Molecular surveillance and dissemination of *Klebsiella pneumoniae* on frequently encountered surfaces in South African public hospitals

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A dissertation submitted to the School of Laboratory Medicine and Medical Sciences, College of Health Science, University of KwaZulu-Natal, Westville Campus, for the degree of Master of Medical Science (Medical Microbiology).

This is a dissertation by manuscript with an overall introduction and final summary.

This is to certify that the content of this dissertation is the original research work of Miss Nongcebo Zuzile Zekhethelo Malinga, supervised by:

Supervisor: Signed: - [redacted]------- Name: **Dr L.A. Bester** Date: 10/04/2021----------

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DECLARATION

I, Miss Nongcebo Zuzile Zekhethelo Malinga, declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or other any tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.
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Signed: 

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Any omissions and shortcomings that may be identified in this work remain the sole responsibility of the researcher.

Nongcebo Zuzile Zekhethelo Malinga

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LIST OF ABBREVIATIONS AND ACRONYMS

AAC	Aminoglycoside *N*-acetyltransferase
ANT	Aminoglycoside *O*-nucleotidyltransferase
APH	Aminoglycoside *O*-phosphotransferase
API	Analytical profile index
ATCC	American Type Culture Collection
BP	Blood pressure
CAT	Chloramphenicol acetyltransferase
CHC	Community healthcare centre
CLSI	Clinical Laboratory Standards Institute
CPS	Capsular polysaccharide synthesis
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
ERIC	Enterobacterial repetitive intergenic consensus
ESBL	Extended-spectrum β-lactamase
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species
GFP	Green fluorescent protein
HAI	Hospital-acquired infection
HGT	Horizontal gene transfer
ICU	Intensive care unit
IMP	Imipenemase
IPC	Infection prevention control
IVF	*In-vitro* fertilisation
KPC	*K. pneumoniae* carbapenemase
LGT	Lateral genetic transfer
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time of flight
MDR	Multidrug-resistant
ME-AFLP	Multienzyme amplified length polymorphism
MGEs	Mobile genetic elements

MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
NDM-1	New Delhi metallo-β-lactamases-1
NICU	Neonatal intensive care unit
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PHC	Primary healthcare
PMQR	Plasmid-mediated quinolone resistance
QRDR	Quinolone resistance determining region
REP	Repetitive extragenic palindromic
rRNA	Ribosomal ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
SPSS	Statistical package for social sciences
ST	Sequence type
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TLR4	Toll-like receptor 4
UPGMA	Unweighted pair group method with arithmetic averages
USA	United States of America
UTIs	Urinary tract infections
VIM	Verona-integron metallo-β-lactamase
VRE	Vancomycin-resistant *Enterococcus faecium*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WGS	Whole-genome sequencing
WHO	World Health Organization

ABSTRACT

Hospital equipment and surfaces can harbour *Klebsiella pneumoniae*. In the absence of effective cleaning, anyone who encounters these surfaces can unknowingly spread this opportunistic pathogen throughout the hospital. This study aimed to investigate the prevalence of *K. pneumoniae* on inanimate surfaces and evaluate the genetic diversity, antibiotic resistance and virulence profile of the recovered isolates. Overall, 777 swab samples were collected from four different South African public hospitals classified as central (A), tertiary (B), regional (C) and district (D). These samples were taken from 11 predetermined surfaces present in three different wards: the intensive care unit (ICU), paediatric and general. *K. pneumoniae* was identified using polymerase chain reaction (PCR) followed by antibiotic susceptibility testing using disk diffusion. Extended-spectrum β-lactamases (ESBL) producers were characterised using the combination disc method. Six resistance and three virulence genes were screened using PCR. The genetic diversity of the isolates was examined using enterobacterial repetitive intergenic consensus (ERIC)-PCR. Collectively, 75 (10%) *K. pneumoniae* isolates were recovered from the collected samples. The isolates recovered were equally abundant in tertiary hospital B and district hospital D. The recovery of *K. pneumoniae* was highest in the paediatric ward. Six sites harboured *K. pneumoniae* wherein the occupied beds were the most heavily contaminated. Thirty (40%) isolates were identified as ESBL producers and detected in high quantities in tertiary hospital B and the ICU. The ESBLs were mostly classified as multidrug-resistant (MDR), displaying higher resistance levels to the antibiotics screened than non-ESBLs. Majority of the ESBLs harboured the $bla_{\text{CTX-M}}$ group one resistance gene, which was significantly ($p<0.05$) associated with the aminoglycoside [*aac*(3')-*II and aac*(6')-*Ib*] and fluoroquinolone genes (*qnrB*) screened. The prevalence of virulence genes was high, *mrkD* (95%), *wabG* (93%) and *entB* (92%). ERIC-PCR demonstrated that clonally related isolates were recovered from different sites within the same hospital suggesting bacterial transmission. This study demonstrated that *K. pneumoniae* could contaminate diverse surfaces, and the persistence allowed for dissemination within the public hospital environment. The study's findings highlighted the importance of regularly monitoring hospital surfaces and emphasised on the need to strengthen current infection prevention and control (IPC) measures in hospitals to reduce the spread of bacteria.

CHAPTER ONE:

INTRODUCTION AND LITERATURE REVIEW

1.1 Background information

The terms 'hospital-acquired infections' (HAIs) and 'nosocomial infections' can be used interchangeably to describe infections that arise in patients 48 hours or more after being admitted into a healthcare facility or within 30 days after being discharged (Revelas, 2012). These infections are non-existent when a patient is first admitted. Thus, they result from exposure to infectious agents within the healthcare facility (Ali *et al*., 2018; Revelas, 2012). The leading infectious agents are bacteria; however, viruses and fungal parasites can also be a likely cause (Khan *et al*., 2017). Six bacterial species commonly cause HAIs. These six species are collectively known as the ESKAPE pathogens, and it includes *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species (Santajit & Indrawattana, 2016).

Antibiotics are essential in treating and preventing infections. However, a rise in antibiotic-resistant bacteria has been observed due to selective pressures from the misuse and overuse of antibiotics (Zhen *et al*., 2019). ESKAPE pathogens, in particular, are notoriously known for 'escaping' the action of antibiotics, thus posing as a risk (Zhen *et al*., 2019). This increased resistance has reduced the number of effective antibiotics; hence the World Health Organization (WHO) has declared that specific pathogens require new antibiotics. Based on importance, these pathogens are grouped into three categories: critical, high and medium priority (Mulani *et al*., 2019). Gram-negative bacteria such as extended-spectrum β-lactamase (ESBL) resistant *K. pneumoniae* and *Enterobacter* species along with carbapenem-resistant *K. pneumoniae*, *Enterobacter* species, *A. baumannii* and *P. aeruginosa*, are listed under critical (Mulani *et al*., 2019). Whereas, Gram-positive bacteria, namely methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecium* (VRE) and *S. aureus* (VRSA), are classified as high priority (Mulani *et al*., 2019). This suggests that the four Gram-negative ESKAPE pathogens are a greater concern since some bacterial strains are resistant to all or nearly all antibiotics (Wyres & Holt, 2018). Nonetheless, the detrimental effects associated with HAIs include a prolonged hospital stay, higher healthcare costs and an increase in morbidity and mortality (Ali *et al*., 2018; Loftus *et al*., 2019).

Bacteria can be transmitted to patients in various ways. Firstly, through the hands of healthcare workers who may have encountered an infected patient during daily care. These contaminated hands can contact another patient, thus facilitating bacterial transmission (Facciolà *et al*., 2019). Secondly, a patient can shed microorganisms onto surrounding surfaces where they can persist. When healthcare workers touch these surfaces, their hands become contaminated with bacteria which may spread to other patients (Facciolà *et al*., 2019; Suleyman *et al*., 2018). Lastly, direct transmission of bacteria can occur when a patient encounters a contaminated surface (Suleyman *et al*., 2018).

Maryam *et al*. (2014) detected *Klebsiella* on *in-vitro* fertilisation (IVF) stands and stethoscopes in a Nigerian teaching hospital. Likewise, Ayatollahi *et al*. (2017) reported the recovery of *Klebsiella* from 17 different medical and non-medical surfaces in Iran. Examples of contaminated surfaces included laryngoscopes, ventilators, telephones, handsets, surgical instruments, patients' beds and oxygen masks. Given this information, it is essential to regularly monitor hospital surfaces to detect pathogens (Galvin *et al*., 2012). Such data can help recognise which surfaces are easily contaminated with bacteria, thus assisting with infection prevention and control (IPC) measures in place (Otter *et al*., 2011).

1.2 Taxonomy and classification of *Klebsiella*

The *Klebsiella* genus was named in 1885 as a tribute to the late Edwin Klebs, who was a German microbiologist (Martínez *et al*., 2004). *Klebsiella* is classified under the *Enterobacteriaceae* family that consists of other Gram-negative bacteria such as *Escherichia coli*, *Yersinia*, *Shigella* and *Salmonella* species (Barrios-Camacho *et al*., 2019; Martin & Bachman, 2018). *Klebsiella* species have been detected in various niches such as water, soil, plants, animals and humans (Merla *et al*., 2019).

K. pneumoniae is the dominant species in this genus; thus it is the most common cause of infections in humans (Garza-Ramos *et al*., 2018; Merla *et al*., 2019). Within this species, several different phylogroups exist. Each phylogroup consists of distinct species that are closely related (Barrios-Camacho *et al*., 2019; Garza-Ramos *et al*., 2018; Passet & Brisse, 2018). The first phylogroup (KpI) is the most common and designated as *K. pneumoniae*, which has three subspecies, namely *K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *rhinoscleromatis* and *K. pneumoniae* subsp. *ozaenae* (Brisse *et al*., 2014; Garza-Ramos *et al*., 2018; Martin & Bachman, 2018). The two latter subspecies are not frequently detected since

they cause distinct diseases in humans, namely rhinoscleroma and ozaena, respectively (Fevre *et al*., 2011). The rarely isolated second phylogroup (KpII) is *Klebsiella quasipneumoniae*. This species has two subspecies known as *K. quasipneumoniae* subsp. *quasipneumoniae* (KpII-A) and *K. quasipneumoniae* subsp. *similipneumoniae* (KpII-B) (Garza-Ramos *et al*., 2018; Martin & Bachman, 2018). The third phylogroup (KpIII) is *Klebsiella variicola* (Garza-Ramos *et al*., 2018). KpII and KpIII are also responsible for causing infections in humans. However, the latter phylogroup has also been detected in various environmental sources (Holt *et al*., 2015; Martin & Bachman, 2018).

Klebsiella oxytoca is the second most frequently encountered species within this genus that causes human infections (Merla *et al*., 2019). This species also consists of several closely related but distinct species that form different phylogroups. *K. oxytoca*, *Klebsiella michiganensis* and *Klebseilla grimontii* belong to phylogroup two (KoII), one (KoI) and six (KoVI), respectively (Merla *et al*., 2019). Based on *rpoB* gene sequences, other species such as *Klebsiella planticola*, *Klebsiella ornithinolytica* and *Klebsiella terrigena*, have been reclassified and now belong to the genus *Raoultella* (Martínez *et al*., 2004).

