriostatic antibiotics inhibit bacteria from growing or slow down their growth rate, effectively halting their reproduction. Upon removal of the bacteriostatic antibiotic, bacteria usually start to grow again. In contrast, bactericidal antibiotics kill bacteria (Lobritz et al., 2015; Odonkor, 2014). In addition to grouping antibiotics into bacteriostatic and bactericidal, antibiotics can be categorized into classes such as aminoglycosides, beta-lactams (including cephalosporins, carbapenems and penicillins), glycopeptides, lincosamides, macrolides, quinolones, sulfonamides, and tetracyclines (Odonkor, 2014).

2.5. Global priority pathogen list (PPL) of multi-drug-resistant bacteria

Multidrug-resistant (MDR) bacteria have become a health priority and efforts have been made to prevent colonization, infection and decrease mortality. The World Health Organization (WHO) proposed a global priority pathogen list (PPL) of MDR bacteria to guide research, discovery and development of new antibiotics (Rello et al., 2019). The global PPL classify bacterial pathogens into three priority tiers: critical, high and medium. Carbapenem-resistant *A. baumannii*, *P. aeruginosa* and Enterobacteriaceae spp., which includes *K. pneumoniae*, feature in the critical priority tier. Methicillin-resistant, vancomycin-intermediate and resistant *S. aureus*, in addition to *E. faecium*, are featured in the high-priority tier (Ramsamy et al., 2018).

2.5.1. *Enterococcus faecium*

Enterococci can obtain resistance to erythromycin, glycopeptides, tetracycline, vancomycin (vancomycin-resistant enterococci; VRE), aminoglycosides (high-level resistance; HLR), gentamicin (high-level resistance gentamicin; HLGR) and streptomycin (high-level resistance streptomycin; HLSR (Navidinia, 2016).

2.5.2. *Staphylococcus aureus*

S. aureus, mainly Methicillin-resistant *S. aureus* (MRSA) is one of the most important MDR human pathogens. The number of MRSA infections, both hospital and community-acquired, is growing worldwide and they are frequently difficult to treat. More than 90% of *S. aureus* clinical isolates are resistant to penicillin and 60% of them are MRSA. Some strains of *S. aureus* with reduced susceptibility to vancomycin or even resistant to glycopeptides have been reported (Nakonieczna et al., 2019).

2.5.3. *Klebsiella pneumonia*

The resistance of *Klebsiella pneumoniae* is mainly due to the production of extended-spectrum beta-lactamases (ESBLs), which are enzymes that hydrolyse and inactivate beta-lactam drugs like penicillin, third-generation cephalosporins and aztreonam. Recent reports record ESBL-producing *Klebsiella* spp being resistant to aminoglycosides, fluoroquinolones, tetracycline, chloramphenicol, and sulphonamides (Asha et al., 2017).

2.5.4. *Acinetobacter baumannii*

The epidemic potential and the clinical severity of *A. baumannii* infections are primarily related to its capability to survive and proliferate in the healthcare setting and to harbour resistance to a diversity of antimicrobial agents, such as those containing fluoroquinolones, broad-spectrum beta-lactams, and carbapenems (Navidinia, 2016).

2.5.5. *Pseudomonas aeruginosa*

P. aeruginosa is the second most commonly reported Gram-negative pathogen associated with UTI in intensive care units. Less frequent, but much more severe are bloodstream infections with carbapenem-resistant *P. aeruginosa* contributing to mortality up to 18.4%. Moreover, *P. aeruginosa* can manifest resistance to quinolones, aminoglycosides or polymyxins, as well as multiple drugs (MDR strains) with no drugs available in clinical development (Nakonieczna et al., 2019).

2.5.6. *Enterobacter spp.*

Many of the *Enterobacter* species have multiple antibiotic resistance that is undetectable *in vitro*, which makes it difficult to treat patients who are infected with these microbes. Based on existing literature, *E. cloacae* is resistant to aminopenicillins, aztreonam and broad-spectrum cephalosporins (refs). The organisms are capable of overproducing AmpC β -lactamases by derepression of a chromosomal gene conferring antibiotic resistance (Trivedi et al., 2015).

2.6. Antimicrobial Resistance Mechanisms of ESKAPE Pathogens

Mechanisms of drug resistance encompass several broad categories such as drug inactivation/alteration, modification of drug binding sites/targets, changes in cell

permeability resulting in reduced intracellular drug accumulation, and biofilm formation (Santajit & Indrawattana, 2016). Antimicrobial resistance is mediated by genes that are found on the bacterial chromosome, plasmid, or transposons (Carattoli, 2003).

2.6.1. Drug Inactivation or Alteration

Many bacteria produce enzymes such as β -lactamases, aminoglycoside-modifying enzymes, or chloramphenicol acetyltransferases that irreversibly modify and inactivate antibiotics. One of the well-characterized enzymes is β -lactamases. They are highly prevalent and act by hydrolysing the β -lactam ring present in all β -lactams (Santajit and Indrawattana, 2016). β -lactam is the most widely used class of cell-wall-targeting antibiotics since the 1920s. β -lactamase inhibitors, identified in the 1970s, are the most successful and clinically used antibiotic adjuvants to curb resistance to β -lactam antibiotics (Ma et al., 2020).

2.6.2. Modification of Drug Binding Sites

Some resistant bacteria avoid recognition by antimicrobial agents by changing their drug-binding sites. The mutation of the gene encoding for penicillin-binding proteins (PBPs), which are enzymes typically anchored on the cytoplasmic membrane of the bacterial cell wall and function in assembly and control of the latter stages of the cell wall building, results in the expression of unique penicillin-binding proteins (Santajit & Indrawattana, 2016). For instance, the expression of a unique PBP2a in *S. aureus*, which is the most dominant PBP in the MRSA cell compared to the native PBPs (PBP1–4) (Santajit & Indrawattana, 2016). PBP2a has a low affinity for all β -lactam antibiotics and acts as a substitute for the other PBPs and that results in the ability of *S. aureus* to

survive in the presence of high concentrations of β -lactam drugs including methicillin (Santajit & Indrawattana, 2016).

2.6.3. Reduced Intracellular Drug Accumulation

The difference between antibiotic uptake and elimination determines the susceptibility of bacteria to a particular drug. Thus, bacteria use several methods such as reducing the amount of antibiotic able to pass through the bacterial cell membrane to develop antibiotic resistance. Mechanisms by which bacteria achieve this include the occurrence of diminished protein channels on the bacterial outer membrane to decrease drug entry and the presence of efflux pumps to decrease the amount of drug accumulated within the cells (Santajit & Indrawattana, 2016).

2.6.4. Efflux Pumps

Efflux pumps expel the drug from the bacterial cell at a high rate, which means that the drug concentrations are never sufficiently high to produce an antibacterial effect (Santajit & Indrawattana, 2016). They serve as a source of multi-drug resistance in ESKAPE pathogens. Efflux pumps are of considerable interest for the development of novel adjunct therapies. Potent efflux pump inhibitors may be used to counteract the prevalence of MDR bacteria and increase the efficacy of existing antibiotics. Through competitive inhibition of antibiotics on the efflux pumps of *P. aeruginosa*, a series of pyridopyrimidine compounds such as phenylalanine arginyl β -naphthylamide and other derivatives have been introduced as efflux pump inhibitors. Pyranopyridine EPI, MBX2319, was reported with better activity against *Enterobacteriaceae* than *P. aeruginosa* (Ma et al., 2020).

2.6.5. Biofilm Formation

Biofilms are complex microbial communities living as thin layers on biotic or abiotic surfaces, implanted in a matrix of extracellular polymeric substances created by the biofilms themselves. Microorganisms within the biofilm can interact with each other, as well as the environment (Santajit & Indrawattana, 2016). One of the most important features of microbial biofilms is their intrinsic antibiotic tolerance. For example, *P. aeruginosa* can survive antibiotic treatment because of its capability to form biofilms which display both intrinsic tolerance and mutational resistance. Biofilm formation and dispersal are highly controlled processes regulated at the genetic level and by environmental signals (Ma et al., 2020).

2.7 Emerging methods to fight ESKAPE pathogens

The general method to effectively treat infections caused by bacteria involves the use of antibiotics either singly or in combination. With every passing year, the overall number of antibiotics effective against ESKAPE is decreasing, which is predisposing us toward a future with ineffective antibiotics. It is, therefore, pivotal to find alternative ways to treat infections especially those caused by ESKAPE pathogens (Mulani et al., 2019). Alternative therapies that are currently in practice or under trial include the use of antibiotics in combination with adjuvants, bacteriophage therapy, use of antimicrobial peptides, photodynamic therapy, antibacterial antibodies, phytochemicals and nanoparticles as antibacterial agents (Wang et al., 2020; Ma et al., 2020).

2.7.1. Antibiotics in combination

Antibiotics in combination have been tested and proved to have potential advantages when it comes to treating infections caused by MDR bacteria. One of the advantages is

that combination therapy increases the spectrum of coverage, allowing a higher probability of appropriate initial therapy and reducing mortality (Vazquez-Grande & Kumar, 2015). Secondly, combination therapy reduces the risk of the emergence of resistance when compared with the use of a single drug (Mulani et al., 2019). Lastly, potential additive or synergistic effects leading to more rapid pathogen clearance may be the most directly clinically relevant advantage of combination therapy (Vazquez-Grande & Kumar, 2015).

2.7.2. Bacteriophage therapy

Bacteriophages are viruses that infect and kill bacterial cells and, in the case of lytic phages, the bacterial metabolism is disrupted and which causes the bacterium to lyse. They were discovered by Ernest Hankin (1896) and Frederick Twort (1915) who described their antibacterial activity. However, Felix d'Herelle (1919) was probably the first scientist who used bacteriophages as a therapy to treat severe dysentery (Garcia et al., 2008). Several strategies and applications of phage therapy have been reported to fight MDR pathogens, especially *P. aeruginosa*, *E. coli*, and *S. aureus* in clinical trials (Ma et al., 2020). Phages were used in the past in Europe for the treatment of infections caused by bacteria such as osteomyelitis, skin/wound infections, UTIs, and ear infection (Yaneja and Kaur, 2016). Phages are more advantageous than antibiotics due to their ability to mutate at least as fast as their hosts, giving them the ability to evolve new infectivity resulting in them regaining the “upper hand” over bacteria (Pirnay et al., 2018). Several studies carried out *in vitro* have proven phages to be effective as antibacterial agents against biofilm and planktonic cells of ESKAPE (Mulani et al., 2019).

2.7.3. Antimicrobial peptides (AMPs) in therapy

AMPs are also known as host defence peptides because they are important components of the innate immunological defence system. They are expressed by the host to defend against invading pathogens and boost the immune response in most living species such as protozoa, bacteria, archaea, fungi, plants and animals (Mulani et al., 2019; Ma et al., 2020). AMPs are considered to be the potential alternative to combat MDR pathogens due to their ability to interact with bacterial cell membranes which result in cell lysis. Moreover, AMPs have more advantages than antibiotics because they physically damage the bacterial cell through electrostatic interactions thereby making it difficult for bacteria to develop resistance against them (Mulani et al., 2019). Figure 2.1. shows mechanisms of action of antimicrobial peptides.

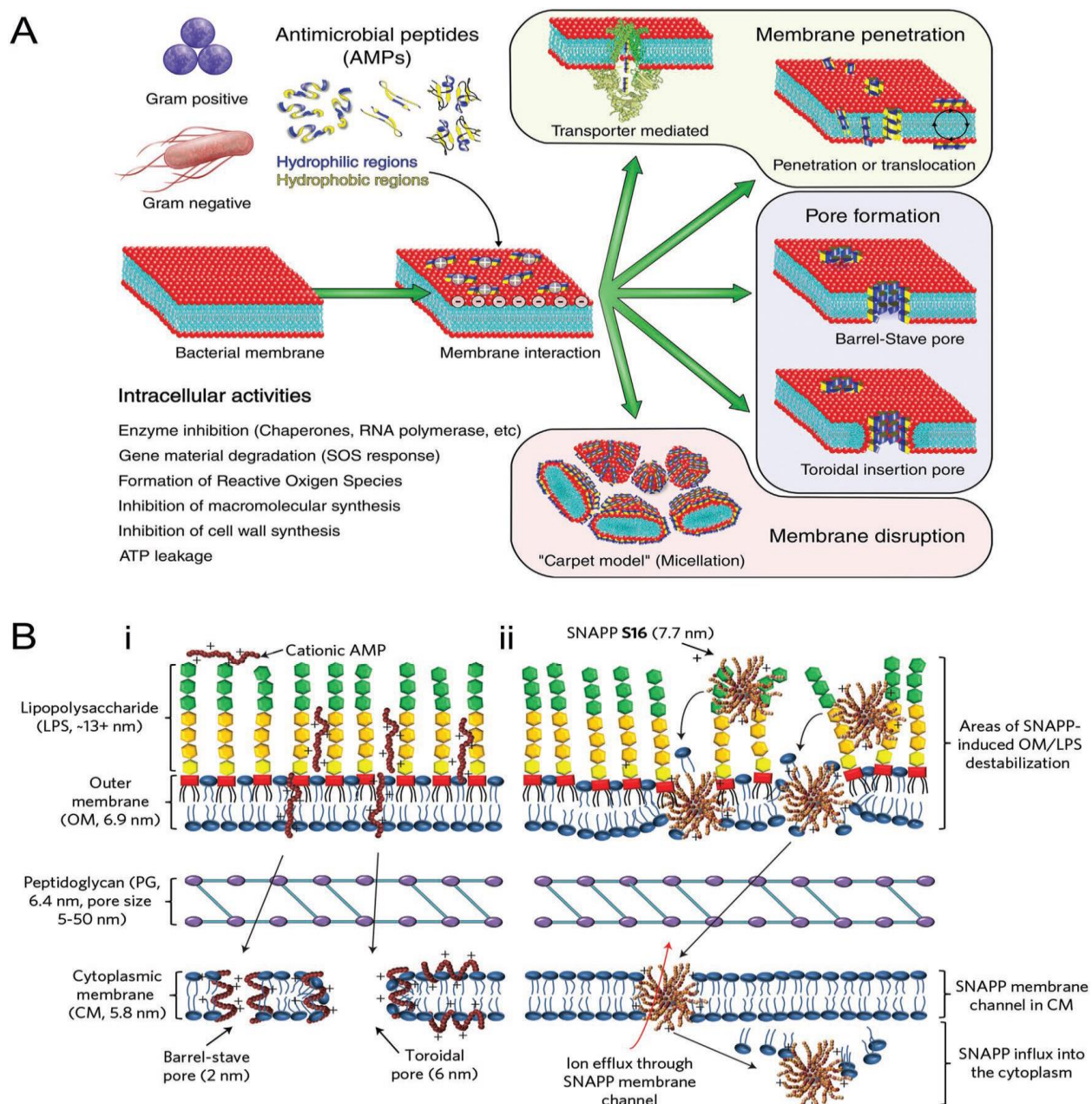


Figure 2.1: Antimicrobial peptides. A) Mechanisms of action of antimicrobial peptides (AMPs). B) Comparison between the antimicrobial mechanism of AMPs. Source adapted from (Ma et al., 2020)

2.7.4. Photodynamic light therapy

Antimicrobial photodynamic inactivation (aPDI) is a therapeutic option used in the treatment of infectious diseases such as dental, skin, and soft tissue infections. It is based on a combination of a photosensitizer (PS), light and oxygen to remove highly

metabolically active cells such as fungi, viruses or bacteria (Mulani et al., 2019; Nakonieczna et al., 2019). The technique employs harmless white light to photo-inactivate both antibiotic-sensitive and antibiotic-resistant pathogens via the generation of cytotoxic reactive oxygen species resulting in a photo-oxidative stress response that leads to the death of microbes. Excitation of PS with the light of an appropriate wavelength leads to the formation of an excited triplet state which can transfer electrons or energy to biomolecules or molecular oxygen, resulting in the formation of reactive oxygen species (ROS) or single oxygen radicals, which are toxic to cellular targets such as nucleic acids, proteins and lipids (Mulani et al., 2019; Ma et al., 2020). Antimicrobial photodynamic inactivation has several advantages when compared with other methods of treatment because it allows local treatment, which reduces side effects. Furthermore, aPDI has several cellular targets and therefore it is highly unlikely that the microbes can develop resistance to the treatment (Nakonieczna et al., 2019).

2.7.5. Silver nanoparticles in therapy

The problem of drug-resistant pathogens can be resolved by using nano medicine which is one of the emerging branches. Metal nanoparticles have wide biomedical applications as antimicrobial agents due to their unique physical and chemical properties (Mulani et al., 2019). Amongst metal nanoparticles, silver nanoparticles (AgNPs) synthesized using physical, chemical or biological methods have shown promising bactericidal activity due to their multi-targeted approach which reduces the probability of resistance (Mulani et al., 2019). Furthermore, silver-containing nanoparticles show inhibitory activities against ESKAPE bacteria and reduced toxicity to mammalian cells (Ma et al., 2020). The ability of silver nanoparticles to kill the pathogenic, MDR as well as multidrug susceptible strains of bacteria was studied by many scientists, and it was proved that silver

nanoparticles are the powerful methods which can be used against the MDR bacteria such as *Pseudomonas aeruginosa*, ampicillin-resistant *Escherichia coli*, erythromycin-resistant *Streptococcus pyogenes*, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) (Rai et al., 2012). Figure 2.2 shows the mechanisms of action of nano delivery strategies.

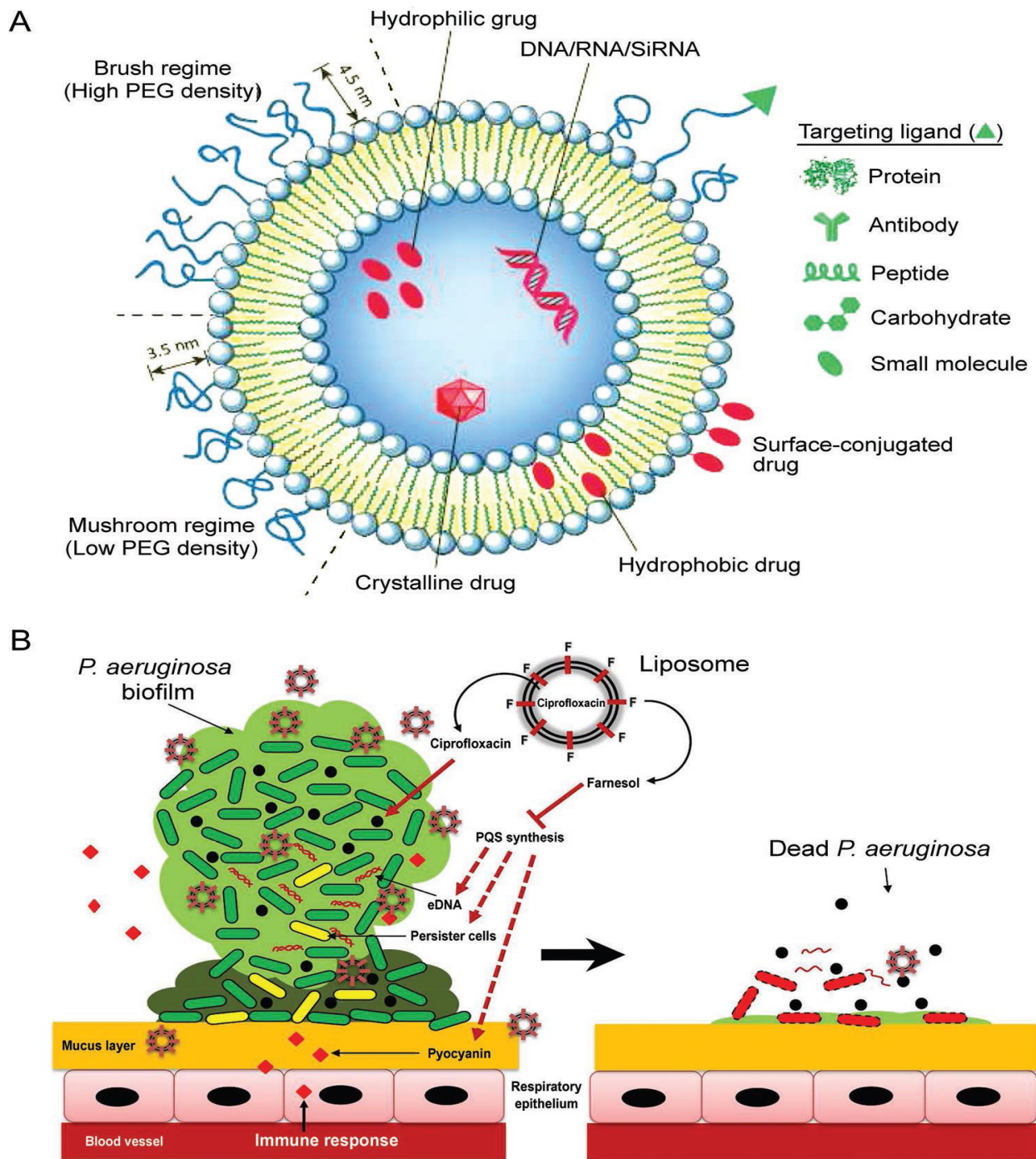


Figure 2.2.: Nanodelivery strategies. A) General structure of a liposome. B) Proposed killing mechanisms and summary of disruption of *P. aeruginosa* by farnesol and ciprofloxacin loaded liposomes. Source: adapted from (Ma et al., 2020)

2.8. Taxonomy of ESKAPE pathogens

2.8.1. *Enterococcus faecium*

The genus *Enterococcus* comprises Gram-positive, catalase-negative, non-spore-forming, facultative anaerobic bacteria that can occur both as single cocci and in chains. These organisms belong to a group of organisms known as lactic acid bacteria (LAB) that produce bacteriocins (Fisher & Phillips, 2009). The history of enterococci cannot be considered separately from that of the genus *Streptococcus*. Previously, scientists have combined all streptococci and enterococci species, which means they were all under the genus *Streptococcus* (Javed et al., 2011; Maheux et al., 2011). Enterococci were previously included in a group known as faecal streptococci, which are now regrouped into the genus *Enterococcus* (Maheux et al., 2011). The genus *Enterococcus* has been reviewed several times by a large number of scientists (Schleifer & Kilpper-Balz, 1987; Stackebrandt & Teuber, 1988; Devriese & Pot, 1995; Hardie & Whiley, 1997; (Moreno et al., 2006). In 1984, methods such as DNA–DNA hybridisation and 16S rRNA sequencing studies indicated that the species *Streptococcus faecium* and *S. faecalis* were sufficiently different from other streptococci, and Schleifer and Kilpper-Balz (1984) proposed their transfer to the genus *Enterococcus* (Moreno et al., 2006; Fisher & Phillips, 2009). To date, 28 species, namely *E. asini*, *E. avium*, *E. canis*, *E. casseliflavus*, *E. cecorum*, *E. columbae*, *E. dispar*, *E. durans*, *E. faecalis*, *E. faecium*, *E. flavescens*, *E. gallinarum*, *E. gilvus*, *E. haemoperoxidus*, *E. hirae*, *E. malodoratus*, *E. moraviensis*, *E. mundtii*, *E. pallens*, *E. phoeniculicola*, *E. pseudoavium*, *E. raffinosus*, *E. ratti*, *E. saccharolyticus*, *E. saccharominimus*, *E. solitarius*, *E. sulfureus*, and *E. villorum*, have been added to the genus based on phylogenetic evidence strengthened by 16S rRNA DNA sequencing and DNA–DNA hybridization studies (Moreno et al., 2006).

2.8.2. *Staphylococcus aureus*

The term “Staphylococcus” was introduced by Sir Alexander Ogston because the masses looked like bunches of grapes which differentiates these organisms from streptococci (Licitra, 2013; Cole et al., 2019). In 1884, the bacteria were differentiated based on the colour of their colonies by German physician Friedrich Julius Rosenbach into *S. aureus* (from the Latin aurum, gold) and *S. albus* (Latin for white). *S. albus* was later renamed *S. epidermidis* because of its ubiquitous nature on human skin (Licitra, 2013). The *Staphylococcus* genus is widely spread in the environment due to its ability to adapt easily to various environmental conditions. This genus contains 32 species, whereby 16 of which are found in humans as opportunistic microbe, forming part of the skin’s microbiota and the mucous of mammals and birds (De Jesús Cortés Sánchez et al., 2017). Species which are infectious and frequently isolated on humans include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus capitis*, *Staphylococcus hominis*, *Staphylococcus cohnii*, *Staphylococcus lugdunensis*, *Staphylococcus schleiferi*, *Staphylococcus saprophyticus*, *Staphylococcus simulans*, *Staphylococcus warneri* and *Staphylococcus xylosus* (Lindsay, 2011). *Staphylococcus aureus* is regarded as species of medical importance because of its known ability to cause several pathological conditions in humans and animals such as mild skin infections, bacteremia, systemic diseases, osteomyelitis as well as the more complicated toxic shock syndrome and staphylococcal food poisoning (SFP) (Akindolire et al., 2015).