1.3 Genome composition of *Klebsiella*

The *K. pneumoniae* genome consists of approximately 5000-6000 genes. Around 2000 of those genes form part of the core genome; thus, they are usually conserved and present in more than 95% of isolates (Martin & Bachman, 2018). The core genome encodes genes that contribute to the survival of *K. pneumoniae* in different niches. The remaining genes (approximately 3500) are a part of the accessory genome that varies between isolates (Wyres & Holt, 2018). Genes from the accessory genome can be chromosomally encoded or plasmid-borne. Besides their role in specific processes, these genes can also encode different virulence factors and antibiotic-resistant mechanisms (Martin & Bachman, 2018).

1.4 Characteristics associated with *Klebsiella*

Species under the genus *Klebsiella* are Gram-negative, non-motile, non-spore-forming and medium-size (0.4-0.6 x 2-3 μm) rod-shaped bacteria (Aher *et al*., 2012; Barrios-Camacho *et al*., 2019). These microorganisms are facultative anaerobes that are lactose fermenting, catalase-positive and oxidase-negative (Aher *et al*., 2012; Barrios-Camacho *et al*., 2019; Martin & Bachman, 2018). *Klebsiella* species do not have distinctive growth requirements. Thus, they

can grow on ordinary laboratory solid media where they form circular, convex, smooth, glistening and mucoid colonies (Barrios-Camacho *et al*., 2019; Martin & Bachman, 2018; Ristuccia & Cunha, 1984). The optimal growth conditions are 35 °C to 37 °C and a pH of 7.2 (Ristuccia & Cunha, 1984).

1.5 The different phenotypic and genotypic methods used to identify *Klebsiella*

Conventional phenotypic methods commonly used to identify bacteria include observing specific growth patterns on laboratory media, performing Gram staining tests and taking note of specific biochemical characteristics (Van Veen *et al*., 2010). These phenotypic methods are labour intensive, time-consuming and utilise excessive laboratory material (Franco-Duarte *et al*., 2019; Van Veen *et al*., 2010). Furthermore, the outcomes obtained from phenotypic tools may be difficult to interpret or inconclusive (Van Veen *et al*., 2010). This is because phenotypic and biochemical characteristics are unstable and very similar amongst certain species within the *Klebsiella* genus (Barrios-Camacho *et al*., 2019; Fonseca *et al*., 2017).

Genotypic methods such as polymerase chain reaction (PCR), real-time (RT)-PCR and whole-genome sequencing (WGS) have been used to identify bacteria to address some of the shortcomings associated with phenotypic methods (Franco-Duarte *et al*., 2019; Váradi *et al*., 2017). Genotypic methods are sensitive, discriminatory and the results can be generated quickly. Furthermore, genotypic tools can identify un-culturable bacteria, thus identifying more diverse microbial species (Franco-Duarte *et al*., 2019).

1.5.1 The cultivation of *Klebsiella* using laboratory media

MacConkey agar provides ideal conditions for the growth of *Klebsiella*, where it forms pink mucoid bacterial colonies (Aher *et al*., 2012). Lactose is one of the critical components of this agar. Therefore, bacterial species fermenting lactose (e.g. *E. coli*, *Enterobacter* species and *Klebsiella* species) will display pink colonies. Whereas species not fermenting lactose (e.g. *Pseudomonas*, *Salmonella* and *Shigella* species) will appear as colourless colonies (Elazhary *et al*., 1973; Humphries & Linscott, 2015). MacConkey agar also contains crystal violet and bile salt. These two components impede the growth of most Gram-positive bacteria (Bonnet *et al*., 2019).

MacConkey-Inositol-Carbenicillin agar is also an alternative growth media used to detect *Klebsiella* (Ohtomo & Saito, 2003). This agar differs from MacConkey agar in that the lactose is substituted with inositol, and the media is supplemented with carbenicillin (Gao *et al*., 2010). Most *Klebsiella* strains are highly resistant to carbenicillin and can ferment inositol, resulting in red-pink colonies (Bagley & Seidler, 1978; Gao *et al*., 2010).

1.5.2 Analytical profile index

The analytical profile index (API) 20 E system is also used to identify species belonging to the *Enterobacteriaceae* family. This identification method involves a plastic strip with capsules containing 20 different dehydrated biochemical substrates that are inoculated with a bacterial suspension (Maina *et al*., 2014; O'Hara, 2005). After an 18 to 24 hour incubation, outcomes are analysed, and a seven-digit number is generated to allow species identification using a database (O'Hara, 2005; Washington, 1976).

1.5.3 Mass spectrometry

Klebsiella species can be identified using matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry (Barrios-Camacho *et al*., 2019). For each microorganism, this method generates a unique mass spectral fingerprint that will be compared to a known mass spectral fingerprint stored on the database (Croxatto *et al*., 2012; Singhal *et al*., 2015). Although this technique is simple, fast and cost-effective, misidentifications have been reported (Barrios-Camacho *et al*., 2019). Using WGS, Long *et al*. (2017) discovered that isolates previously described as *K. pneumoniae* using MALDI-TOF, were in fact, *K. variicola* and *K. quasipneumoniae* (Long *et al*., 2017). The misidentification probably occurred because the database may lack well-characterised data for all the distinct species in the *K. pneumoniae* phylogroup (Barrios-Camacho *et al*., 2019; Rodrigues *et al*., 2018).

1.5.4 Polymerase chain reaction-based methods

PCR was developed in the 1980s and has been used to identify bacteria (Franco-Duarte *et al*., 2019). *K. oxytoca* and *K. pneumoniae* have been identified with PCR targeting the *pehX* and *khe* gene, respectively. These genes encode for polygalacturonase and haemolysin, respectively (Kovtunovych *et al*., 2003; Yin-Ching *et al*., 2002).

K. pneumoniae, K. quasipneumoniae and *K. variicola* have the *bla*$_{SHV}$, *bla*$_{OKP}$ and *bla*$_{LEN}$ genes encoded on their chromosomes, respectively (Fonseca *et al*., 2017). These three β-lactamase chromosomal genes have resulted in the development of PCR primers that could be utilised for identification purposes. These primers target the β-lactamase genes along with the flanking chromosomal encoded *deoR* gene. The latter gene's inclusion avoids detecting any plasmid-encoded β-lactamase genes (Fonseca *et al*., 2017).

1.5.5 Sequencing-based methods

The direct sequencing of specific deoxyribonucleic (DNA) markers is one of the most reliable methods to identify microorganisms (Rodrigues *et al*., 2018). The most popular marker used is the 16S ribosomal ribonucleic acid (rRNA) gene; however, other markers such as the *rpoB* gene can also be used (Franco-Duarte *et al*., 2019). The resulting sequence generated is compared to other reference sequences present in private or publicly available databases. This gives rise to a similarity percentage which allows the isolate in question to be identified to species or genus level (Petti, 2007).

Alternatively, WGS can also identify bacteria and serve as a molecular typing method (Kwong *et al*., 2015; Váradi *et al*., 2017). This high-resolution method reveals detailed genetic information concerning the virulence and antibiotic resistance determinants linked with microorganisms of interest (Aliyu, 2014; Kwong *et al*., 2015). Despite these advantages, the limitations affiliated with WGS include high costs and the data generated is difficult to interpret (Aliyu, 2014; Kwong *et al*., 2015).

1.6 Infections and carriage of *Klebsiella*

Bacteria colonise a variety of bodily surfaces without causing infection or disease (Dani, 2014). In humans, *K. pneumoniae* commonly colonise the gastrointestinal tract as well as the nasopharynx. It is also a transient member of the skin (Martin & Bachman, 2018). Once this bacterium establishes a route of entry from the colonisation sites into tissues, an infection can occur (Paczosa & Mecsas, 2016). Infections can either be hospital-acquired or community-acquired. HAIs such as urinary tract infections (UTIs), pneumonia and bacteremia occur mostly in immunocompromised individuals. Such medical conditions can typically be caused by classical *K. pneumoniae* strains which may be multidrug-resistant (MDR) (Paczosa & Mecsas, 2016).

Community-acquired infections such as pneumonia, UTIs, pyogenic liver abscess, meningitis and endophthalmitis are caused by classical and hypervirulent strains of *K. pneumoniae* (Navon-Venezia *et al*., 2017; Paczosa & Mecsas, 2016). Hypervirulent strains are concerning since they can elicit infections in immunocompromised individuals and those in good health. Furthermore, infections caused by these strains occur in unusual sites and can spread to multiple other sites (Shon *et al*., 2013). The most frequent sites of infection include the liver, eyes, lungs and central nervous system. However, these strains have also been infrequently linked to infections occurring in other sites such as the spleen, kidney and prostate (Choby *et al.*, 2020). Hypervirulent strains are seldomly resistant to antibiotics; however, resistance has been detected due to the dissemination of mobile genetic elements (MGEs) harbouring antibiotic resistance genes (Lee *et al*., 2017).

1.7 Virulence factors associated with *Klebsiella*

For bacteria to cause an infection or disease, it must have different virulence factors expressed at different stages (Ramachandran, 2014). These virulence factors allow the bacterium to thrive during infection by providing functions such as evasion from the host's immune system and allowing bacteria to travel across barriers, which aids in their replication and dissemination (Ramachandran, 2014). The four virulence factors in *K. pneumoniae* (Martin & Bachman, 2018) are described below.

1.7.1 Capsule

The capsule is an imperative virulence factor in *K. pneumoniae* (Huynh *et al*., 2017). This structure is located outside of the bacterial cell and comprises of polysaccharides containing various sugars (Schembri *et al*., 2005; Wilson *et al*., 2002). The capsule plays an essential role in protecting the bacteria from phagocytosis, opsonisation and antibiotics (Struve *et al*., 2008; Wilson *et al*., 2002).

The capsule is synthesised by the capsular polysaccharide synthesis (*cps*) gene cluster found on the chromosome (Hsu *et al*., 2016). The 5′ end region of the gene cluster has six different genes: *galF*, *cpsACP*, *wzi*, *wza*, *wzb* and *wzc* (Pan *et al*., 2015). *Wzi* is one of the most critical genes as it encodes a protein responsible for facilitating the attachment of the capsule to the outer membrane (Paczosa & Mecsas, 2016). Furthermore, *wzi* is often used for capsular typing to differentiate between the different capsular types. This genotypic method looks at the allelic

differences in the gene making it more specific and more sensitive compared to previously used techniques such as serotyping (Martin & Bachman, 2018; Paczosa & Mecsas, 2016; Pan *et al*., 2015). Currently, 79 different capsular types (K antigens) have been described for *Klebsiella,* and the most common include K1, K2, K5, K20, K54 and K57 (Catalan-Najera *et al*., 2017; Hsu *et al*., 2016). The 3′ end of the *cps* gene cluster consists of two genes, namely *gnd* and *ugd*. The gene cluster's middle region is highly variable, and the genes located within encode proteins involved in polymerisation. An example is *wzy*, a gene also used for capsular typing (Pan *et al*., 2015).