2.8.3. *Klebsiella pneumoniae*

The genus *Klebsiella*, of the family Enterobacteriaceae, was named by Trevisan (1885) to honour the German microbiologist Edwin Klebs (Modh et al., 2021). Bacteria that belong to the genus *Klebsiella* are Gram-negative, encapsulated, non-motile, rod-shaped, lactose fermenting and facultative anaerobic bacterium (Aly et al., 2014). There are five species under the genus *Klebsiella*, namely, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Klebsiella ornithinolytica*, *Klebsiella terrigena* and *Klebsiella planticola* (Asha et al., 2017). *Klebsiella pneumoniae* as well as *Klebsiella oxytoca* are the medically-important species in the genus and are responsible for nosocomial infections in humans (Harada et al., 2016). *Klebsiella pneumoniae* is a species of medical importance because it is an opportunistic pathogen responsible for hospital-acquired infections such as pneumonia, pyogenic infections, meningitis, urinary tract infections (UTI) and rarely diarrhoea which attack immunocompromised, hospitalized individuals associated with diabetes mellitus, chronic pulmonary, cardiac, renal and neoplastic diseases (Asha et al., 2017). The type strain of *K. pneumoniae* is ATCC 13883 (=NCTC 9633, CDC 298-53, CIP 82.91) and contains three subspecies namely, *K. pneumoniae* subsp *pneumoniae*, *K. pneumoniae* subsp *ozaenae* and *K. pneumoniae* subsp *rhinoscleromatis* (Hæggman, 2010).

2.8.4. *Acinetobacter baumannii*

The genus *Acinetobacter* was discovered in 1911 by Beijernick as he isolated the organism from the soil and named it *Micrococcus calcoaceticus* (Al-Hasnawy & Al-Harmoosh, 2021). The genus was first proposed by Brisou and Pre´vot (1954) in the phylum Proteobacteria under the family Moraxellaceae (Li et al., 2014). The genus

Acinetobacter was widely accepted in 1968 after Baumann et al. (1968) published a comprehensive study of organisms such as *Micrococcus calco-aceticus*, *Alcaligenes hemolysans*, *Mima polymorpha*, *Moraxella lwoffii*, *Herellea vaginicola* and *Bacterium anitratum*, which concluded that they belonged to a single genus and could not be further sub-classified into different species based on phenotypical characteristics (Howard et al., 2012). In 1971, the sub-committee on the Taxonomy of Moraxella and Allied Bacteria officially acknowledged the genus *Acinetobacter* based on the results of Baumann's 1968 publication (Howard et al., 2012). Currently, 59 different species with distinct names are found under the genus *Acinetobacter*. A total of 11 effectively but not validly published named species and nearly 15 tentative species with designation have been reported (Vijayakumar et al., 2019). The term '*Acinetobacter calcoaceticus*–*Acinetobacter baumannii* (ACB) complex' was initially described by Gerner-Smidt and Tjernberg in 1993 for a group of four phenotypically similar species. These included *A. calcoaceticus*, *A. baumannii* and genomic species (gen. sp.) 3 and gen. sp. 13, which were later named *Acinetobacter pittii* and *Acinetobacter nosocomialis*, respectively (Nemec et al., 2015).

Currently, five *Acinetobacter* species are associated with human diseases (*A. baumannii*, *A. pittii*, *A. nosocomialis*, *A. seifertii* and *A. dijkshoorniae*) and one environmental species (*A. calcoaceticus*) are very closely related and display similar phenotypic and biochemical properties and thus, it is difficult to differentiate them at the individual species level. Accurate identification of the clinically important members of this group is only possible by molecular methods (Vijayakumar et al., 2019). Bacteria belonging to the genus *Acinetobacter* are non-motile, strictly aerobic, oxidase-negative, catalase-positive and Gram-negative coccobacilli and they have other characteristics

which include their ability to utilize a wide range of compounds as sources of carbon and to grow on minimal salt medium (Malhotra et al., 2012). Out of all acinetobacter species, *Acinetobacter baumannii* is regarded as the medically important species because of its opportunistic nature to cause various problems such as pneumonia (commonly ventilator-associated), urinary tract and bloodstream infections as well as meningitis and wound infections in immunocompromised patients suffering from various underlying diseases (Müller et al., 2014).

2.8.5. *Pseudomonas aeruginosa*

The genus *Pseudomonas* belonging to the family Pseudomonadaceae in the class Gammaproteobacteria was described by Migula (1894). The taxonomic position of members of the genus *Pseudomonas* has changed several times based on physiological, molecular and phenotypic features, DNA–DNA hybridization, 16S rRNA gene sequence similarity and chemotaxonomic data (Amoozegar et al., 2014; Hameed et al., 2014). Some of the former *Pseudomonas* species have been redistributed into other genera which include the alpha, beta or gamma subclasses of Proteobacteria due to the sequencing of the 16S rDNA gene (*rrs*) (Mulet et al., 2010). The members of the genus *Pseudomonas* (*sensu stricto*) belong to Palleroni's RNA group I, in the Gammaproteobacteria (Mulet et al., 2010). More than 220 species with validly published taxonomic names have been characterized, and approximately 10 new species in the genus have been described yearly in the last 10 years (Lalucat et al., 2020). The genus *Pseudomonas* was classified by Mulet et al. (2010) into two main lineages, namely, the *Pseudomonas aeruginosa* lineage and the *Pseudomonas fluorescens* lineage, based on partial sequences of four core housekeeping genes (16S rRNA, *gyrB*, *rpoB* and *rpoD*) of the type strains of 107 species of the genus *Pseudomonas* (Zhong et al., 2015).

Members of the genus *Pseudomonas* are rod-shaped, Gram-negative, non-spore-forming, catalase positive, oxidase positive and have one or more polar flagella which provide motility (Yang et al., 2013). Within the *Pseudomonas* genus, *Pseudomonas aeruginosa* is a bacterium which is regarded as an opportunistic pathogen being frequently associated with causing nosocomial infections, such as respiratory tract infections, skin and soft tissue infections, bacterial keratitis as well as 'swimmers ear' infections in immunocompromised patients (Streeter & Katouli, 2016).

2.8.6. *Enterobacter* species

The genus *Enterobacter* was initially described by Hormaeche and Edwards in 1960 and its taxonomy has gone through several significant changes over the last 50 years (Mezzatesta et al., 2012). The taxonomy of *Enterobacter* has had a complicated history, with several species transferred to and from this genus, for example, *Enterobacter agglomerans* has been transferred to the genus *Pantoea*, and *Enterobacter sakazakii* was recently reassigned to the newly proposed genus *Cronobacter* (Mezzatesta et al., 2012; Davin-Regli et al., 2019). To date, twenty-two species are found in the genus *Enterobacter*, which includes *E. aerogenes*, *E. amnigenus*, *E. arachidis*, *E. asburiae*, *E. carcinogenus*, *E. cloacae*, *E. cowanii*, *E. dissolvans*, *E. gergoviae*, *E. helveticus*, *E. hormaechei*, *E. kobei*, *E. ludwigii*, *E. mori*, *E. nimipressuralis*, *E. oryzae*, *E. pulveris*, *E. pyrinus*, *E. radicincitans*, *E. soli*, *E. taylorae* and *E. turicensis* (Davin-Regli et al., 2019). Methods like whole genome DNA–DNA hybridizations and phenotypic characteristics have led to a group called *Enterobacter cloacae* complex, which includes seven species, namely, *E. cloacae*, *E. asburiae*, *E. hormaechei*, *E. kobei*, *E. ludwigii*, *E. mori* and *E. nimipressuralis* (Mezzatesta et al., 2012; Davin-Regli et al., 2019). The genus *Enterobacter* belongs to the Enterobacteriaceae family, bacteria belonging to this genus

are facultatively anaerobic, rod-shaped, and motile due to peritrichous flagella and their glucose fermentation occurs with acid and gas production (Ghazvini & Farsiani, 2020). *Enterobacter aerogenes* and *Enterobacter cloacae* are regarded as clinically important species because of their opportunistic nature to cause a wide range of nosocomial infections such as bloodstream, respiratory tract, and central nervous system infections as well as endocarditis (Davin-Regli, 2015; Ghanavati et al., 2018).

2.9. OCCURRENCE OF ESKAPE PATHOGENS IN AQUATIC ENVIRONMENTS

2.9.1. *Enterococcus faecium*

Bacteria of the genus *Enterococci* are common components of the normal flora of the intestinal tracts of all warm-blooded animals. They can enter streams or coastal waters through animal inputs worsening bacteriological quality (Caruso et al., 2008). *Enterococci* can be found in different aquatic habitats such as waste water and coastal marine environments, pristine waters and aquaculture (Valenzuela et al., 2010). The ability of *Enterococcus faecalis* and *Enterococcus faecium* to survive longer in a marine environment has been demonstrated (Caruso et al., 2008; Valenzuela et al., 2010). *Enterococci* may survive longer in marine environments due to their capacity to tolerate high salt concentrations (Caruso et al., 2008; Valenzuela et al., 2010). The prevalence and abundance of enterococci in faeces have resulted in these bacteria being used for decades as indicators of sewage or faecal contamination (faecal indicator bacteria; FIB) in recreational waters (Staley et al., 2014).

Faecal indicator bacteria (FIB) were chosen to assess the potential presence of pathogens in water bodies. Traditionally, *Escherichia coli* and faecal coliforms have been used as FIB in freshwater systems, while enterococci were initially used as

indicators in marine waters (Ran et al., 2013). The use of *E. coli* as an indicator of water quality is likely to be limited because this bacterium can become naturalized to the microbial community in tropical, subtropical and temperate soil and sand (Ran et al., 2013). The US Environmental Protection Agency (EPA) has suggested that coastal states and those bordering the Great Lakes states use enterococci as an alternative indicator of faecal contamination (Ran et al., 2013; Staley et al., 2014). Identification of enterococci in beach water renders many advantages over *E. coli*, such as having the ability to determine the main sources of faecal contamination because species from this genus are found in varying proportions in animal and human faeces (Dada et al., 2013).

2.9.2. *Staphylococcus aureus*

The presence of *Staphylococcus aureus* in drinking water is a concern because of its potential to cause human infection and also because of its multiple antimicrobial resistance (Santos et al., 2020). *S. aureus* is not typically considered a waterborne pathogen, but recent research has demonstrated that both clinical and environmental strains of this pathogen can survive in marine and freshwaters for up to ten days. Waterborne *S. aureus* has been detected in recreational marine and fresh waters (Hatcher et al., 2016). Studies of *S. aureus* in marine environments have concluded that swimmers are linked to the dissemination of *S. aureus* in marine water through the shedding of the bacterium from their noses, skin, and respiratory tract (Akanbi et al., 2017). *S. aureus* is occasionally found in high abundance in recreational waters as well as in sand, and the abundance is related to bather density and attributed to human activity around the beach (Goodwin & Pobuda, 2009; Goodwin et al., 2012). *Staphylococcus aureus* in potable water may be regarded as a natural flora or one of the genera that are commonly found in water supplies known as heterotrophic plate

count (HPC) bacteria (Chouhan, 2015; Adesoji et al., 2019). The incidence of high concentrations of *S. aureus* in water intended for human consumption may represent potential health hazards and consumption of such water may result in disease manifestations such as boils, skin sepsis, post-operative wound infections, enteric infections, septicaemia, endocarditis, osteomyelitis and pneumonia (Chouhan, 2015). Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) have been detected in air samples from many environments, including wastewater treatment plants, and the fact that these bacteria can be aerosolized from water and are capable of colonizing skin and soft tissues, exposure through inhalation is of concern (Goldstein et al., 2014).

2.9.3. Klebsiella pneumoniae

Klebsiella pneumoniae is an organism that is ubiquitous in the environment and has been isolated from a variety of habitats such as surface water, lakes, salt water, fresh water, brackish water, sewage, soil, vegetation, grain, land mammals, sea mammals and importantly gastrointestinal tract of humans (Van Ginkel, 2017; Modh et al., 2021). This bacterium is included in a group of bacteria called faecal coliforms with insignificant public health risk but Padschun and co-workers isolated potentially pathogenic *K. pneumoniae* from the aquatic environment in Germany that possess virulence factors such as pili, serum resistance and siderophore (Barati et al., 2016). The presence of these virulence factors which are commonly present in the clinical isolates of *Klebsiella pneumoniae* isolated from water suggests that the *K. pneumoniae* of environmental origin could have public health implications (Barati et al., 2016). *Klebsiella pneumoniae* is one of the valuable contamination indexes of water, food and agricultural products (Maal et al., 2014).

2.9.4. *Acinetobacter baumannii*

Acinetobacter baumannii is considered to be ubiquitous as it can be recovered from various environments, including soil or surface water, human skin, and inorganic surfaces (Tsai et al., 2018). Water and soil are regarded as major habitats for *A. baumannii* but the pathogen has been isolated from other sources such as foods, arthropods, animals, and humans (Karumathil et al., 2014). The organism has been isolated from raw sewage and wastewater treatment plants as well as from the activated sludge, and the ability of this organism to grow on crude oil has also been demonstrated (Gallego, 2016). One of the reasons *Acinetobacter baumannii* has gained increasing attention in recent years is because of its role in the removal of phosphorus or the degradation of polycyclic aromatic hydrocarbons from wastewater (Narciso-da-Rocha et al., 2013; Doughari et al., 2009). The growth of *A. baumannii* has been reported to be supported by Protozoans such as *Acanthamoeba* which act as its reservoir in water (Karumathil et al., 2014). Unpolluted sites such as ground, surface, or tap water are also places where *Acinetobacter* spp. are frequently found and these habitats constitute important sources of bacteria with the potential to infect humans, particularly immune-compromised ones (Narciso-da-Rocha et al., 2013).

2.9.5. *Pseudomonas aeruginosa*

P. aeruginosa is one of the most diverse and ecologically important groups of bacteria playing crucial roles in the carbon and nitrogen cycles in many natural environments (Khan et al., 2009). The bacterium is highly ubiquitous in water systems because its polysaccharide capsule enables it to adhere to surfaces where the cells multiply rapidly

on surfaces in contact with water delivery systems under favourable conditions to form a biofilm (Lutz & Lee, 2011; Loveday et al., 2014). *P. aeruginosa* is commonly detected in both natural and man-made water ecosystems since it is a strong biofilm producer (Simcox et al., 2019). Furthermore, it is often found in the drinking water supply and this contamination is difficult to remove, even by backwashing at free chlorine concentrations of >2 mg/L (Zhang et al., 2013). This bacterium thrives best in the distal parts of the water distribution system, such as taps and sinks where there is sufficient access to oxygen since it is an aerobic bacterium that relies on oxidation to metabolize carbohydrates (Loveday et al., 2014). Unlike other waterborne pathogens, *P. aeruginosa* thrives in the environment over a wide range of temperatures and is capable of exploiting relatively nutrient-poor circumstances (Loveday et al., 2014). Studies have shown that *Pseudomonas* spp. was one of the most frequently identified agents associated with waterborne outbreaks of dermatitis (rash or folliculitis), conjunctivitis, otitis externa and other symptoms in recreational water in the United States of America (Tirodimos et al., 2010). Moreover, studies have well described the role of *P. aeruginosa* in outbreaks related to contaminated tap or bottled water (Kauppinen et al., 2021). Pseudomonads can survive in whirlpools, hot tubs and indoor pools because of the warm water temperatures and these waters are prone to contamination during periods of high use when it is difficult to maintain adequate disinfection levels (Tirodimos et al., 2010). *P. aeruginosa* is found also in freshwater environments such as rivers, bottled mineral water, holy water, tap water, water baths, humidifiers and distilled water and can grow relatively fast in distilled water obtained in hospitals (Khan et al., 2009).

2.9.6. *Enterobacter species*

Members of the genus *Enterobacter* are ubiquitous and are found in habitats such as water, sewage, vegetables and soil (Grimont & Grimont, 2006). These microorganisms are saprophytic in the environment, as they are found in soil and sewage, and are also part of the commensal enteric flora of the human gastrointestinal tract (Mezzatesta et al., 2012; Akbari et al., 2016). The bacteria can also be regarded as pathogens of plants, insects, and humans and are ubiquitous in terrestrial and aquatic environments (Akbari et al., 2016; Hoffmann & Roggenkamp, 2003a). Studies indicate that *E. amnigenus* biogroups 1 and 2 have been isolated from drinking water, surface water and wild soils whereas *E. asburiae* has been isolated from water, soils, plants, food, hospital environments and health care staff equipment, such as probes and catheters (Davin-Regli et al., 2019). Furthermore, *E. cloacae* has been isolated from food, especially from samples of a formula containing plants, raw vegetables and roots, as well as from drinking water (Davin-Regli et al., 2019).

2.10. Detection of ESKAPE pathogens

The first important step in effective patient care is to accurately identify the causative agent responsible for an infection. This first step guides patient treatment strategies and effective usage of antibiotics in cases where an infection is caused by bacteria (Renner et al., 2017). The current gold standard for clinical diagnostics includes microbiological culture followed by the biochemical identification of bacteria where bacterial isolates are routinely identified by morphological and biochemical methods in the clinical laboratory (Leung et al., 2017). Culture-based methods within clinical laboratories are typically used to identify and detect several infections such as

nosocomial infections caused by ESKAPE pathogens (Ternus et al., 2021). Nucleic acid amplification and molecular diagnostic techniques, such as PCR, are displacing laboratory culture-based methods of bacterial identification because of their ability to offer increased accuracy (Leung et al., 2017; Renner et al., 2017). Once the identities of bacteria are confirmed, additional testing such as antibiotic susceptibility can be performed to guide definitive antibiotic treatment (Leung et al., 2017).

2.11. Pathogenicity and virulence determinants of ESKAPE pathogens

Bacterial virulence factors give bacteria the ability to survive the selection pressure and adapt to new pathogenic niches, these genes comprise a wide array of molecules interacting directly with eukaryotic cells, ranging from the membrane to secretory proteins (toxins, exoenzymes, type I to VI secretion systems), biofilm-forming proteins, siderophores as well as polysaccharides that compose the capsules and exhibit anti-phagocytic properties (Bakour et al., 2016). For example, *Enterococcus faecium* displays several virulence factors which include pheromone-inducible multifunctional aggregation substance (AS) (*asa1*, *prgB*, *asp1* gene variants and *asc10* on pathogenicity island), extracellular surface protein (Esp) (encoded by *esp* gene on pathogenicity island), a group of hydrolytic enzymes including hyaluronidase and gelatinase (encoded by the plasmid *hylEfm* gene and chromosomal *gelE* gene), secreted virulence factor such as cytolysin (encoded by *cytLL* *cytLs* genes on pathogenicity island or the plasmid) and *fsr* locus, which regulates other genes important for virulence (Seputiene et al., 2012).

Staphylococcus aureus expresses several cell surface factors that play a role in its virulence which include microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), capsular polysaccharides, and staphyloxanthin (carotenoid

pigment) as well as secreted factors (exotoxins) which can be divided into four categories, namely, superantigens, cytolytic (pore-forming) toxins, various exoenzymes and miscellaneous proteins (Costa et al., 2013). *Klebsiella pneumoniae* has many virulence genes that play a significant role in its pathogenicity of strains isolated from nosocomial infections such as coding regulators of mucoid phenotype A (rmpA), type 1 and type 3 adhesins (fimH-1, mrkD), aerobactin (iron siderophore) synthase (iucC), bacteriocin biosynthesis [enterobactin (entB) and yersiniabactin (irP-1)], and serum-dependent outer membrane lipoprotein (traT) (Ahmadi et al., 2022).

Acinetobacter baumannii possesses important virulence factors which include phospholipase C activity, serum resistance, hemolytic activity, siderophore activity, and acinetobactin production which play a role in assisting the organism to survive within a host and produce an infection, as well as the biofilm which is formed by a bacterial community in response to an adverse environment (Ali et al., 2017). In cases of *Pseudomonas aeruginosa*, several toxins and proteases are secreted, namely, exotoxin A, exoenzyme S, pyocyanin, elastase as well as lipopolysaccharide (LPS) components of the bacterial cell membrane which injure mammalian hosts by increasing endothelial permeability (Azghani et al., 2000). Several factors contribute to the pathogenesis of *Enterobacter cloacae* such as biofilm formation, secretion of multiple cellular toxins such as enterotoxins, hemolysins, and pore-forming toxins as well as type III secretion system and curli fimbriae (Ghazvini and Farsiani, 2020).

2.11.1. Pheromone-inducible multifunctional aggregation substance (AS)

Aggregation substance (AS) is responsible for the formation of larger vegetations and mediates adherence to neutrophils (polymorphonuclear leukocytes [PMNs]) through a preferential route, allowing resistance to inhibition or destruction (Lucilla et al., 2004;

Chuang et al., 2009). It has been suggested that the ability to synthesize extracellular polysaccharides (slime) might mediate such alternate phagocytosis leading to enterococcal survival within peritoneal macrophages (Lucilla et al., 2004).

2.11.2. Extracellular surface protein (Esp)

Extracellular surface protein (esp) is a protein associated with the cell wall which was first described in *Enterococcus* species by Shankar et al. (1999). It is thought to be responsible for the promotion of adhesion, colonization and evasion of the immune system, and to play some role in antibiotic resistance. It also contributes to the formation of biofilm of Enterococci, which accounts for resistance to environmental stresses, and adhesion to eukaryotic cells, such as those of the urinary tract (Kafil & Mobarez, 2015).

2.11.3. Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)

Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) on the *S. aureus* surface are responsible for mediating staphylococcal adherence to components of the extracellular matrix of the host. These components are attached covalently to peptidoglycan by sortase enzymes and they also participate in biofilm formation (Ghasemian et al., 2015).

2.11.4. Staphyloxanthin

Staphyloxanthin is an orange-red triterpenoid carotenoid whose biosynthesis and structure have been recently clarified. It is well known that the role of carotenoids is to function as antioxidants, and it has been suggested that staphyloxanthin can protect

Staphylococcus aureus against oxidative stress (Clauditz et al., 2006). Furthermore, carotenoid pigment staphyloxanthin is an important virulence factor in *S. aureus* which plays a role in protecting *S. aureus* from innate immune defence systems (Xue et al., 2019).

2.11.5. Type 1 and type 3 adhesines (*fimH-1*, *mrkD*)

The fimbriae or pili are non-flagellar, filamentous projections on the bacterial surface that are responsible for mediating the attachment of the organism to respiratory, gastrointestinal, and urinary tract mucosal cells (Gupta et al., 2003). There are two fimbrial adhesions expressed by *K. pneumoniae*, namely, type 1 and 3 fimbriae. Type 1 fimbriae are important for enabling *K. pneumoniae* to cause urinary tract infection (UTI) and are found on all members of Enterobacteriaceae. Whereas type 3 fimbriae have been shown to promote biofilm formation on biotic as well as abiotic surfaces. Moreover, type 3 fimbriae are responsible for mediating binding to extracellular matrix proteins such as collagen and epithelial cells of respiratory and urinary tracts (Abdulhasan, 2015).

2.11.6. Siderophores

Microorganisms growing under aerobic conditions require iron for a wide range of functions including the reduction of oxygen for the synthesis of ATP, the formation of heme and other essential purposes (Ali & Vidhale, 2013). In most aerobic microbial habitats, iron exists as insoluble oxide hydrate complexes and during infections, this mineral is similarly not freely accessible for the pathogen due to the fact that it is tightly sequestered by proteins or haems in the host. Therefore, the free iron concentrations are always extremely low in the environment of microorganisms (Schalk et al., 2011).

Siderophores are defined as low-molecular-mass microbial compounds with a very high affinity for iron with functions that include mediating iron uptake by microbial cells (Winkelmann, 2002). Chelating the ferric iron [Fe(III)] from different terrestrial and aquatic habitats and thereby making it available for microbial and plant cells is the primary function of these compounds (Ahmed & Holmström, 2014). In other words, the siderophores play an important role in the extracellular solubilization of iron from minerals to make it available to microorganisms (Schalk et al., 2011).

2.11.7. Phospholipase C activity

Phospholipases are a heterogeneous group of enzymes that share the ability to hydrolysing one or more ester bonds in glycerophospholipids (Ghannoum, 2000). Phospholipases which are important virulence factors in an increasing number of intra- and extracellular bacterial pathogens can be divided into three groups depending on the position of the bond they hydrolyse on the phospholipid substrate: phospholipase A (PLA), phospholipase C (PLC), and phospholipase D (PLD) (Raynaud et al., 2002). PLA is responsible for hydrolysing fatty acids from the glycerol backbone, whereas PLC cleaves the phosphorylated head group from the phospholipid. PLD is a transphosphatidylase that only cleaves off the head group (Lee et al., 2017). One method by which bacterial pathogens can invade host cells is by producing phospholipases, which act on phospholipids in host membranes resulting in membrane destabilizing products thereby leading to cytolysis and the release of host intracellular contents (Fiester et al., 2016). Destruction of phospholipids affects the stability of host cell membranes, and the cleaved head group can interfere with cellular signalling, resulting in changes in the immune response of the host (Lee et al., 2017).