Hypervirulent *K. pneumoniae* strains overproduce polysaccharides resulting in a larger capsule (Lee *et al*., 2017). The enhanced capsule is activated by chromosomal mutations that occur in the *rcsA* and *rcsB* genes that encode a signalling system responsible for regulating the synthesis of the capsule (Arena *et al*., 2017; Paczosa & Mecsas, 2016). Transcriptional regulators initiate the synthesis of the capsule. These regulators are encoded by the *rmpA* and *rmpA2* genes located on chromosomes or plasmids (Arena *et al*., 2017; Paczosa & Mecsas, 2016). The *magA* gene encodes a polymerase enzyme involved in capsule synthesis. However, this gene is only associated with highly pathogenic K1 hypervirulent strains (Catalan-Najera *et al*., 2017).

1.7.2 Pili (Fimbriae)

For bacteria to cause an infection, it must be capable of adhering to the host structures (Struve *et al*., 2008). Adherence is achieved through pili defined as small protein filamentous structures that protrude from the bacteria's surface (Martin & Bachman, 2018). In *K. pneumoniae,* type one and type three pili are the two most common, and the genes that encode them form part of the core genome (Alcántar-Curiel *et al*., 2013; Martin & Bachman, 2018). Pili is essential in biofilm formation on living and non-living surfaces (Paczosa & Mecsas, 2016).

Type one pili are encoded by the *fimABCDEFGHIK* gene cluster (Stahlhut *et al*., 2012). A large proportion of the structure is the major subunit known as FimA, encoded by the *fimA* gene. The tip of the pili is the minor subunit known as FimH, encoded by the *fimH* gene (Alcántar-Curiel *et al*., 2013; Paczosa & Mecsas, 2016). Type one pili are detected in most *K. pneumoniae* isolates and other species in the *Enterobacteriaceae* family (Paczosa & Mecsas, 2016).

Type three pili are encoded by the *mrkABCDF* gene cluster (Ares *et al*., 2016). The *mrkA* gene encodes the major subunit known as mrkA, and it forms the majority of the structure. On the

other hand, the minor subunit known as mrkD is encoded by the *mrkD* gene, which forms the tip of the structure (Alcántar-Curiel *et al*., 2013; Paczosa & Mecsas, 2016). Type three pili are expressed in most *K. pneumoniae* isolates (Paczosa & Mecsas, 2016).

1.7.3 Siderophores

K. pneumoniae needs iron to grow and thrive during an infection (Paczosa & Mecsas, 2016). However, there is often a meagre supply available in the host since it is bound to various other proteins such as haemoglobin and transferrin (Podschun & Ullmann, 1998). *K. pneumoniae* can secrete siderophores to acquire bound and unbound iron present in the host environment (Paczosa & Mecsas, 2016).

There are four different types of siderophores (Martin & Bachman, 2018). Enterobactin is synthesised by the *entABCDEF* gene cluster located on the chromosome. The proteins responsible for transporting this siderophore are encoded by the *fepABCDG* gene cluster (Paczosa & Mecsas, 2016). As the most common siderophore, enterobactin has the highest iron affinity; furthermore, it is present in most classical and hypervirulent strains (Paczosa & Mecsas, 2016). Other siderophores encoded by genes belonging to the accessory genome include salmochelin, yersiniabactin and aerobactin. These siderophores are predominantly found in hypervirulent strains and rarely detected in classical strains (Martin & Bachman, 2018; Paczosa & Mecsas, 2016).

The *iucABCD* gene cluster is responsible for synthesising aerobactin, whereas the associated transport proteins are encoded by the *iutA* gene (Paczosa & Mecsas, 2016). The synthesis of salmochelin is encoded by the *iro* loci (Russo & Marr, 2019). The synthesis of yersiniabactin is encoded by the *irp* genes, and the transport molecules are encoded by the *ybt* and *fyu* genes (Paczosa & Mecsas, 2016). This siderophore cannot acquire iron in the presence of transferrin. Transferrin is a protein concentrated in blood plasma, as such, infections caused solely by yersiniabactin-producing strains will not proliferate to other regions of the body, but they remain in the lung resulting in lung infections (Paczosa & Mecsas, 2016).

1.7.4 Lipopolysaccharide

The lipopolysaccharide is a significant constituent of the outer membrane. This structure is composed of three different elements namely lipid A, core oligosaccharide and an O antigen

(Huynh *et al*., 2017; Steimle *et al*., 2016) coded by the genes in the *lpx*, *waa* and *wb* gene clusters, respectively (Paczosa & Mecsas, 2016). Nine different O antigens are reported in *Klebsiella* species, and the most common are O1, O2 and O3 (Martin & Bachman, 2018). Lipid A is the most toxic and crucial component because the host's innate immune system recognises it through toll-like receptor 4 (TLR4). Once the TLR4 is activated, it causes the release of pro-inflammatory cytokines which induce inflammation (Ramachandran, 2014; Wilson *et al*., 2002). When the host's immune cells are overstimulated, septic shock can occur (Shapira *et al*., 1996).

1.8 Antibiotic resistance

The rise in antibiotic resistant bacteria is attributable to the use and misuse of antibiotics in the medical and agricultural industries (Von Wintersdorff *et al*., 2016). A microbe is considered resistant when it can grow and survive despite being exposed to an antibiotic at a particular concentration that would normally inhibit or kill a microorganism of the same species (Sabtu *et al*., 2015).

Resistance can occur in several ways. Firstly, bacteria can be naturally resistant to some antibiotics. This is known as intrinsic resistance (Blair *et al*., 2015). Secondly, through chromosomal mutatations. Lastly, via the acquisition of resistance genes (Blair *et al*., 2015; Sabtu *et al*., 2015). Resistance genes are present on different MGEs that can be shared between related and unrelated species. This genetic sharing occurs through horizontal gene transfer (HGT) or lateral genetic transfer (LGT) defined as the movement of DNA from one cell to another (Lerminiaux & Cameron, 2019; Stokes & Gillings, 2011; Sultan *et al*., 2018). This movement occurs via conjugation, transduction or transformation (Von Wintersdorff *et al*., 2016). Once movement occurs, the different MGEs are integrated into the genome via autonomous replication, transposition, site-specific recombination or homologous recombination (Stokes & Gillings, 2011). The different MGEs that exist include plasmids, transposons, insertion sequences, integrons, integrative conjugative elements as well as gene cassettes (Stokes & Gillings, 2011).

1.9 The mechanism of action of the different antibiotics and how resistance is expressed

1.9.1 β-lactams

The β-lactams have been used in clinical settings since the 1940s (Navon-Venezia *et al*., 2017). Currently, this antibiotic class is the most prescribed worldwide due to its effectiveness in treating a variety of infections and being well tolerated by most patients (Bush & Bradford, 2016; Navon-Venezia *et al*., 2017). β-lactam antibiotics are classified into four groups: penicillins (e.g. ampicillin), cephalosporins (e.g. ceftriaxone), carbapenems (e.g. meropenem) and monobactams (e.g. aztreonam) (Bush & Bradford, 2016; Worthington & Melander, 2013). Structurally, all β-lactams contain a β-lactam ring and function by inhibiting bacterial cell wall synthesis by binding to penicillin-binding proteins (PBPs). This prevents these enzymes from executing their role in cross-linking peptidoglycan (a key component of the cell well), leading to cell lysis (Bush & Bradford, 2016; Drawz & Bonomo, 2010; Worthington & Melander, 2013). The expression of β-lactamases is the most imperative resistance mechanism in Gram-negative bacteria to evade the inhibitory action of β-lactam antibiotics (Cantón *et al*., 2012; Drawz & Bonomo, 2010).

β-lactamases can enzymatically hydrolyse the β-lactam ring, thereby inactivating the antibiotic (Worthington & Melander, 2013). Several different enzymes have been detected over the years and grouped according to two various schemes: The Bush-Jacoby-Medeiros functional classification and the Amber molecular classification (Shaikh *et al*., 2015). The former utilises four groups (1,2,3,4) to classify the enzymes based on functional properties. In contrast, the latter utilises four classes (A, B, C, D), basing the classification on the enzyme's protein homology (Drawz & Bonomo, 2010; Shaikh *et al*., 2015). Enzymes that fall under class A, C and D, are regarded as serine β-lactamases. In contrast, enzymes in class B are regarded as metallo-β-lactamases (Shaikh *et al*., 2015). Listed below are the different β-lactamases enzymes detected in *Klebsiella* species.

1.9.1.1 Classical β-lactamases and extended-spectrum β-lactamases

TEM-1, TEM-2 and SHV-1 classical enzymes were the first class A serine β-lactamases detected (Drawz & Bonomo, 2010; Martin & Bachman, 2018; Pitout & Laupland, 2008; Shaikh *et al*., 2015). In *K. pneumoniae*, the latter enzyme is encoded by a gene located in the core genome, and as a result, this species is often intrinsically resistant to ampicillin (Wyres & Holt,

2018). The frequent usage of β-lactams has led to the evolution of mutated β-lactamases, known as ESBLs (Shaikh *et al*., 2015).

ESBL-producing isolates are resistant to third and fourth-generation cephalosporins along with monobactams (Cantón *et al*., 2012). The 1980s and 1990s, the TEM and SHV type ESBLs were very popular, and research suggests that they originated from point mutations within classical enzymes (Cantón *et al*., 2012; Zeynudin *et al*., 2018). Currently, the CTX-M type is the most popular and most extensive group of ESBLs (Cantón *et al*., 2012; Zeynudin *et al*., 2018).

The CTX-M family originated from the chromosome of *Kluyvera* species; however, several studies have identified this family in various other microorganisms, including *Klebsiella* species due to HGT (Shaikh *et al*., 2015; Zeynudin *et al*., 2018). The CTX-M family consists of more than 170 variants which are classified into five major groups (Zeynudin *et al*., 2018). CTX-M-15 is classified under group one and is the most dominant variant in Africa. Furthermore, it has been frequently observed in different environments and hosts (Cantón *et al*., 2012; Zeynudin *et al*., 2018). Overall, these plasmid-encoded ESBLs are worrisome particularly in clinical settings because these strains are often associated with additional antibiotic resistance genes, conferring resistance to other clinically important antibiotic classes (Martin & Bachman, 2018; Rawat & Nair, 2010).

A military hospital in Tunisia reported the recovery of 28 ESBL isolates from different abiotic surfaces (e.g. beds, treatment tables, sinks) and the hands of patients and sanitary staff (Dziri *et al*., 2016). Eleven and two ESBL isolates were identified as *K. pneumoniae* and *K. oxytoca*, respectively. All the *Klebsiella* isolates recovered, harboured the $bla_{\text{CTX-M-15}}$ gene. Other β-lactamase genes detected include $bla_{\text{TEM-1}}$, $bla_{\text{SHV-11}}$, $bla_{\text{SHV-28}}$, $bla_{\text{SHV-1}}$ and $bla_{\text{SHV-12}}$ (Dziri *et al*., 2016).