2.11.8. Elastase (*LasB*)

Elastase B is a 33-kDa endopeptidase belonging to the thermolysin family (M4 peptidase) which hydrolyses the peptide bond at the amino side of the P1' amino acid residue, usually a hydrophobic one (Andrejko et al., 2013). As a major virulence factor of *Pseudomonas aeruginosa*, the enzyme elastase (*LasB*) supports the infection and colonization process by damaging tissue and degrading immune proteins (Szamosvári et al., 2016). It is proven that *LasB* destroys complement components, cytokines, immunoglobulins IgA and IgG, human airway lysozyme, proteinase-activated receptors and surfactant proteins A and D (Andrejko et al., 2013). Furthermore, *LasB* has been reported to be involved in the degradation of mucins and surfactant proteins which collectively function to promote bacterial clearance (Casilag et al., 2016). Lastly, the pathogenicity of elastase B to the host has been well defined, causing tissue damage, disruption of intercellular junctions of lung epithelia and degradation of components of the pulmonary innate immune system (Yu et al., 2014).

2.11.9. Lipopolysaccharide (*LPS*)

The outer cell wall of Gram-negative bacteria consists of lipopolysaccharide, which is the predominant inducer of inflammatory responses to these bacteria (Knapp et al., 2006). *LPS* induces an inflammatory response in the infected host due to its ability to bind membrane factors present on the surface of macrophages and other cells essential to host defence (Vreugdenhil et al., 2003). Overwhelming inflammation in response to *LPS* can be harmful and may cause septic shock with organ failure (Knapp et al., 2006). Bacterial lipopolysaccharide (*LPS*) is a toxic substance (endotoxin) with various biological activities *in vivo* and *in vitro*. When an excess amount of *LPS* is released *in*

vivo from infecting organisms, severe syndromes such as multiple organ failure (MOF), disseminated intravascular coagulation (DIC), endotoxic shock and abortion may be manifested (Kohmura et al., 2000). The syndromes are thought to occur mainly because of a set of biologically active mediators, including tumour necrosis factor (TNF) and nitric oxide (NO), which are released from various cells, especially macrophages, activated by LPS (Kohmura et al., 2000).

2.11.10. Pore-forming toxins (PFTs)

Pore-forming toxins (PFTs) are proteins that possess the ability to switch from a water-soluble form to a membrane-inserted pore form. There are over 80 different families of PFTs that have been characterized, including short peptides and large globular proteins (Rosado et al., 2008). PFTs which have been considered traditionally as virulence factors comprise almost one-third of all the characterized bacterial toxins, and are found across both Gram-positive and Gram-negative bacterial species (Rosado et al., 2008). PFTs promote pathogen growth and dissemination during bacterial infection through the disruption of epithelial barriers and interactions with the immune system. Moreover, PFTs change the plasma membrane permeability of their target cells which potentially leads to cell death, but may also lead to a more subtle cellular functions manipulation (Dal Peraro & Van Der Goot, 2016). PFTs create pores in the membranes of host cells, predominantly the plasma membrane but also intracellular organelle membranes to directly kill target cells for intracellular delivery of other bacterial or external factors to release nutrients or for phagosomal escape (Los et al., 2013). PFTs are secreted by the pathogens in a water-soluble form that attaches to the target cell and generally polymerizes into an amphipathic structure that finally inserts in the target

cell membrane and forms a pore which leads to cellular ion imbalance, and that is a widely used form of attack (Gonzalez et al., 2008).

2.11.11. Type III secretion systems (TTSS)

A wide range of Gram-negative pathogens of plants and animals possess type III secretion systems (TTSS) that can inject bacterial virulence “effector” proteins into host cells (Alfano & Collmer, 2004). Bacterial type III secretion systems which contribute a lot to the virulence of a wide spectrum of medically important pathogens are conserved membrane-embedded nanomachines that deliver virulence-effector proteins directly from the bacterial cytosol into the host cell (Nans et al., 2015). The TTSS apparatus is a structure that looks like a needle that includes the inner and outer membranes of the bacterial envelope and which is responsible for the secretion of translocon and effector proteins (Waterman & Holden, 2003). Translocon proteins enable effector proteins to gain access to the eukaryotic cell, probably by causing perforation in the host cell membrane and in some cases a connecting channel between the bacterium and the eukaryotic membrane, whereas the effector proteins destabilize different aspects of the host cell physiology and immunity, thus promoting bacterial virulence (Waterman & Holden, 2003).

2.12 Possible Reservoirs and Modes of Transmission of ESKAPE pathogens

The ESKAPE bacteria are regarded as clinically important species because they are the most common causes of life-threatening nosocomial infections amongst critically ill and immunocompromised individuals with characteristic potential drug resistance (Singh, 2018). The fact that these ESKAPE bugs represent new paradigms of pathogenesis, transmission, and resistance makes them extraordinarily important (Rice, 2008). Microorganisms such as ESKAPE pathogens may reach the potential host through different methods including via aerosols (inside droplets of water), direct contact

between two animated objects, and fomites (a contaminated inanimate object), vector transmission as well as through airborne transmission (Cunliffe et al., 2021; Ramsden, 2019).

2.13. Infectious syndromes of ESKAPE pathogens

ESKAPE pathogens with multiple drug resistance mechanisms have been implicated in a wide range of life-threatening hospital-acquired infections, especially among critically ill individuals (Ramsamy et al., 2018). For example, *Enterococcus faecium* is part of the intestinal microbiota of warm-blooded animals, including humans. However, it is associated with various extraintestinal diseases such as urinary tract, surgical wounds, and heart infections (Morrone et al., 2016). Furthermore, *E. faecium* is also responsible for nosocomial infections which include endocarditis, bacteremia, urinary tract infections as well as neonatal sepsis (Dutka-Malen et al., 1995). *Staphylococcus aureus* is both a commensal bacterium and a human pathogen which is a leading cause of bacteremia, infective endocarditis (IE) as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections (Tong et al., 2015). Another bacterium under the ESKAPE group is *Klebsiella pneumoniae*, which is a causative agent of many diseases such as pneumonia, urinary tract infections, bacteremia, burns and wounds infections and pyogenic liver abscesses (Aljanaby and Alhasani, 2016). Moreover, *Acinetobacter baumannii* is also an important opportunistic pathogen responsible for nosocomial infections such as bacteraemia, pneumonia, meningitis, urinary tract infections and wound infections (Sato et al., 2017). *Pseudomonas aeruginosa* is associated with a spectrum of diseases, including urinary tract infections, wound infections, septicemia, neonatal infections as well as pneumonia, keratitis and folliculitis in immune-deficient and healthy individuals (Trautmann et al., 2008; Faraji et al., 2016). Lastly, In human beings, multiple Enterobacter species such as *E. cloacae* and *E.*

aerogenes are known to act as opportunistic pathogens which can cause numerous infections, including eye and skin infections, meningitis, bacteremia, pneumonia, urinary tract infections, wound, intestinal infections and surgical site infections (Alammar, 2013).

2.14. Therapeutic options for ESKAPE pathogens

Therapeutic options for ESKAPE pathogens are so extremely limited in such a way that clinicians are forced to use older, previously discarded drugs, such as colistin, that are associated with nephrotoxicity and neurotoxicity and for which there is a lack of robust data to guide the selection of dosage regimen or duration of therapy (Boucher et al., 2009; Yaneja and Kaur, 2016). Colistin is a concentration-dependent bactericidal drug which is a cationic peptide that interacts and destabilizes the negatively charged bacterial cell membranes, leading to leakage of intracytoplasmic material. The drug acts against Extended-spectrum beta-lactamases (ESBLs) and carbapenemase-producing Enterobacteriaceae (CPE), *P. aeruginosa*, and *A. baumannii* which are the most worrisome pathogens (Yaneja & Kaur, 2016). A group of drugs called glycopeptide antibiotics which can inhibit bacterium peptidoglycan synthesis is considered drugs of the last resort to combat drug-resistant bacteria. Dalbavancin, oritavancin, and telavancin are second-generation glycopeptides which are semi-synthetic derivatives with superior pharmacokinetic and target engagement profiles that target vancomycin-resistant infections. Furthermore, dalbavancin and oritavancin demonstrate to be effective in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections (Ma et al., 2020). When it comes to carbapenem-resistant *K. pneumoniae* combination therapy with drugs such as tigecycline and gentamicin has been shown to be effective (Yaneja and Kaur, 2016). Oxazolidinone antibiotics (e.g. linezolid) have

shown efficacy against Gram-positive bacteria, including MRSA, vancomycin-resistant *S. aureus*, vancomycin-resistant enterococci (VRE), penicillin-resistant pneumococci, and anaerobes because of their ability to inhibit protein synthesis via binding to the 50S ribosome (Ma et al., 2020). Temocillin which is a drug derived from ticarcillin has shown the ability to act against ESBL- and AmpC-producing Enterobacteriaceae, but is ineffective in treating *Pseudomonas*, *Acinetobacter*, and anaerobic bacteria (Yaneja & Kaur, 2016). Fluoroquinolones directly target DNA gyrase and/or topoisomerase IV which are essential in DNA replication. Ciprofloxacin has been the most widely used fluoroquinolone for treating infections caused by Gram-negative bacteria, but because of increasing resistance towards it, FDA approved the newest fluoroquinolone by the name of delafloxacin in 2017, which is responsible for the treatment of complicated “acute bacterial skin and soft tissue structure infections” (ABSSSI) (Ma et al., 2020).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study area

This study was conducted in the Northwest province, South Africa with samples collected from different villages around Mahikeng and Zeerust. Individuals in these villages most often lack access to treated water and rely on other sources such as boreholes for their daily needs.

3.2. Ethical Clearance

Ethical clearance for the study was obtained from the Faculty of Natural and Agricultural Sciences Research Ethics Committee. An ethics number NWU-00502-21-A9) was assigned to the study.

3.3. Sample collection

Random sampling that was based on the availability or access was used to collect samples from different selected villages around Mahikeng and Zeerust. Sixty water samples were collected aseptically into 1-litre sterile bottles from municipal taps and boreholes taps in homes from different villages. The number of samples collected from the different villages is shown in Table 3.1. The samples were labelled properly and transported on ice to the Antimicrobial Resistance Phage Biocontrol Research Laboratory in the Department of Microbiology, North-West University for analysis immediately after collection.

Table 3.1.: Sample collection sites, sample source and number of samples collected

Sampling Area	Source and number of samples collected	
	Taps	Boreholes
Moshana	1	4
Rietpan	1	3
Motswedi	1	4
Mosweu	0	5
Ntsweletsoku	0	5
Dinokana	0	5
Lehurutshe	1	0
Dibate	1	4
Motlhabeng	1	4
Phola	1	4
Seweding	1	4
Ramosadi	1	4
Mocoseng	1	4
Total number of samples 60		

3.4. Sample processing

Aliquots of 50 ml of each water sample were filtered through a 0,45 µm grid filter unit (Type HA) using a Gelman Little Giant Pressure/vacuum pump machine (model 13156-Gelman Sciences, Michigan-USA). The filter papers were placed on MacConkey agar plates for Gram-negative bacteria (*K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* spp), Mannitol salt agar (MSA) for (*S. aureus*) and Bile Esculin agar (BEA) for (*E. faecium*) using sterile forceps. The plates were incubated aerobically at 37 °C for 24 hours. The preliminary ESKAPE pathogens were purified by sub-culturing on the respective media and plates were incubated aerobically at 37 °C for 24 hours to obtain discrete pure colonies.

3.5. Cellular morphology

The cellular morphologies of pure colonies were determined using Gram-staining (Cruickshank et al., 1975).

3.6. Bacterial control strains

Enterococcus faecium (ATCC 700221), *S. aureus* (ATCC 25923), *K. pneumoniae* (ATCC 13883), *P. aeruginosa* (ATCC 27853) and *E. cloacae* (ATCC 13047) obtained from BioMérieux, Marcy l'Etoile, France were used as positive control strains in all experiments. In addition, a previously confirmed and sequenced environmental *A. baumannii* isolate provided by the Antimicrobial Resistance and Phage Biocontrol Research Laboratory at the North West University, South Africa, was used as a positive control.

3.7. Molecular Characterization

Molecular characterization tests were performed on ESKAPE presumptive isolates to confirm their identities.

3.7.1. Extraction of genomic DNA

Prior to identification, each isolate was cultured on nutrient agar and incubated aerobically at 37 °C for 24 hours. Pure bacteria colonies were inoculated in 15 mL of Luria–Bertani (LB) broth (Merck, South Africa) and incubated aerobically at 37 °C for 24 hours. Bacterial DNA was extracted using the boiling cell method. A 500 µl culture broth of each isolate containing bacterial cells was centrifuged for 5 minutes at 10,000 rpm. The pellet was then resuspended in 100 µl of Tris EDTA (TE) buffer and boiled for 10 minutes. The sample was cooled to -20 °C for 10 minutes and centrifuged at 10,000

rpm for 10 minutes. The supernatants were stored in a freezer at -20 °C and used as DNA templates for PCR amplification.

3.7.2. PCR for the identification of ESKAPE pathogens

3.7.2.1. Amplification of bacterial 16S rRNA gene fragments

A 1420-bp fragment of 16S rRNA gene sequence was amplified using the universal bacterial primer sets Eubac 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The amplifications were performed in a mini cycler (model CG1 -96 Corbett Research, Australia). PCR reaction mixtures were prepared as 25 µl volumes containing 12,5 µl of 2X master mix (BioLabs, New England), 0,25µl each of the forward and reverse primers, 1 µl of template DNA and 11 µl of nuclease-free water. PCR conditions included an initial denaturation temperature of 94 °C for 4 minutes, 35 cycles of denaturation step of 94 °C for 1 minute, annealing for 1 minute at 55 °C, and an extension at 72 °C for 2 min; and a final extension at 72 °C for 10 minutes. The PCR products were stored at 4 °C until electrophoresis.

3.7.2.2. Species-specific PCR for the identification of ESKAPE pathogens

The identities of potential ESKAPE isolates were achieved through species-specific PCR assays using oligonucleotide primer sequences and conditions listed in Table 3.2 (Mahmudunnabi et al., 2018).

Table 3.2: Oligonucleotide primer sequences for species-specific PCR

Primer	Sequence (5'-3')	Target gene	Amplicon size (bp)	PCR cycling conditions	Reference
<i>Enterococcus faecium</i>	F:TTGAGGCAGACCAGATTGACG R:TATGACAGCGACTCCGATTCC	<i>ddlE.faecium</i>	658	Initial denaturation for 5 minutes at 95°C, 30 cycles of denaturation at 95°C for 60s, annealing at 56°C for 60s, extension at 72°C for 60s and final extension at 72°C for 7minutes	(Saleem, 2020)
<i>Staphylococcus aureus</i>	F: GCGATTGATGGTGGATACGGT R: AGCCAAGCCTTGACGAACTAAAGC	<i>nuc</i>	279	Initial denaturation for 5 minutes at 94°C, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 1 minutes and final extension at 72°C for 5 minutes	(Akindolire et al., 2015; Igbinosa et al., 2016)
<i>Klebsiella pneumoniae</i>	F: ATTTGAAGAGGTTGCAAACGAT R:TTCACCTCTGAAGTTTTCTTGTGTTC	<i>Klebsiella pneumoniae</i> 16s	130	Initial denaturation for 4 minutes at 94°C, 35 cycles of denaturation at 94°C for 1 minutes, annealing at 55°C for 1minutes, extension at 72°C for 1,5	(Mahmudunnabi et al., 2018; Turton et al.,

				minutes and final extension at 72°C for 10 minutes	2010)
sp4-F sp4-R	CACGCCGTAAGAGTGCATTA AACGGAGCTTGTCAGGGTTA	<i>A. baumannii</i>	490	Initial denaturation for 5 minutes at 95°C, 30 cycles of denaturation at 95°C for 20s, annealing at 66°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 7 minutes	(Li et al., 2016)
<i>Pseudomonas aeruginosa</i>	F: GGGGGATCTTCGGACCTCA R: TCCTTAGAGTGCCACCCG	<i>PA-SS</i>	956	Initial denaturation for 5 minutes at 95°C, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 60 seconds and final extension at 72°C for 5 minutes	(Spilker et al., 2004)
<i>Enterobacter</i> spp	F: GGTAAGAAGAAGGCGTGGTTGC R: ATGCATTCGGTGGTGATCATCAG	<i>Hsp60</i>	341	Initial denaturation for 5 minutes at 94°C, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 60s and final extension at 72°C for 5 minutes	(Akbari et al., 2016; Hoffmann and Roggenkamp, 2003b)

3.7.2.3. Determination of virulence genes of ESKAPE pathogens

Multiplex PCR assays were used to determine the virulence potentials of the isolates using specific primers sequences (Table 3.3.) following previous protocols.

Table 3.3: oligonucleotide primers sequences for virulence genes.

Organism	Target gene	Sequence (5'-3')	Amplicon size (bp)	PCR cycling conditions	Reference
<i>Staphylococcus aureus</i>	<i>Sea</i>	F: GGTTATCAATGTGCGGGTGG R: CGGCACTTTTTTCTCTTCGG	102	Initial denaturation for 5 minutes at 94°C, 35 cycles of denaturation at 94°C for 2 minutes, annealing at 57°C for 2 minutes, extension at 72°C for 60 seconds and final extension at 72°C for 7 minutes	(Akindolire et al., 2015)
	<i>Sec</i>	F: AGATGAAGTAGTTGATGTGTATGG R: CACACTTTTAGAATCAACCG	451	Initial denaturation for 5 minutes at 94°C, 35 cycles of denaturation at 94°C for 2 minutes, annealing at	(Akindolire et al., 2015)

				57°C for 2 minutes, extension at 72°C for 60 seconds and final extension at 72°C for 7 minutes	
<i>Klebsiella pneumoniae</i>	<i>fimH-1</i>	F: ATGAACGCCTGGTCCTTTGC R: GCTGAACGCCTATCCCCTGC	688	Initial denaturation for 4 minutes at 94°C, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 40 s, extension at 72°C for 60 seconds and final extension at 72°C for 10 minutes	(Aljanaby and Alhasani, 2016)
	<i>ycfm</i>	F: ATCAGCAGTCGGGTCAGC R: CTTCTCCAGCATTCAGCG	160	Initial denaturation for 4 minutes at 94°C, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 40 seconds, extension at 72°C for 60 seconds and final extension at 72°C for 10 minutes	(Aljanaby and Alhasani, 2016)
<i>Pseudomonas aeruginosa</i>	<i>alg D</i>	F: CGTCTGCCGCGAGATCGGCT R: GACCTCGACGGTCTTGCGGA	313	Initial denaturation for 5 minutes at 95°C, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 60 seconds and final extension at 72°C for 5 minutes	(Faraji et al., 2016)

	<i>las B</i>	F: GGAATGAACGAAGCGTTCTCCGAC R: TTGGCGTCGACGAACACCTCG	284	Initial denaturation for 5 minutes at 95°C, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 60 seconds and final extension at 72°C for 5 minutes	(Faraji et al., 2016)
<i>Enterobacter cloacae</i>	<i>rpoS</i>	F: CACTTCACGCTGTTTGGCG R: CGCGAGTTGTCCCATAAACTG	273	Initial denaturation for 5 minutes at 95°C, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 60 seconds and final extension at 72°C for 5 minutes	(Girlich et al., 2015)
	<i>fliI</i>	F: ATACGGCGCAGTGCGTTAC R: ACCAAAGAGAGGACACAATGC	154	Initial denaturation for 5 minutes at 95°C, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 60 seconds and final extension at 72°C for 5 minutes	(Girlich et al., 2015)

3.8. Antibiotic susceptibility test

Confirmed ESKAPE isolates were subjected to an antibiotic susceptibility test on Muller Hinton agar using the Kirby-Bauer disc diffusion method according to the CLSI guidelines (CLSI, 2017). The antibiotics Ampicillin (10 µg), Chloramphenicol (30 µg), Kanamycin (30 µg), Amoxicillin (10 µg), Piperacillin (100 µg), Aztreonam (30 µg), Gentamycin (30 µg), Cefuroxime (30 µg), and Trimethoprim-sulfamethoxazole (23,75 µg) all obtained from Davies Diagnostics, South Africa was used to determining the antibiotic resistance profiles of the isolates. Overnight cultures of ESKAPE isolates suspended in nutrient broth were transferred to nutrient agar in quantities of 100 µl. The inoculated plates were incubated at 37 °C for 24 hours. Pure colonies were picked and re-suspended in phosphate-buffered saline solution (pH=7.4). The optical density of the suspension was adjusted to a 0.5 McFarland turbidity standard. Aliquots of 100 µl of the inoculum were sub-cultured on Mueller Hinton agar and the antibiotics discs were placed on the inoculated plates. The plates were incubated at 37 °C for 24 hours. The bacteria growth inhibition zone diameter data were measured in millimetres and standard reference values from the CLSI, (2017) were used to classify the isolates as either sensitive(S), intermediate resistant (IR) or resistant (R) to specific antibiotics.

CHAPTER FOUR

RESULTS

4.1 Molecular identification of ESKAPE pathogens

4.1.1. Amplification of bacterial 16s rRNA by universal PCR

The bacterial 16s rRNA fragment was successfully amplified in all the presumptive ESKAPE isolates. All of the 158 isolates had the 16S rRNA sequences. Figure 4.1 indicate a 2 % agarose gel of the 16s rRNA gene fragments which yielded product sizes of 1420bp.

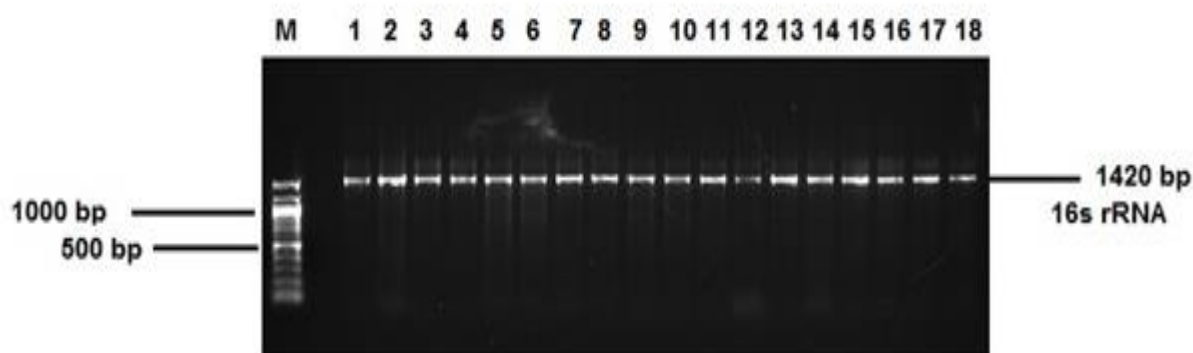


Figure 4.1.: Agarose gel (2% w/v) image showing 16S rRNA gene fragments amplified from *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *E. cloacae* isolates. Lane M - 100 base pairs DNA molecular ladder, Lane 0-No template DNA negative control, Lane 1 - 18= 16S rRNA gene fragments from isolates.

4.1.2. PCR analysis to confirm the identities of ESKAPE isolates.

PCR results revealed that none of the isolates amplified for the *E. faecium* and *A. baumannii* species-specific genes. A total number of 5/71 (7 %), 16/71 (23%), 18/71 (25%) and 32/71 (45%)isolates amplified for the species-specific genes of *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *E. cloacae* respectively. Figure 4.2. depicts a 2% agarose gel of the *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, and *E. cloacae* gene

fragments which yielded product sizes of 279 bp, 130 bp, 956 bp and 341 bp respectively.

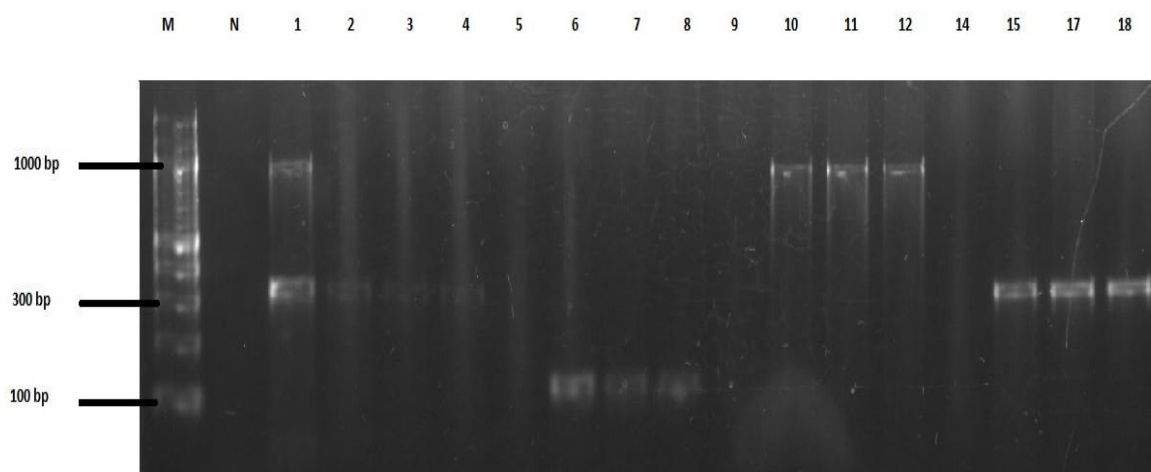


Figure 4.2: Agarose gel (2% w/v) image showing species specific gene fragments amplified from *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *E. cloacae* isolates. Lane M =100 base pairs DNA molecular ladder. Lane N = no template DNA negative control. Lane 1= *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27853) and *E. cloacae* (ATCC 13047) positive controls. Lane 2-4= *S. aureus* *nuc* gene. Lane 6= *K. pneumoniae* (ATCC 13883). Lane 7-8 *K. pneumoniae* 16S rRNA. Lane 10 -12 = *P. aeruginosa* *PA-SS* gene. Lane 15-18 = *E. cloacae* *Hsp60* gene.