1.9.1.2 Carbapenemases

Carbapenems were initially used to treat infections caused by ESBL-producing bacteria. However, carbapenem resistance has been detected (Martin & Bachman, 2018; Navon-Venezia *et al*., 2017). Resistance is mainly through the acquisition of plasmid-borne carbapenemases which hydrolyse all β-lactams, including carbapenem (Martin & Bachman, 2018; Navon-Venezia *et al*., 2017). In *Klebsiella*, the most prevalent carbapenemase is the *K. pneumoniae*

carbapenemase (KPC) enzyme classified under class A (Martin & Bachman, 2018; Navon-Venezia *et al*., 2017).

Class B consists of zinc-dependent carbapenemases such as the plasmid-encoded New Delhi metallo-β-lactamases-1 (NDM-1) enzyme (Martin & Bachman, 2018). Other enzymes of class B are Verona-integron metallo-β-lactamases (VIM) and imipenemase (IMP). The genes that encode these two enzymes are located on integrons inserted into the chromosome or carried on plasmids. Lastly, OXA-48 is a plasmid-encoded enzyme belonging to class D (Martin & Bachman, 2018).

Three different hospitals located in Iran recovered 37 *K. pneumoniae* isolates from hospital surfaces and equipment in the intensive care unit (ICU) (Moghadampour *et al*., 2018). Thirty-four isolates exhibited high resistance to carbapenem antibiotics, and the most frequently identified carbapenem resistance genes amongst these isolates was the $bla_{\text{OXA-48}}$ gene followed by bla_{NDM} and then bla_{IMP}. The carbapenem-resistant *K. pneumoniae* isolates also harboured several ESBL encoding genes such as bla_{SHV}, bla_{TEM} and $bla_{\text{CTX-M}}$, in high frequencies (Moghadampour *et al*., 2018). This study highlighted that surfaces in the hospital environment could harbour highly resistant *K. pneumoniae*. Therefore, it is essential to ensure that IPC measures regarding the disinfection of surfaces and equipment are implemented efficiently (Moghadampour *et al*., 2018).

1.9.1.3 AmpC β-lactamases

Class C enzymes are known as AmpC β-lactamases and confer resistance to penicillins, β-lactam/β-lactamases inhibitor combinations as well as first, second and third-generation cephalosporins (Drawz & Bonomo, 2010). The most commonly detected bla_{AmpC} gene families in *K. pneumoniae* are CMY, DHA, FOX and MOX types whereas infrequently detected gene families include ACT, MIR, ACC and LAT (Navon-Venezia *et al*., 2017).

1.9.2 Aminoglycosides

Aminoglycosides have been actively used in clinical settings since the 1940s. This class is highly effective against species belonging to the *Enterobacteriaceae* family such as *K. pneumoniae* (Krause *et al*., 2016). Despite the clinical success, aminoglycosides are associated with adverse effects such as nephrotoxicity and ototoxicity (Ramirez & Tolmasky, 2010).

Aminoglycosides bind to the A-site situated on the 16S rRNA of the 30S ribosome, thereby obstructing protein synthesis (Krause *et al*., 2016). Several resistance mechanisms have been described in bacteria; however, enzymatic inactivation of the antibiotic is the most popular in clinical settings (Ramirez & Tolmasky, 2010).

1.9.2.1 Modification of antibiotic using enzymes

The structure of aminoglycosides consists of several exposed amino and hydroxyl groups, making them prone to modification by enzymes known as aminoglycoside modifying enzymes resulting in high-level resistance (Blair *et al*., 2015). The modification of the antibiotic by these enzymes prevents the antibiotic from effectively binding to the target site, thus rendering the antibiotic ineffective at disrupting protein synthesis (Blair *et al*., 2015; Krause *et al*., 2016). Currently, more than 100 enzymes discovered are classified into three families (Krause *et al*., 2016). Most of the enzymes belong to the aminoglycoside *N*-acetyltransferase (AAC) family, and they acetylate amino groups found at different positions on the aminoglycoside structure (Krause *et al*., 2016). Enzymes capable of phosphorylating hydroxyl groups form part of the aminoglycoside *O*-phosphotransferase (APH) family. Lastly, aminoglycoside *O*-nucleotidyltransferase (ANT) is the smallest group of enzymes, and they adenylate hydroxyl groups (Krause *et al*., 2016).

A study conducted in Egypt detected various genes encoding aminoglycoside modifying enzymes amongst *K. pneumoniae* isolates obtained from clinical samples (El-Badawy *et al*., 2017). The most frequently observed gene was *aac*(6')-*II* followed by *aac*(3')-*II, aph*(3')-*IV* and *ant*(3")-*I.* These genes were mostly noted in isolates showing reduced susceptibility to gentamicin and amikacin (El-Badawy *et al*., 2017).

1.9.2.2 Modification of target sites

Different bacteria can express enzymes known as 16S rRNA methylases (Navon-Venezia *et al*., 2017). Such enzymes methylate different nucleotides in the A site of the 16S rRNA, thus hindering the antibiotic from binding to its target site (Garneau-Tsodikova & Labby, 2016; Krause *et al*., 2016). An example of a plasmid-borne gene encoding this type of enzyme is *armA* (Krause *et al*., 2016; Navon-Venezia *et al*., 2017), observed in a study involving the recovery of *K. pneumoniae* from hospital surfaces in Algeria (Zenati *et al*., 2017).

1.9.3 Quinolones and fluoroquinolones

Quinolones and fluoroquinolones are a group of clinically relevant antibiotic agents that consist of four generations. Ciprofloxacin, a second-generation fluoroquinolone, is one of the most frequently used (Correia *et al*., 2017; Redgrave *et al*., 2014). As a result, it is classified as a crucial antibiotic by the WHO. Overall, fluoroquinolones are used globally due to their potency, oral bioavailability and effectiveness against both Gram-positive and Gram-negative bacteria (Correia *et al*., 2017; Redgrave *et al*., 2014).

DNA supercoiling is an important process carried out by DNA gyrase and topoisomerase IV. Quinolones and fluoroquinolones act by disrupting these two crucial enzymes (Correia *et al*., 2017; Redgrave *et al*., 2014). The subunits that form DNA gyrase are encoded by the *gyrA* and *gyrB* genes. Whereas, topoisomerase IV constitutes of subunits coded by *parC* and *parE* genes (Cattoir & Nordmann, 2009).

1.9.3.1 Mutations in genes that encode the target enzymes

The genes encoding DNA gyrase and topoisomerase IV have a short segment of DNA known as the quinolone resistance determining region (QRDR), where mutations take place (Redgrave *et al*., 2014). These mutations cause amino acid substitutions resulting in structural changes in the target, thus altering the antibiotic's ability to bind to the enzymes. This action results in high-level resistance to fluoroquinolones (Redgrave *et al*., 2014). In *K. pneumoniae,* mutations commonly occur in *gyrA* and *parC* compared to *gyrB* and *parE* (Navon-Venezia *et al*., 2017).

1.9.3.2 Protection proteins

Plasmid-mediated quinolone resistance (PMQR) genes usually result in low-level resistance (Correia *et al*., 2017). An example is the *qnr* genes encoding proteins that protect the target enzymes, thus preventing quinolones from inhibiting (Blair *et al*., 2015; Navon-Venezia *et al*., 2017). *QnrA* and *qnrB* genes were first described in *K. pneumoniae* strains derived from the United States of America (USA) and India, respectively (Jacoby *et al*., 2006; Navon-Venezia *et al*., 2017; Wyres & Holt, 2018; Yanat *et al*., 2017).

1.9.3.3 Enzymatic inactivation

The *aac*(6')-*Ib-cr* gene is another type of PMQR gene observed in *K. pneumoniae*, encoding an enzyme known as AAC(6')-Ib-cr. This variant enzyme differs from the original AAC(6')-Ib enzyme as it has two distinct amino acid substitutions (Correia *et al*., 2017; Machuca *et al*., 2016; Navon-Venezia *et al*., 2017). Unlike the original enzyme that only confers resistance to aminoglycosides, the variant enzyme can confer resistance to both aminoglycosides and two fluoroquinolones: ciprofloxacin and norfloxacin (Machuca *et al*., 2016; Navon-Venezia *et al*., 2017). The structure of these two fluoroquinolones enables the variant enzyme to acetylate the antibiotics resulting in decreased activity (Correia *et al*., 2017; Navon-Venezia *et al*., 2017).

1.9.3.4 Efflux pumps

Another two PMQR genes reported are *qepA* and *oqxAB* encoding efflux pumps that remove quinolones from bacterial cells (El-Badawy *et al*., 2017). The efflux pump encoded by the former gene only reduces susceptibility to certain fluoroquinolones. In comparison, the efflux pump coded by the latter gene confers resistance to quinolones, tetracycline, chloramphenicol and trimethoprim (Correia *et al*., 2017; El-Badawy *et al*., 2017).

In India, a study found that most *K. pneumoniae* isolates retrieved from clinical samples displayed resistance to ciprofloxacin and levofloxacin (Dehnamaki *et al*., 2020). This phenotypic resistance was attributable and significantly ($p<0.05$) associated with the molecular amplification of the *oqxA* and *oqxB* genes. Notably, the *qepA* gene was detected at a lower prevalence amongst isolates and only showed significant ($p<0.05$) association with the levofloxacin resistance phenotype. Overall, the study demonstrated that these efflux pumps could play a role in quinolone resistance in *K. pneumoniae* (Dehnamaki *et al*., 2020).

1.9.4 Tetracycline

Tetracycline is an antibiotic class first introduced in the 1940s. Antibiotics belonging to this class are broad-spectrum and are commonly used therapeutically (Roberts, 2003; Shankar *et al*., 2017). Apart from clinical usage, tetracycline has also been used extensively worldwide in the animal industry as growth promoters as well as for therapeutic and prophylaxis treatment (Kang *et al*., 2018). The extensive usage in both humans and animals is dangerous because it has resulted in widespread tetracycline resistance (Markley & Wencewicz, 2018).

Tetracyclines disrupt protein synthesis by binding to the 16S rRNA of the 30S ribosomal subunit, thus preventing aminoacyl-t-RNA from binding to the A site located on the ribosome (Adesoji *et al*., 2015; Markley & Wencewicz, 2018). Bacteria have developed and acquired resistance mechanisms to tetracycline. Efflux pumps are one of the most commonly detected mechanisms in human pathogens (Markley & Wencewicz, 2018). Overall, most of the *tet* genes are located on plasmids, transposons, conjugative transposons and integrons (Shankar *et al*., 2017).

There are 30 different tetracycline specific efflux pumps (Grossman, 2016)**.** These 46 kDa protein structures are membrane-bound and responsible for removing antibiotics from the cell. As a result, the antibiotic concentration in the bacterium is never high enough to cause detriment (Santajit & Indrawattana, 2016; Shankar *et al*., 2017). The 30 different efflux pumps are categorised into seven different groups. Group one and group two efflux pumps are the most prevalent in clinical settings (Grossman, 2016). Group one contains several pumps commonly detected in Gram-negative bacteria, however, the Tet (A) and Tet (B) pumps encoded by the *tetA* and *tetB* genes respectively, are the most frequently encountered (Grossman, 2016; Shankar *et al*., 2017). These aforementioned genes have been described in *Klebsiella* (Roberts & Schwarz, 2016). Alternatively, group two contains Tet (K) and Tet (L) pumps, commonly found in Gram-positive bacteria (Grossman, 2016; Shankar *et al*., 2017).

1.9.5 Phenicols

Chloramphenicol is used sparingly in developing countries to treat serious infections when antibiotic treatment options are limited. This antibiotic is not used in developed countries due to the adverse effects, such as fatal aplastic anaemia (Čivljak *et al*., 2014). Its action mechanism is based on inhibiting protein synthesis by binding to the peptidyl transferase cavity of the 23S rRNA of the 50S ribosomal subunit (Kapoor *et al*., 2017; Schwarz *et al*., 2004).

1.9.5.1 Enzymes that inactivate antibiotics

The enzyme chloramphenicol acetyltransferase (CAT) is encoded by the *cat* gene. This enzyme can acetylate chloramphenicol, thereby inactivating it and hindering binding to the target site (Huang *et al*., 2017; Yoneyama & Katsumata, 2006). There are two different types of CAT enzymes, namely, CAT-A and CAT-B, encoded by different genes. Generally, the genes *catA1*, *catA2*, *catA3*, *catB2* and *catB3* are commonly detected in species belonging to the

Enterobacteriaceae family and have been noted in *Klebsiella* species (Roberts & Schwarz, 2016; Williams *et al*., 2019).

1.9.5.2 Efflux transporters

Several efflux pumps are effective at exporting chloramphenicol out of the bacterial cell. Efflux pumps encoded by the *cml*A1 and *flo*R genes have been described in *Klebsiella* (Roberts & Schwarz, 2016).

1.9.6 Folate pathway inhibitors

Since 1968, sulfamethoxazole and trimethoprim have been used in combination, to treat UTIs, enteric bacterial infections and respiratory tract infections (Frank *et al*., 2007; Manyahi *et al*., 2017). This antibiotic combination is known as cotrimoxazole, and each antibiotic disrupts a different enzyme involved in distinct steps in the folic acid pathway (Frank *et al*., 2007; Huovinen, 2001). Sulfamethoxazole inhibits dihydropteroate synthase (DHPS) whereas trimethoprim inhibits dihydrofolate reductase (DHFR). Although resistance can happen in various ways, in Gram-negative enteric bacteria, acquisition of drug-resistant enzymes is the most common mechanism (Frank *et al*., 2007; Manyahi *et al*., 2017).

Resistance is gained by acquiring genes that encode enzymes that are naturally resistant to the antibiotics, thus making them ineffective (Frank *et al*., 2007). These modified enzymes (DHFR and DHPS) are encoded by the *dfr* and *sul* genes for trimethoprim and sulfamethoxazole, respectively (Manyahi *et al*., 2017). More than 30 *dfr* genes have been described compared to only three *sul* genes (Shin *et al*., 2015). Amongst species within the *Enterobacteriaceae* family, the *dfr* and *sul1* genes are associated with class 1 integrons that may be present on plasmids, transposons or the chromosome. The *sul2* gene is found on small non-conjugative plasmids or large conjugative plasmids (Manyahi *et al*., 2017). Lastly, the rarely detected *sul3* gene is located on plasmids (Shin *et al*., 2015).

Genes encoding these modified enzymes have been detected in *K. pneumoniae*. For instance, D'Souza *et al*. (2019) conducted a study involving two tertiary hospitals in Pakistan and USA. The sampling of five different surfaces (light switch, nurses call button, sink handles, bed-side rail and dispenser) in the ICU led to the recovery of many bacterial species. *K. pneumoniae*

was only present in samples from Pakistan, and these isolates harboured the *sul1, sul2, dfrA27* and *dfrA12* resistance genes (D'Souza *et al*., 2019).

1.10 The different molecular typing methods used for *K. pneumoniae*

Typing methods are employed to differentiate bacterial isolates belonging to the same species (Foxman *et al*., 2005). The application of these tools can help describe the sources and routes of bacterial transmission and identify pathogenic strains or clones. This is essential when investigating outbreaks and performing infectious disease-related surveillance studies (Van Belkum *et al*., 2007). Initially, the differences between isolates were evaluated using phenotypic characteristics based on results obtained from biotyping, serotyping and antibiotic susceptibility testing (Singh *et al*., 2006). However, due to the inability to differentiate between closely related strains, these phenotypic methods have been replaced with genotypic ones that focus on the genetic content of bacteria (Li *et al*., 2009). None of the genotypic methods are designated as universally ideal (Li *et al*., 2009). Instead, their performance is evaluated based on typeability, discriminatory power, reproducibility and convenience (Van Belkum *et al*., 2007).

Typeability refers to the ability to produce results that can be interpreted, whereas discriminatory power is based on the ability to differentiate between genetically unrelated isolates (Foley *et al*., 2009; Van Belkum *et al*., 2007). Reproducibility is the ability to obtain the same result if the experiment is repeated at a different time and place. The convenience criteria is based on several factors such as costs involved, time taken to generate results and resources required. The resource component includes the skills necessary to execute the test and the required reagents and equipment (Foley *et al*., 2009; Van Belkum *et al*., 2007).

1.10.1 Repetitive sequence-based polymerase chain reaction

Throughout the bacterial genome, several dispersed repeated DNA sequences can be used for molecular typing. The different repeat sequences have unique complementary primers to amplify fragments between the repetitive sequences (Li *et al*., 2009). There are three types of repeat sequences: the enterobacterial repetitive intergenic consensus (ERIC) sequence, the repetitive extragenic palindromic (REP) sequence and the BOX sequence. Depending on the target repeat sequence, the procedure is named ERIC-PCR, REP-PCR and BOX-PCR (Foley *et al*., 2009; Li *et al*., 2009). The resulting PCR products are subjected to gel electrophoresis to

separate the fragments according to size. The resulting banding pattern formed is used to differentiate between isolates (Foley *et al*., 2009; Li *et al*., 2009).

There are several advantages associated with the above-mentioned PCR-based typing methods. Firstly, outcomes are generated in a short space of time (Foley *et al*., 2009). Secondly, the technique is able to differentiate between two closely related strains (Hashemi & Baghbani-Arani, 2015). Lastly, the procedure is not labour intensive and the different materials required are of low cost (Li *et al*., 2009). However, the reproducibility is low because the banding patterns formed may vary depending on the protocol, equipment and reagents used (Foley *et al*., 2009; Foxman *et al*., 2005). Nonetheless, PCR-based methods are beneficial because they serve as an excellent feature for research purposes and can be used to support pulsed-field gel electrophoresis (PFGE) in reference centres (Neoh *et al*., 2019).

Otman *et al*. (2002) aimed to investigate the genetic relationship between *Klebsiella* isolates recovered from an outbreak in the neonatal ICU (NICU) ward of a healthcare facility situated in Brazil. Seventeen isolates were collected from May 1998 to March 1999. ERIC-PCR revealed that 14 isolates were 100% similar thus clustered (designated as cluster E1) together on the dendrogram (Otman *et al*., 2002). These identical isolates were derived from patients and one staff member. The remaining three isolates, which clustered together to form cluster E2 were obtained from one unspecified environmental source and patients. All isolates from E2 shared more than 90% similarity with isolates from E1 (Otman *et al*., 2002). This study suggested that clonally related isolates were involved in the outbreak.

1.10.2 Pulsed-field gel electrophoresis

PFGE utilises specific enzymes to cut chromosomal DNA (Singh *et al*., 2006). These enzymes have uncommon recognition sites; thus, the DNA will be cleaved less frequently, resulting in the formation of large DNA fragments (Li *et al*., 2009). The fragments generated range between 20-600 kb. Thus, they cannot be separated using conventional gel electrophoresis which utilises a unidirectional electrical current (Adzitey *et al*., 2013; Singh *et al*., 2006; Van Belkum *et al*., 2007). Instead, the electric field's direction across the gel is changed periodically (Singh *et al*., 2006). The resulting banding pattern is analysed and compared between isolates (Foley *et al*., 2009). Although PFGE has moderate to high reproducibility and discriminatory power, it is also labour intensive as the time taken to generate and analyse the results varies from two to

four days. Furthermore, the costs associated with this technique are high due to the equipment and supplies needed (Foley *et al*., 2009; Foxman *et al*., 2005; Van Belkum *et al*., 2007).

During 2001, a university hospital situated in New York City reported an outbreak of ESBL-producing *K. pneumoniae* affecting infants in the NICU (Gupta *et al*., 2004). Using PFGE, the outbreak strain (clone A) was found to be genetically similar to surveillance cultures obtained from a stethoscope and the hands of two healthcare practitioners working within the affected ward. The affected healthcare workers either had artificial nails or a professional manicure coupled with a long nail length. No positive cultures were obtained after removing artificial nails and reducing the length of the nails (Gupta *et al*., 2004).

1.10.3 Multilocus sequence typing

Bacteria have specific housekeeping genes that can be used in multilocus sequence typing (MLST) (Foley *et al*., 2009). The commonly used genes for *K. pneumoniae* when performing this technique include *rpoB, phoE, gapA, tonB, mdh, infB* and *pgi* (Diancourt *et al*., 2005). MLST uses DNA sequencing to identify any genetic polymorphisms present in the sequence of each gene. Every unique sequence detected in a gene is given a number. Thus, each isolate will have a seven-numbered allelic profile representing the sequence type (ST) (Li *et al*., 2009; Singh *et al*., 2006). When isolates are the same ST or have the same allelic profile, they are regarded as clonally related (Foley *et al*., 2009).

MLST has high reproducibility and repeatability. Furthermore, the discriminatory power ranges from moderate to high, depending on the genes chosen (Foxman *et al*., 2005). However, since multiple genes need to be sequenced, MLST is very time-consuming and expensive (Li *et al*., 2009). The housekeeping genes used in MLST are often highly conserved and do not undergo rapid genetic changes, which is advantageous when conducting phylogenetic studies. However, this may be also problematic as the genetic variation between two closely related strains may not be detected (Foley *et al*., 2009; Li *et al*., 2009).