4.1.3. Grouping of ESKAPE pathogens according to localities

Isolates were grouped according to localities based on the PCR protocol. ESKAPE pathogens were most frequently isolated from Dibate where 17 /71 (24%) isolates were confirmed by PCR. Detailed results are shown in Table 4.2.

Table 4.2.: The number of samples collected from various villages and the names of ESKAPE species identified.

Area	Sample source	No of samples collected	ESKAPE pathogens identified	No positive for the different species in samples
Dibate	Tap Borehole	12 5	<i>P aeruginosa</i> <i>K pneumoniae</i> <i>E cloacae</i> <i>S aureus</i>	3 5 8 1
Motlhabeng	Tap Borehole	7 4	<i>P aeruginosa</i> <i>K pneumoniae</i> <i>E cloacae</i> <i>S aureus</i>	3 4 4 0
Phola	Tap Borehole	0 15	<i>P aeruginosa</i> <i>K pneumoniae</i> <i>E cloacae</i> <i>S aureus</i>	5 4 5 1
Ramosadi	Tap Borehole	0 14	<i>P aeruginosa</i> <i>K pneumoniae</i> <i>E cloacae</i> <i>S aureus</i>	2 1 10 1
Seweding	Tap Borehole	0 10	<i>P aeruginosa</i> <i>K pneumoniae</i> <i>E cloacae</i> <i>S aureus</i>	4 1 3 2
Mocoseng	Tap Borehole	0 1	<i>P aeruginosa</i> <i>K pneumoniae</i> <i>E cloacae</i> <i>S aureus</i>	0 0 1 0
Rietpan	Tap Borehole	0 1	<i>P aeruginosa</i> <i>K pneumoniae</i> <i>E cloacae</i> <i>S aureus</i>	1 0 0 0
Mosweu	Tap Borehole	0 2	<i>P aeruginosa</i> <i>K pneumoniae</i> <i>E cloacae</i> <i>S aureus</i>	0 1 1 0
Total		71		71

4.1.4. Prevalence of virulence-associated genes

All the virulence genes tested were present in the isolates. A large proportion of 3 (60%) of *S. aureus* isolates harboured the *Sea* gene. While a large proportion of 10

(63%) of *K. pneumoniae* isolates harboured a *ycfm* gene. *las B* gene was most frequently isolated from 11 (61%) of *P. aeruginosa* isolates. Whereas a *fliI* gene was most frequently harboured by *E. cloacae* species in 30 (94%) of the isolates. A summary of the number of isolates that harboured the different virulence genes appear in Table 4.3. Figure 4.3 depicts a 2% (w/v) agarose gel of the virulence gene fragments amplified from *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *E. cloacae*.

Table 4.3: Prevalence of virulence genes

Species	Virulence genes	Number tested	Number amplified
<i>S. aureus</i>	<i>Sea</i>	5	3 (60%)
	<i>Sec</i>	5	2 (40%)
<i>K. pneumoniae</i>	<i>fimH-1</i>	16	6 (38%)
	<i>ycfm</i>	16	10 (63%)
<i>P. aeruginosa</i>	<i>alg D</i>	18	9 (50%)
	<i>las B</i>	18	11 (61%)
<i>E. cloacae</i>	<i>rpoS</i>	32	7 (22%)
	<i>fliI</i>	32	30 (94%)

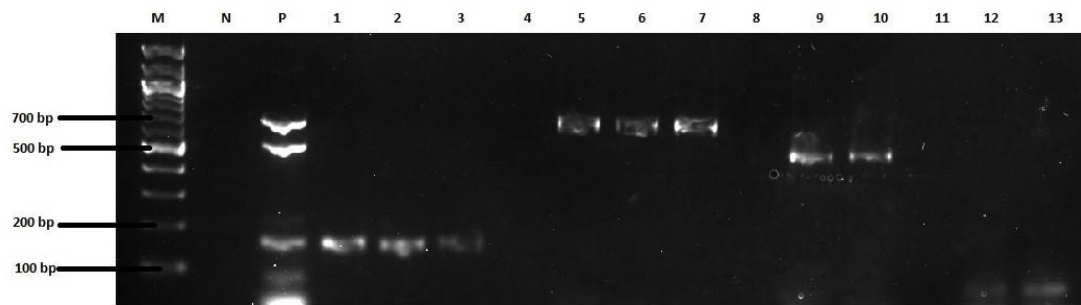


Figure 4.3: Agarose gel (2% w/v) image showing virulence gene fragments amplified from *S. aureus* and *K. pneumoniae* isolates. Lane M =100 base pairs DNA molecular ladder. Lane N = no template DNA negative control. Lane P = *S. aureus* (ATCC 25923) and *K. pneumoniae* (ATCC 13883) positive controls amplified for different virulence genes. Lane 1-3 = *ycfm* gene. Lane 5-7 = *fimH-1* gene. Lane 9-10 = *sec* gene. Lane 12-13 = *sea* gene.

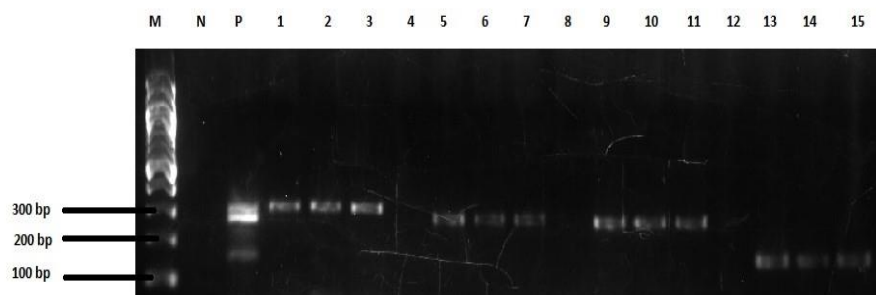


Figure 4.4: Agarose gel (2% w/v) image showing virulence gene fragments amplified from *P. aeruginosa* and *E. cloacae* isolates. Lane M =100 base pairs DNA molecular ladder. Lane N = no template DNA negative control. Lane P = *P. aeruginosa* (ATCC 27853) and *E. cloacae* (ATCC 13047) positive controls amplified for different virulence

genes. Lane 1-3 = *alg D* gene. Lane 5-7 = *las B* gene. Lane 9-11 = *rpoS* gene. Lane 13-15 = *fliI* gene.

4.2. Antimicrobial Resistance

S. aureus, *K. pneumoniae*, *P. aeruginosa* and *E. cloacae* isolates demonstrated different resistant patterns towards different antibiotics, with *S. aureus* isolates showing complete (100%) resistance to multiple antibiotics, including ampicillin, piperacillin, amoxicillin and aztreonam, whereas 94% of *K. pneumoniae* were resistant to amoxicillin, cefuroxime, aztreonam antibiotics. *P. aeruginosa* isolates showed some resistance (83%) towards amoxicillin and cefuroxime antibiotics, whereas 78% were resistant to ampicillin. More than 90% of *E. cloacae* isolates were resistant to antibiotics such as ampicillin, amoxicillin, cefuroxime and aztreonam. When it comes to antibiotic susceptibility, gentamicin seemed to be the only effective drug that inhibited the growth of most bacteria. Overall, the antimicrobial susceptibility pattern to gentamicin revealed *S. aureus* (100%), *K. pneumoniae* (94%), *P. aeruginosa* (94%) and *E. cloacae* (97%). A summary of antibiogram results is listed in table 4.3.

Table 4.4: Percentage of antibiotic resistance of ESKAPE pathogens. Isolates that demonstrated intermediate resistance to the different antibiotics were considered as resistant in the analysis.

Antimicrobial class	Antibiotics (disc content)	<i>S. aureus</i> (NT= 5) R	<i>K. pneumoniae</i> (NT=16) R	<i>P. aeruginosa</i> (NT= 18) R	<i>E. cloacae</i> (NT= 32) R
Penicillins	Ampicillin (10 µg)	5 (100%)	16 (100%)	17 (94%)	30 (94%)
	Piperacillin (100µg)	5 (100%)	15 (94%)	13 (72%)	29 (91 %)
	Amoxicillin (10µg)	5 (100%)	16 (100%)	18 (100%)	32 (100%)
Cephems	Cefuroxime (30µg)	5 (100%)	16 (100%)	18 (100%)	32 (100%)
Monobactams	Aztreonam (30µg)	5(100%)	15 (94%)	16 (88%)	31 (97%)
Phenicol	Chloramphenicol (30µg)	2(40 %)	11 (69%)	11 (61%)	25 (78%)

Antimicrobial class	Antibiotics (disc content)	S. aureus (NT= 5) R	K. pneumoniae (NT=16) R	P. aeruginosa (NT= 18) R	E. cloacae (NT= 32) R
Aminoglycosides	Gentamicin (30µg)	0 (0 %)	1 (6%)	01 (6%)	1 (3%)
	Kanamycin (30µg)	Not tested	13 (81%)	9 (50%)	26 (81%)
Folate pathway antagonists	Trimethoprim sulfamethoxazole (23,75µg)	2 (40 %)	14 (88%)	9 (50%)	14 (44%)

NT=Number Tested; **R**=Resistant;

4.3. Antibiotic resistance profiles of isolates.

A total of 71 isolates that were genotypically detected as ESKAPE pathogens by PCR were subjected to the disc diffusion antimicrobial susceptibility test. The test was done to assess the resistance patterns of isolates from the different sampling sites. A large proportion of isolates (100%) from Mocoseng were resistant to all antibiotics used in the study and all isolates were resistant to amoxicillin and cefalexin. The data in Table 4.5 indicate the percentages of resistance against a panel of nine antibiotics.

Table 4.5.: Proportion of isolates from the different sampling sites that were resistant to antibiotics tested

Sampling area	C30	K30	A10	PRL 100	GM 30	ATM 30	CXM 30	AP 10	TS 25
Dibate	9 (53%)	11 (65%)	17 (100%)	12 (71%)	0 (0%)	14 (82%)	17 (100%)	15 (88%)	11 (65%)
Motlhabeng	9 (82%)	9 (82%)	11 (100%)	9 (82%)	1 (9%)	11 (100%)	11 (100%)	11 (100%)	10 (91%)
Phola	12 (80%)	10 (67%)	15 (100%)	15 (100%)	1 (7%)	15 (100%)	15 (100%)	15 (100%)	11 (73%)
Ramosadi	12 (86%)	11 (79%)	14 (100%)	14 (100%)	0 (0%)	14 (100%)	14 (100%)	14 (100%)	11 (79%)
Seweding	5 (50%)	5 (50%)	10 (100%)	9 (90%)	1 (10%)	9 (90%)	10 (100%)	10 (100%)	6 (60%)
Mocoseng	1 (100%)	1 (100%)	1 (100%)	1 (100%)	0 (0%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)
Rietpan	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	1 (100%)	1 (100%)	0 (0%)	0 (0%)
Mosweu	0 (0%)	1 (50%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)

CHAPTER FIVE

DISCUSSION

5.1. General discussions

One of the pivotal factors for survival is access to safe drinking water which should not be a source of danger to people at any stage of life, including children, the elderly, and vulnerable subpopulations such as immunocompromised people (Hamad et al., 2022). The quality of drinking water is a very important issue of concern worldwide, especially in semi-arid countries such as South Africa where groundwater is the most important water resource (Nkuna et al., 2014a). It has been reported that the presence of microbial contaminants in groundwater such as viruses, bacteria and protozoa exposes consumers to a variety of waterborne infections when drinking water supplies are untreated or inadequately treated (Nkwe et al., 2015b). According to WHO, about 9.1% of the global burden of disease and 6.3% of all deaths are caused by unsafe water, inadequate sanitation, and poor hygiene (Pal et al., 2018).

The primary aim of this study was to investigate the presence of ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) pathogens from groundwater sources in some areas of Northwest province, South Africa. Even though these pathogens are frequently isolated from clinical environments and have been proven to have implications in a variety of life-threatening, hospital-associated infections, antibiotic-resistant ESKAPE strains have been isolated from environmental reservoirs such as surface water, wastewater, food, and soil (Denissen et al., 2022). In recent years, a study has been conducted in Kwa Zulu Natal, South Africa, where ESKAPE pathogens were isolated from clinical specimens such as blood, urine, catheters and respiratory specimens (Ramsamy et al., 2018). This study expands on the previous studies by characterising ESKAPE pathogens from groundwater, where 60 water samples from different groundwater sources in some areas of the Northwest

province, South Africa were collected and analysed for the presence of ESKAPE pathogens.