Between January 2013 and February 2014, 41 carbapenem-resistant *K. pneumoniae* isolates were collected from a Chinese tertiary hospital (Hu *et al*., 2016). The outbreak was linked to an environmental component as clonally related isolates were recovered from 13 ventilators and 25 patients hospitalised in different wards. All these isolates were classified as ST11 using MLST and clustered together (≥80% similarity) on the dendrogram obtained by PFGE (Hu *et al*., 2016). Notably, most isolates obtained from patients in the surgical ICU ward were

identical (100% similarity) to isolates derived from ventilators. The remaining three isolates retrieved from patients belonged to novel ST1844, novel ST1855 and ST1244. The two latter isolates belonging to different STs did not cluster with ST11 isolates; instead, each isolate clustered independently, thus demonstrating genetic unrelatedness (Hu *et al*., 2016).

1.11 *Klebsiella* outbreaks in South Africa

The most recent outbreak took place at Thelle Mogoerane regional hospital situated in Vorsloorus, Gauteng. The outbreak involved antibiotic-resistant *K. pneumoniae* and resulted in six infants' death between July and September 2018. An investigation was initiated, and several IPC measures were implemented to prevent further cross-infection (Petersen, 2018).

In 2012, a tertiary hospital based in Cape Town described an outbreak of $bla_{\text{OXA-181}}$ producing *Klebsiella* in a haematology unit. *K. pneumoniae* was determined as the causative species in seven of the eight positive cases (Jacobson *et al*., 2015). Investigations suggested that inadequate IPC measures could have resulted in the spread of *Klebsiella* amongst affected patients as healthcare workers practised poor hand hygiene. Furthermore, healthcare workers often breached contact precaution protocols involving the usage and appropriate disposal of apron and gloves when entering a patient room (Jacobson *et al*., 2015).

In 2005, Mahatma Gandhi Memorial Hospital situated in Durban, KwaZulu-Natal reported an outbreak of ESBL-producing *K. pneumoniae* in the neonatal ward resulting in the fatality of 22 infants. Investigations linked the source of the outbreak to medication, as opened vamin and glucose preparations were contaminated and used for multiple dosing, while unopened vials of the preparation remained sterile (Moodley *et al*., 2005). The researchers suggested that a contaminated hand touched the rubber stoppers of the vial where the bacterium persisted. Due to multiple usages, an entry passage for the bacterium was established, and the bacteria thrived in the glucose-rich medium (Moodley *et al*., 2005). During the outbreak period, the affected hospital was understaffed and had insufficient sinks and space, which adversely affected the implementation of IPC measures. The banning of multiple dosing and emphasising good hand hygiene helped eradicate the outbreak (Moodley *et al*., 2005).

Coovadia *et al*. (1992) described an outbreak of amikacin and cephalosporin-resistant *K. pneumoniae* during 1989, affecting nine infants (six colonised and three infected) in a nursery of a hospital situated in Durban, South Africa. The outbreak strain was detected in the nose and the hands of a doctor as well as the hands of a nurse and an infected child's mother (Coovadia

et al., 1992). Furthermore, the outbreak strain was also detected from a few environmental sources such as bassinettes, suction apparatus, thermometers, pedal bins, wooden shelves, sinks and a specimen forms box. Reinforcing strict IPC measures helped terminate the outbreak (Coovadia *et al*., 1992).

1.12 The dissemination of bacteria within the hospital environment

1.12.1 The role of healthcare workers

The most significant way bacteria can spread to patients is through the hands of healthcare workers, such as doctors and nurses (Hartmann *et al*., 2004). The hands of healthcare workers are in constant contact with patients; hence they can easily become colonised with bacteria during patient care. Once hand hygiene is ignored, bacteria will survive for a few minutes on the skin, which has undesirable growth conditions for Gram-negative bacteria. *Klebsiella* is considered a transient microbe, thus it can persist on the skin, but it does not multiply (Mathur, 2011; Pittet *et al*., 2006; Podschun & Ullmann, 1998).

A tertiary hospital situated in India investigated the prevalence of bacteria on the hands of 44 doctors working in the medicine and dermatology ward (Paul *et al*., 2011). Upon entry into the wards, no *Klebsiella* was detected although this bacterium was later recovered from the hands of four doctors when they exited the wards (Paul *et al*., 2011). After handwashing with tap water and after sanitisation with alcohol swabs, the contamination was eradicated as no *Klebsiella* was detected on their hands afterwards. This study highlighted the importance of practising good hand hygiene as it may help reduce hand contamination which can contribute to disseminating bacteria (Paul *et al*., 2011).

1.12.2 The role of the hospital environment

The hospital environment can harbour multiple bacterial species with the ability to survive on surfaces for various periods (Doll *et al*., 2018). When a healthcare worker's hands encounter contaminated surfaces, they can transmit bacteria to patients if hand hygiene is disregarded. Patients can also become colonised with bacteria if they encounter contaminated surfaces (Suleyman *et al*., 2018). These abovementioned transmission pathways are illustrated in Figure 1.1.

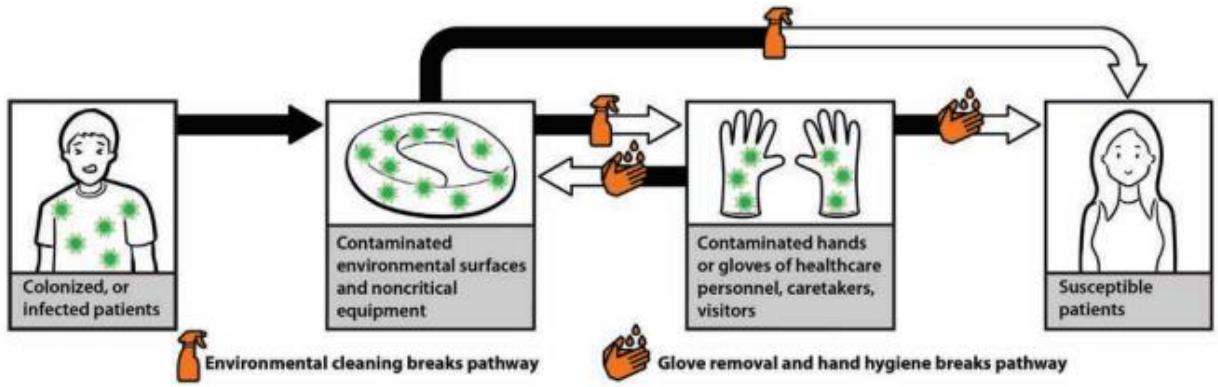

Figure 1.1: The various routes of transmission in the hospital environment (CDC & ICAN, 2019).

Moist and soiled environments such as sinks, toilets and showering facilities, favour the growth and persistence of Gram-negative bacteria (Muzslay *et al*., 2017; Suleyman *et al*., 2018). During a non-outbreak period, a study involving 13 French ICUs was conducted to examine the role of handwashing sinks as a source of contamination (Roux *et al*., 2013). A total of 60 ESBLs were isolated from the sink drains. Twenty-nine and four of these recovered ESBL isolates were identified as *K. pneumoniae* and *K. oxytoca*, respectively (Roux *et al*., 2013). *Klebsiella* can also survive on dry surfaces (Suleyman *et al*., 2018). Worku *et al*. (2018) detected *Klebsiella* isolates on the door handle, thermometer, floor, table-top, window handle, stethoscope and wall of a University hospital in Ethiopia.

Apart from prevalence studies, *Klebsiella* species have been involved in outbreaks linked to an environmental component (wet and dry). During 2000, an outbreak of MDR ESBL-producing *K. pneumoniae* occurred in a Netherlands teaching hospital. Through environmental surveillance, the outbreak strain was recovered from patient transfer roller boards (Van't Veen *et al*., 2005). Using, multienzyme amplified length polymorphism (ME-AFLP), the environmental isolates were considered identical (90-100% similarity) to the clinical isolates recovered from the affected patients. The outbreak was eradicated after rigorous sterilisation and replacement of the roller boards (Van't Veen *et al*., 2005).

A minor outbreak of ESBL-producing *K. pneumoniae* occurred in the neurosurgical ICU ward of a tertiary hospital in Sweden between 2009 to 2010 (Starlander & Melhus, 2012). The patients affected (colonised and infected) seemed to have shared the same room in the ICU but at different times. Environmental sampling of the sink plug hole followed by PFGE analysis showed that the isolates were clonally related to isolates obtained from the affected patients

(Starlander & Melhus, 2012). Further investigations showed that apart from being used to access clean water, the sink was also used for waste disposal. The waste disposal procedure was abolished to terminate the outbreak effectively. Furthermore, the sinks and plumbing system was replaced (Starlander & Melhus, 2012).

1.13 The importance of implementing infection prevention and control measures in the hospital environment

IPC measures are crucial in all healthcare facilities to ensure a safe environment for all individuals within (Arbee *et al*., 2012; Storr *et al*., 2017). However, several factors may negatively influence the implementation of IPC measures. These factors include limited financial resources, overcrowding, insufficient staff, inadequate medical and medicinal resources (Dusé, 2005).

Five crucial elements of IPC are handwashing, isolation, disinfection, sterilisation and surveillance (Arbee *et al*., 2012). Effective hand hygiene is the most critical IPC measure (Loftus *et al*., 2019). Healthcare workers' hands may become contaminated with bacteria due to their exposure to patients during routine care (Allegranzi & Pittet, 2009). Furthermore, since the patients also shed microorganisms onto surrounding surfaces, healthcare workers' hands can also be contaminated upon touching these surfaces (Allegranzi & Pittet, 2009). Contaminated hands can spread bacteria throughout the hospital, and the only way to interrupt this transmission is through practising good hand hygiene (Loftus *et al*., 2019).

The WHO identified five key points (see Figure 1.2) where hand hygiene needs to be practised: prior to patient contact, prior to performing aseptic procedures, after being exposed to bodily fluids, after patient contact and after encountering surfaces that surround the patient (Loftus *et al*., 2019). Generally, hand hygiene adherence is low in both developing and developed countries. The low compliance is due to skin irritation caused by some handwashing agents and lack of time resulting from overcrowding and understaffing. Lastly, the lack of or inconvenient position or location of sinks and handwashing agents may also result in low adherence (Allegranzi *et al*., 2013; Allegranzi & Pittet, 2009).