In our study area, all the ESKAPE pathogens isolated were grouped according to different localities of sample collection with Dibate village having more isolates of 17/71 (24%) followed by 15/71 (21%) from Phola, then 14/71 (20%) from Ramosadi. Rietpan and Mocoseng produced the least number of isolates, 1/71 (1%). The reasons behind this large difference in numbers are probably due to the fact that the quality of groundwater differs from place to place, in some instances depending on seasonal changes, the types of soils, rocks and surfaces through which it moves as naturally occurring contaminants are present in the rocks and sediments (Palamuleni & Akoth, 2015).

The identities of the ESKAPE pathogens were confirmed by PCR analysis. Biochemical characterization and other phenotypic identification such as conventional culture on selective media and Gram-staining often give false results and require a considerable amount of time for confirmation (Aurna, 2017; Mahmudunnabi et al., 2018). Molecular analysis has therefore been reported to be more accurate when compared to other preliminary identification tests (Abd El-Aziz). The present study performed PCR on the isolates for the amplification of 16s rRNA and ESKAPE-specific genes. All the isolates amplified for the 16S rRNA sequences, while (5/71), 16/71, 18/71 and 32/71 amplified for *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *E. cloacae* specific genes respectively. None of the isolates amplified for *A. baumannii* and *E. faecium*. That means out of 71 isolates, a large proportion represented *E. cloacae* (45%), followed by *P. aeruginosa* (25%) then *K. pneumoniae* (23%). Only a small proportion (7%) represented *S. aureus* and that is not surprising because *S. aureus* is primarily found on human skin and in nasal passages. This finding is in contrast with the previous study in which *P.*

aeruginosa was the most frequently isolated organism (33.3%), followed by *S. aureus* (24%), *Enterobacter spp.* (22.2%), *K. pneumoniae* (13%) and *A. baumannii* (7.4%), with no episodes of *E. faecium* isolation (Bodro et al., 2014).

ESKAPE pathogens which are responsible for the majority of nosocomial infections, and are capable of “escaping” the biocidal action of antimicrobial agents, have growing multidrug resistance and virulence (Tuttobene et al., 2021). In this study, PCR was performed on the isolates for the amplification of two virulence genes each for *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *E. cloacae*. The virulence genes included *Staphylococcal* enterotoxins A and C (*Sea* and *Sec* gene) which are emetic toxins responsible for causing food poisoning in humans (Omoe et al., 2002). Out of five *S. aureus* isolates, 2 (40%) were amplified for the *Sec* gene while 3 (60%) were amplified for the *Sea* gene. Previous reports designed to detect these toxin genes in *S. aureus* revealed that 32% of isolates tested possessed the *sec* gene and none of the other virulence determinants was detected (Akindolire et al., 2015). In the present study, 40% of isolates tested amplified for the *Sec* gene, whereas 60% amplified for the *sea* gene.

For *K. pneumoniae* PCR was used to detect genes encoding type 1 adhesin (*fimH-1*) which is responsible for the attachment of *K. pneumoniae* to epithelial cells through the first steps of invasion as well as for colonization of tissues and infection of the body (Aljanaby, 2017). Another virulence gene which was detected in *K. pneumoniae* isolates was *ycfM*, which is the outer membrane lipoprotein that is responsible for protecting *Klebsiella* from drug exposure (Nahar & Rashid, 2017). The *fimH-1* gene was detected in 38% of the isolates while the *ycfM* gene was detected in 63% of the isolates. This

study is in contrast to a previous report which stated that all the isolates amplified for the *fimH-1* and *ycfm* genes (Aljanaby & Alhasani, 2016).

One of the major virulence factors of *Pseudomonas aeruginosa*, the enzyme elastase (*LasB*) which supports the infection and colonization process by damaging tissue and degrading immune proteins was detected in 61% of the isolates (Szamosvári et al., 2016). Another one called *algD* which is a capsule-like polysaccharide called alginate that is responsible for the evasion of host defences was detected in 50% of the isolates (Jain & Ohman, 2005). This study is contrary to the previous reports where the prevalence of these genes was 100% (Mitov et al., 2010).

The previous study indicates that a large proportion of *Enterobacter cloacae* isolates (87.7%) carried *rpos* and *fliI* genes (Ghanavati et al., 2018). In the present study, 94% and 22% of the isolates had *fliI* and *rpos* genes amplified, respectively. *RpoS* gene in *E. cloacae* is responsible for playing an important role in protecting the bacterium from multiple stress conditions such as acid, heat, and oxidative stress, starvation, high osmolarity, and near UV exposure, while the type III secretion system encoded by *FliI* plays a role in delivering a variety of effectors directly into the cells of the host (Ghanavati et al., 2018).

The ESKAPE pathogens are known for their characteristics such as increased levels of resistance towards multiple classes of the first-line and last-resort antibiotics (Denissen et al., 2022). These pathogens are included in the global PPL of antibiotic-resistant bacteria because they have a high prevalence of multidrug resistance (Ramsamy et al., 2018). Another objective of this study was to determine the antibiotic susceptibility

profiles of ESKAPE pathogens and also assess the phenotypic similarities through clustering of antibiotic inhibition zone diameter data.

All ESKAPE pathogen isolates were subjected to antibiotic susceptibility testing against the panel of antibiotics which included Ampicillin 10 µg, Chloramphenicol 30 µg, Kanamycin 30µg, Amoxicillin 10 µg, Piperacillin 100 µg, Aztreonam 30 µg, Gentamycin 30 µg, Cefuroxime 30 µg, and Trimethoprim sulfamethoxazole 23,75 µg. 100% Of *S. aureus* isolates were resistant to multiple antibiotics, including ampicillin, piperacillin, amoxicillin and aztreonam, whereas 60% were resistant to chloramphenicol. All *S. aureus* isolates were susceptible to gentamicin while the previous report indicated that 27,3% were resistant to gentamicin whereas 60% and 50.9% were resistant to chloramphenicol and ampicillin respectively (Singh, 2018).

Some 94% Of *K. pneumoniae* isolates were resistant to amoxicillin, cefuroxime, aztreonam antibiotics whereas 88% were resistant to ampicillin and Trimethoprim sulfamethoxazole and 75% were resistant to Piperacillin. A study performed previously indicate that 97,5%, 18% and 10,3% of the isolates were resistant to ampicillin, gentamicin and chloramphenicol respectively, which is contrary to the present study, where none of the *K. pneumoniae* isolates was resistant to gentamicin and chloramphenicol.

In the present study, 78% and 28% of *P. aeruginosa* isolates were resistant to ampicillin and chloramphenicol respectively, whereas none of the isolates was resistant to gentamicin. This is in contrast with a previous report where 95,8% 18,3% and 80,8% of the isolates were resistant to ampicillin, gentamicin and chloramphenicol respectively (Singh, 2018). About 91%, 3% and 9% of isolates were resistant to ampicillin, gentamicin and chloramphenicol respectively, while another previous report

indicates that 76,2%, 10,6% and 10,9% of the isolates were resistant to ampicillin, gentamicin and chloramphenicol respectively (Singh, 2018).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1. CONCLUSION AND RECOMMENDATIONS

Groundwater samples were collected from different sources to check for the presence of ESKAPE pathogens which are regarded as extremely multi-drug resistant opportunistic pathogens. To the best of our knowledge, this is the first report designed to isolate, characterize and confirm the identities of ESKAPE pathogens from groundwater in the North West Province, South Africa.

Some species of ESKAPE pathogens were isolated with success in this study. Confirmation of the identities of ESKAPE pathogens using molecular techniques such as PCR was done, as well as the assessment of antibiotic-resistant patterns and virulent genes. The isolates were amplified for the four species of the ESKAPE pathogen group, namely: *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterobacter cloacae*, a large proportion of *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterobacter cloacae* isolates exhibited resistance towards the multiple antibiotics tested. The isolates obtained in this study possessed a number of virulence factors such as enterotoxins, adhesin, lipoprotein, elastase, alginate and type III secretion system. The findings of this study indicate contamination. Contaminated water can cause serious health problems in communities because ESKAPE pathogens are known as opportunistic pathogens responsible for multiple infections, especially in immunocompromised individuals. Therefore, we recommend the usage of basic water treatment methods by the communities such as boiling and usage of sodium hypochlorite solutions.

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APPENDIX

APPENDIX A: Materials used during the study

Glutamate Starch Phenol Red (GSP) Agar	g/L
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Sodium L(+) glutamate	10.0
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Starch, soluble	20.0
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Potassium dihydrogen phosphate	2.0
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Magnesium sulfate	0.5
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Phenol red	0.36
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Agar-agar	12.0
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Final pH: 7.2 $\pm$ 0.2 at 25 °C

45 g of these components were suspended in 1 litre and autoclaved at 121 °C for 15 min. After cooling to 45-50 °C, it was mixed well and poured into sterile petri dishes.

Mannitol Salt Agar (MSA)	g/L
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Pancreatic Digest of Casein	5.0 g
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Peptic Digest of Animal Tissue	5.0 g
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Beef Extract	1.0 g
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Sodium Chloride	75.0 g
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D-Mannitol	10.0 g
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Phenol Red	0.025 g
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Agar	15.0 g
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Final pH 7.4 $\pm$ 0.2 at 25°C.

111.025 g of MRS media was suspended in 1000 ml of distilled water. It was boiled in order to dissolve it completely and autoclaved at 121°C for 15-20 minutes. After cooling, it was mixed well and poured into sterile petri dishes.

Bile Esculin Agar	g/L
Peptic digest of animal tissue	5.00
Beef extract	3.00
Esculin	1.00
Bile salts	40.0
Ferric citrate	0.50
Agar	15.00

Final pH (at 25°C): 6.6±0.2

64.5 grams were Suspended in 1000 ml distilled water and boiled to dissolve it completely. It was sterilized by autoclaving at 121°C for 15 minutes. After cooling, it was mixed well and poured into sterile petri dishes.

MacConkey Agar	(g/L)
Peptone	17 g
Polypeptone	3 g
Lactose	10 g
Bile salts	1.5 g
Sodium chloride	5 g
Agar	13.5 g
Neutral red	0.03 g
Crystal violet	0.001 g

Final pH 7.1

50 grams of MacConkey agar powder was suspended in 1 Litre of purified water and mixed thoroughly. It was autoclaved at 121°C for 15 minutes. After cooling to 45-50°C, it was mixed well and poured into sterile Petri dishes and allowed to solidify.

Nutrient Agar	(g/L)
Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0 –

pH 7.4 ± 0.2 at 25°C

28g of nutrient agar powder was suspended in 1L of distilled water. It was mixed and dissolved completely. The medium was sterilized by autoclaving at 121°C for 15 minutes. After cooling, it was mixed well and poured into sterile petri dishes and allowed to solidify.

Nutrient Broth	(g/L)
Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0

pH 7.4 ± 0.2 at 25°C

13g of nutrient broth powder was added in 1L of distilled water and dissolved completely. It was sterilized by autoclaving at 121°C for 15 minutes. After cooling, it was poured into the falcon tubes.µ

Mueller Hinton Agar**g/L**

Peptone 17.5g

Meat extract 2g

Starch 1.5g

Agar 17g

Final pH 7,3 +/- 0,1

CHEMICALS**Buffers (50X TAE)**

Thermo scientific 50 X TAE- Electrophoresis-buffer (40Mm Tris, 20mM Acetic acid and 1Mm EDTA) stock solutions was supplied by Thermo Scientific, Johannesburg, South Africa. 1X TAE buffer was prepared and used for gel electrophoresis.

Ethidium Bromide

A stock solution of 10mg/ml was prepared by dissolving the powder into distilled water and the solution was protected from light by using a masking tape. The solution was stored at room temperature and a final volume of 2 µl was used for staining gel for electrophoresis in order to have clear PCR products.

DNA Ladder

The standard DNA ladders Gene Ruler 100 base pairs was used to determine the relative amplicon sizes after gel electrophoresis. The ladder was obtained from Thermo Scientific, Johannesburg, South Africa.

Oligonucleotide Primers

The primer sets used in this study were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa. A synthesis report was supplied to give a guidance on how to reconstitute a 100 μ M stock solution.

70 % Ethanol

Absolute ethanol (90% v/v) obtained from Merck, Diagnostics, South Africa was used to prepare 70% (v/v) working solution by pouring 700 ml absolute ethanol into 1 litre Duran bottle with 300 ml of distilled water. The solution was kept at room temperature and utilised to disinfect working station

ENZYMES

Master Mix (2X Dream Tag Green)

2X Dream tag master mix (0.4 Mm Datp, 0.4 Mm dGTP and 0.4Mm Dttp, 4 Mm MgCl₂ and loading buffer) was produced by Fermentas, USA and supplied by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside, Pretoria, South Africa. The enzyme was used for PCR amplification of target genes.

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