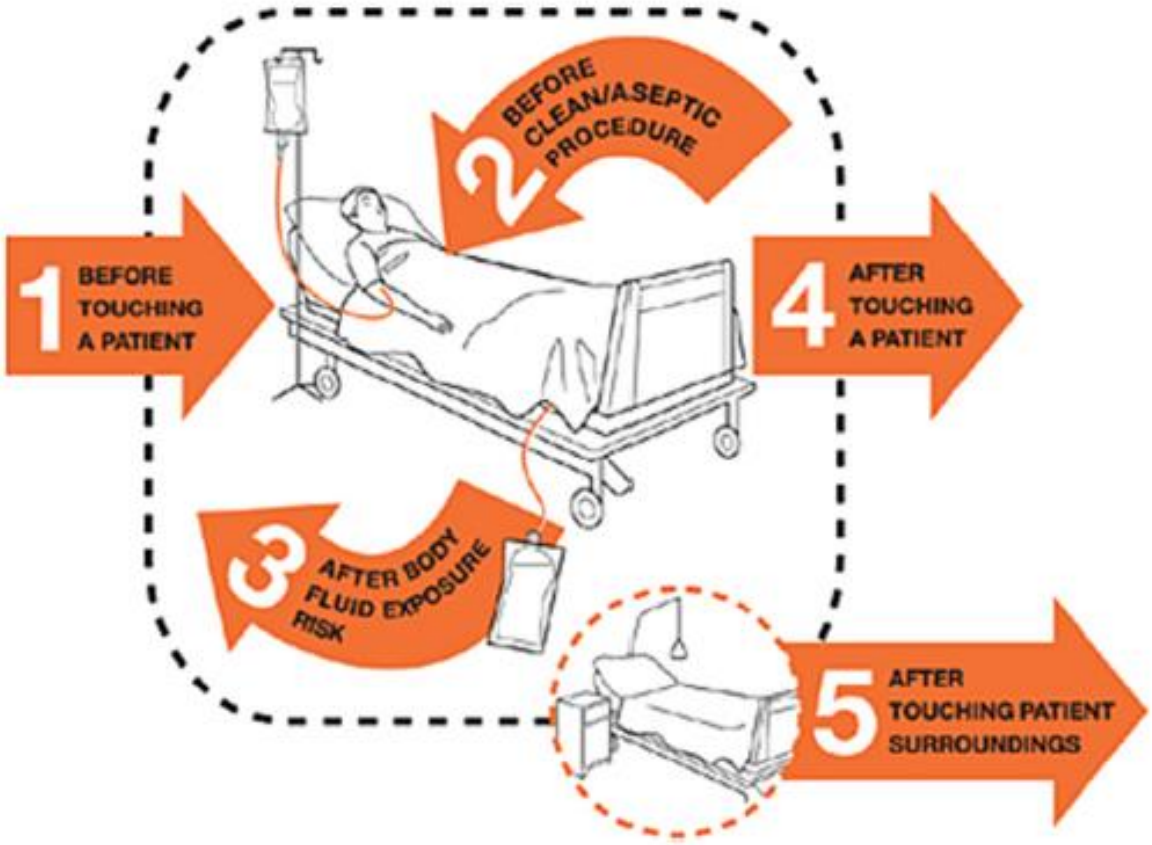

Figure 1.2: The five important points of hand hygiene in a hospital environment (Loftus *et al*., 2019).

There are also specific standard precautions that healthcare workers need to follow, such as the appropriate usage of gloves, gowns, and protective gear (masks, eye and face shields) (Mehta *et al*., 2014). It is essential to wear protective gear and gowns when performing patient activities that may generate splashes from bodily fluids. Gloves are worn when conducting aseptic procedures and when contact needs to be made with mucous membranes and skin that is not intact (Mehta *et al*., 2014). The gloves' surface can still become contaminated during routine care; therefore, it is vital to practice specific measures to prevent cross-transmission. Essential measures include changing gloves when moving from one site to another in the same patient or when moving from one patient to another. Furthermore, it is also essential to safely remove the gloves after usage and practising good hand hygiene after that (Loveday *et al*., 2014; Pittet *et al*., 2006).

To investigate hand hygiene compliance at the five points highlighted by the WHO, a study involving healthcare workers, patients and visitors was conducted in the respiratory medicine

and diabetic care ward of a non-specified teaching hospital (Randle *et al*., 2010). The results showed that amongst healthcare workers, hand hygiene compliance was highest prior to performing sterile procedures (100%), followed by after exposure to bodily fluids (92.5%) and after encountering a patient (80.3%). Lower hand hygiene adherence was observed before patient contact (67.6%) and after touching surfaces surrounding the patient (50.4%) (Randle *et al*., 2010). The latter's low compliance highlights the importance of other IPC measures such as environmental cleaning and the appropriate disinfection and sterilisation of equipment (Allegranzi & Pittet, 2009; Dusé, 2005). These measures can reduce transmission because the cleaning will decrease the bacterial load which will minimise the contamination of healthcare workers' hands (Doll *et al*., 2018).

Surveillance is another important measure that can monitor antibiotic resistance patterns, assess current preventative measures in place and provide information regarding the status (prevalence, cause and type of infection) of HAIs (Ridelberg & Nilsen, 2015; Storr *et al*., 2017). Surveillance studies are neglected and poorly resourced in South Africa; hence the actual prevalence rate of HAIs is unknown. However, it is assumed that the burden of HAIs is more considerable in public healthcare facilities compared to private healthcare facilities (Lowman, 2016).

1.14 Healthcare facilities in South Africa

The healthcare system in South Africa consists of two sectors: private and public (Modisakeng *et al*., 2020). The former sector provides services to less than 20% of the population (Mahomed *et al*., 2017). These healthcare services are funded by medical aids and out-of-pocket payments (Modisakeng *et al*., 2020). On the other hand, more than 80% of the population utilise the government-controlled public sector for healthcare services financed by general tax revenues (Modisakeng *et al*., 2020; Webb *et al*., 2019).

Every healthcare facility within the public sector is required to follow policy guidelines stipulated by the National Department of Health (Gray *et al*., 2016). Every province in South Africa has its own Provincial Department of Health responsible for ensuring that healthcare services are adequately delivered to patients. Each province is further divided into different districts. A total of 52 different health districts exist across the nine provinces in South Africa (Gray *et al*., 2016). The 11 health districts in KwaZulu-Natal include iLembe, eThekwini,

uMgungundlovu, Uthukela, Umkhanyakude, Umzinyathi, Amajuba, Sisonke, Ugu, Uthungulu and Zululand (Department of Health, 2012).

Within the public sector, various healthcare facilities are characterised into four different levels (Pillay & Mahomed, 2019). Level one healthcare facilities include primary healthcare (PHC) clinics, community healthcare (CHC) centres and district hospitals (KwaZulu-Natal Department of Health, 2014). PHC clinics are the first point of entry into the healthcare system. The services provided include immunisation, family planning, prenatal care, treatment and management of communicable diseases (KwaZulu-Natal Department of Health, 2014; Mofolo *et al*., 2019). Suppose the PHC clinic cannot provide the services required by the patient, a referral to a CHC centre will occur due to the additional services they provide such as a 24-hour maternity service, casualty and a short stay ward. If necessary, a patient can subsequently be referred to a district hospital from the CHC centre (KwaZulu-Natal Department of Health, 2014). District hospitals are designed to serve patients within a specific health district, and the services provided are general and include surgery, paediatrics, obstetrics and gynaecology (Department of Health, 2012).

From district hospitals, patients can be referred to regional (level two), tertiary (level three), then central (level four) hospitals, where they can receive specialist and subspecialist services (Pillay & Mahomed, 2019). Regional hospitals receive referral patients from different districts within the same province (Department of Health, 2012). Healthcare services provided include orthopaedics, psychiatry, diagnostic radiology and anaesthesiology (Department of Health, 2017).

Both tertiary and central hospitals can receive referral patients from different provinces (Department of Health, 2012). Tertiary hospitals provide services such as neurology, cardiology and urology. Central hospitals offer services like advanced trauma care and organ transplants (KwaZulu-Natal Department of Health, 2014; Department of Health, 2017). Lastly, specialised hospitals are also classified as level four, providing special services such as rehabilitation, psychiatric and infectious disease (Department of Health, 2012; KwaZulu-Natal Department of Health, 2014).

Overall, healthcare facilities in the public sector are faced with numerous challenges such as long waiting times, suboptimal implementation of IPC measures, shortage of resources, old and poorly maintained infrastructure (Maphumulo & Bhengu, 2019).

1.15 Rationale of the study

Several studies have been conducted on *K. pneumoniae* in hospital settings; however, most reviews have been clinically based. Very few studies have investigated the hospital-environmental component, especially in South Africa where public healthcare facilities are faced with numerous challenges such as the implementation of IPC measures (Maphumulo & Bhengu, 2019). Thus, this study can assist hospital staff as it may reveal which areas are more prone to bacterial contamination, thus aiding in strengthening current IPC interventions. This can potentially reduce bacterial transmission and can impact the occurrence of HAIs which are an economic burden. Furthermore, this study can also examine the burden of antibiotic-resistant *K. pneumoniae* in the hospital environment. This is a crucial aspect because ESBL-producing *K. pneumoniae* is regarded as a critical priority microorganism by the WHO (Mulani *et al*., 2019).

1.16 Aim

- To investigate the prevalence, genetic diversity, antibiotic resistance and virulence profiles of *K. pneumoniae* recovered from frequently touched surfaces in South African public hospitals in a non-outbreak setting.

1.17 Objectives

- To collect environmental samples by swabbing 11 predetermined sites in three different wards of four public hospitals situated in KwaZulu-Natal.
- To use phenotypic and genotypic tools to isolate and identify *K. pneumoniae* from the collected samples.
- To examine the antibiotic susceptibility profiles of the collected *K. pneumoniae* isolates using the Kirby-Bauer disk diffusion method.
- To phenotypically characterise extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* isolates using the combination disk method.
- To screen *K. pneumoniae* isolates for antibiotic resistance and virulence genes using polymerase chain reaction (PCR).
- To assess the clonal relatedness of the collected isolates using enterobacterial repetitive intergenic consensus (ERIC)-PCR.

1.18 Study outline

This thesis is presented according to the guidelines stipulated by the College of Health Science, University of KwaZulu-Natal.

- Chapter 1 provides an in-depth review of the current information available pertaining to this study. It also details the aims and objectives as well as the rationale of the study.
- Chapter 2 presents the findings of this research in manuscript format. This chapter was submitted to Microbial Drug Resistance (MDR-2020-0546) as follows:

  Title: Molecular surveillance and dissemination of *Klebsiella pneumoniae* on frequently encountered surfaces in South African public hospitals

  Authors: Nongcebo Z. Z. Malinga, Christiana O. Shobo, Chantal Molechan, Daniel G. Amoako, Oliver T. Zishiri and Linda A. Bester
- Chapter 3 outlines the main findings, conclusions and implications, limitations and future recommendations associated with the study.

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CHAPTER TWO:

As a requirement by the College of Health Science at the University of KwaZulu-Natal, this dissertation is in manuscript format titled as follows:

Molecular surveillance and dissemination of *Klebsiella pneumoniae* on frequently encountered surfaces in South African public hospitals

Journal submission: Microbial Drug Resistance

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- Miss Nongcebo Z. Z. Malinga, as the investigator, assisted in the conceptualisation of the study, developed the protocols, executed the laboratory work and data analysis, wrote the manuscript.

- Mrs Christiana O. Shobo assisted in the conceptualisation of the study and critically reviewed the manuscript.
- Miss Chantal Molechan assisted with developing the laboratory protocols, helped with data analysis and critically reviewed the manuscript.
- Dr Daniel G. Amoako assisted in the conceptualisation of the study, helped with data analysis and critically reviewed the manuscript.
- Dr Oliver T. Zishiri, as co-supervisor, assisted in the conceptualisation of the study and critically reviewed the manuscript.
- Dr Linda A. Bester, as main supervisor and principal investigator, conceptualised the study, assisted in data analysis and critically reviewed the manuscript.

Running title: *K. pneumoniae* on frequently encountered hospital surfaces.

Abstract

Introduction: Bacteria that cause life-threatening illnesses in humans can also contaminate hospital surfaces, thus posing as a potential source of infection. This study aimed to investigate the prevalence, genetic diversity, virulence and antibiotic resistance profile of *K. pneumoniae* in KwaZulu-Natal, South Africa.

Methods: In a non-outbreak setting involving four public hospitals, 777 samples were collected in three different wards from 11 different sites. Phenotypic and genotypic methods were used for isolation and identification. The Kirby-Bauer disk diffusion method was used to examine antibiotic resistance, followed by the combination disk method to characterise extended-spectrum β-lactamases (ESBLs). Antibiotic resistance and virulence genes were screened using polymerase chain reaction (PCR) and clonality was investigated using enterobacterial repetitive intergenic consensus (ERIC)-PCR.

Results: Seventy-five (10%) *K. pneumoniae* isolates were recovered. These isolates were obtained from all four hospitals and all three wards involved. However, only six frequently touched surfaces were contaminated. These sites included the occupied beds, sinks, mops, unoccupied beds, patient files and blood pressure (bp) monitors. Thirty (40%) isolates were characterised as ESBLs showing high resistance to antibiotics and mostly harbouring the $bla_{\text{CTX-M}}$ group one gene. Virulence genes were highly prevalent amongst all the isolates. ERIC-PCR showed that the isolates recovered from different sites within the same hospital were genetically similar.

Conclusion: The study demonstrated that *K. pneumoniae* can contaminate various surfaces and this persistence allows for the dissemination of bacteria within the public hospital environment when infection prevention and control (IPC) measures are substandard. The information from this study can help hospitals evaluate and improve interventions in place to limit the spread of bacteria.

Keywords: *Klebsiella pneumoniae*; extended-spectrum β-lactamases; virulence; hospital surfaces and equipment; infection prevention and control.

2.1 Introduction

Hospital-acquired infections (HAIs) are infections that occur in a patient while receiving medical care at a healthcare facility. However, HAIs are not initially present when the patient is first admitted; thus, symptoms only appear 48 hours or more after admission or within 30 days of being discharged (Haque *et al*., 2018; Khan *et al*., 2017). HAIs are of global concern; hence, in the United States of America (USA) and Europe, prevalence rates are at 4.5% and 7.1%, respectively (Phu *et al*., 2016). Due to limited resources, the true burden is unestablished in South Africa; however, there are anecdotal reports suggesting that HAIs are more prevalent in public healthcare facilities than private healthcare facilities (Lowman, 2016). Nonetheless, HAIs are associated with increased morbidity and mortality (Ali *et al*., 2018). Furthermore, due to the prolonged hospital stay and the need for additional treatment and diagnostic tests, HAIs increase healthcare costs (Nair *et al*., 2018). A large proportion of HAIs are caused by bacteria (Khan *et al*., 2017). Notably, *Klebsiella pneumoniae* is one of the six bacterial species that commonly cause HAIs (Santajit & Indrawattana, 2016).

Many factors influence a patient's risk of acquiring an infection (Nair *et al*., 2018). Patients who suffer from other underlying conditions such as diabetes and those who have recently undergone medical procedures such as surgery are more vulnerable to infections (Nair *et al*., 2018). Studies have also indicated that a contaminated hospital environment can play a role in transmitting bacteria since medical equipment such as blood pressure cuffs and housekeeping surfaces such as bedrails can harbour bacteria (Suleyman *et al*., 2018). Research has demonstrated that the survival period of *Klebsiella* species on dry surfaces is usually between two hours and more than 30 months. This persistence is influenced by humidity, temperature, inoculum size, surface and strain type (Suleyman *et al*., 2018).

Once the hands of healthcare workers encounter contaminated surfaces, they can transmit bacteria to patients during patient care if hand hygiene protocols are not strictly adhered to. Thus, contaminated hands either from the environment or from direct contact with infected or colonised patients cause 30-40% of the HAIs that occur (Doll *et al*., 2018; Suleyman *et al*., 2018). Infection prevention and control (IPC) measures implemented in healthcare facilities aim to reduce the chances of developing an HAI (Lee *et al*., 2019). Practising good hand hygiene and effectively cleaning surfaces are critical measures that can decrease the bacterial load, thus reducing any chances of bacterial transmission (Doll *et al*., 2018; Loftus *et al*., 2019; Suleyman *et al*., 2018).

The *Klebsiella* genus consists of Gram-negative species that fall under the *Enterobacteriaceae* family (Barrios-Camacho *et al*., 2019). *K. pneumoniae* can cause various infections such as pneumonia, urinary tract infections (UTIs) and bloodstream infections (Martin & Bachman, 2018). These infections are more challenging to treat when antibiotic-resistant bacteria are involved (Paczosa & Mecsas, 2016). *K. pneumoniae* can acquire antibiotic resistance genes encoding extended-spectrum β-lactamases (ESBLs), which confer resistance to important β-lactam antibiotics such as third-generation cephalosporins and monobactams (Paczosa & Mecsas, 2016; Zeynudin *et al*., 2018). The three prominent β-lactamase enzyme families are CTX-M, SHV and TEM, encoded by the *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ genes, respectively (Ojdana *et al*., 2014). *K. pneumoniae* also has four well-established virulence factors. These include the capsule, lipopolysaccharide, siderophores and pili (Martin & Bachman, 2018).

In South Africa, less than 20% of individuals rely on the well-resourced private sector to provide healthcare; thus, a vast majority rely on the under-resourced public sectors (Mahomed *et al*., 2017). The public healthcare system consists of four different levels that cater to various services. District hospitals are level one, and they provide general services to patients. When necessary, a patient can be referred to a regional (level two), tertiary (level three) or central (level four) hospital for specialist and subspecialist services (Pillay & Mahomed, 2019). In South Africa, there is insufficient data investigating the bacterial contamination of surfaces and equipment within the hospital environment. Given this information, the aims of this study were two-fold. Firstly, to examine the prevalence of *K. pneumoniae* on inanimate surfaces in South African public hospitals. Secondly, to determine the genetic diversity, antibiotic resistance and virulence profiles of the collected isolates.

2.2 Materials and methods

2.2.1 Ethical considerations

Ethical clearance to execute this study was granted by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal (Reference number: BE 606/16). The study was registered on the Health Research and Knowledge Management (Reference number: KZ2017RP24 630) database as required by the Department of Health, South Africa. Gatekeepers approval was granted by both district and hospital management.

2.2.2 Sampling sites

Four public hospitals situated in the eThekwini District of KwaZulu-Natal (South Africa) participated in this study. For confidentiality purposes, the different hospitals are denoted as A, B, C and D. Furthermore, the information on each hospital detailed below was obtained from two National Health Act documents which outline the regulation of hospital categories (Department of Health, 2012) and the National Health insurance policy (Department of Health, 2017). Hospital A and hospital B are large, with bed sizes ranging between 800-850 and 900-950, respectively. Hospital A is classified as a central hospital containing highly complex equipment. As a result, it can provide sub-specialist and super-specialist services, such as advanced trauma care and organ transplants. Hospital B is a tertiary hospital offering sophisticated diagnostic and treatment services. Hospital C and hospital D are smaller, with bed sizes ranging between 500-550 and 550-600, respectively. Hospital C is a regional hospital that provides general specialists services in psychiatry, internal medicine, obstetrics and gynaecology, orthopaedic, general surgery, radiology and pathology. Hospital D is a district hospital providing general services such as surgical interventions and emergency services.

2.2.3 Sample collection

In a non-outbreak setting, a total of 777 samples were collected over three months (September - November 2017). Each hospital was sampled before noon once a month on one specific day of the week. Three wards per hospital were selected for sampling, namely the intensive care unit (ICU), general and paediatrics. A total of 11 predetermined frequently touched sampling sites were included in the study namely the patient file, sink, mop, occupied bed (the medical history of the patient occupying the bed was unknown), unoccupied bed (no patient was assigned to that bed before sampling, therefore, it was vacant), blood pressure (BP) monitor, nurse's table, drip stand, telephone, ventilator and the linen room door handle. Amies Agar gel transport swabs (Thermo Fisher Scientific, Waltham, USA) were used to sample ~ 10 cm of the area of interest. The swabs were placed back into the transport media and transported in cooler containers filled with icepacks to the laboratory and processed within four hours after collection. The swabs collected were immersed in 15 ml of tryptic soy broth (Oxoid, Basingstoke, United Kingdom) and incubated. All incubations (Shel lab incubator, Sheldon Manufacturing Inc., Cornelius, USA) described in this study occurred at 35±2 °C for 24 hours.

2.2.4 Isolation and preliminary identification

After incubation, 30 µl of the sample was spread plated onto HiCrome Klebsiella selective agar base supplemented with Klebsiella selective supplement (both from HiMedia Laboratories, Mumbai, India). After incubation, the presumptive purple mucoid colonies formed from each plate were sub-cultured onto MacConkey Agar (Oxoid, Basingstoke, United Kingdom). Following incubation, the pink mucoid lactose fermenting presumptive colonies formed were sub-cultured onto nutrient agar (Oxoid, Basingstoke, United Kingdom) and incubated. The resulting mucoid colonies formed were subjected to several biochemical tests namely catalase (Catalase reagent, Clinical Sciences Diagnostic, Johannesburg, South Africa), oxidase (Oxidase strips, Oxoid, Basingstoke, United Kingdom), motility (Edwards and Ewing Motility Medium, HiMedia Laboratories, Mumbai, India) and triple sugar iron agar (Oxoid, Basingstoke, United Kingdom) test. Further bacterial identification was conducted using analytical profile index (API) 20E (bioMérieux, Marcy-l'Étoile, France) kits. The presumptive *K. pneumoniae* isolates were subsequently stored at -80 °C in tryptic soy broth (Oxoid, Basingstoke, United Kingdom) containing 10% glycerol (VWR International, Radnor, USA). *K. pneumoniae* American Type Culture Collection (ATCC) BAA-1705 was used for quality control purposes.

2.2.5 Deoxyribonucleic acid extraction and molecular confirmation

Stored stock cultures were plated onto nutrient agar (Oxoid, Basingstoke, United Kingdom) and incubated. After incubation, approximately three to five single colonies formed were suspended into 300 µl of 1X Tris-EDTA (TE) buffer (Thermo Fisher Scientific, Waltham, USA). This bacterial suspension was gently vortexed (DLAB MX-S Vortex Mixer, DLAB Scientific, Beijing, China) for 10 seconds and thereafter subjected to deoxyribonucleic acid **(**DNA) extraction using the conventional boiling method following the protocol stipulated by Reddy and Zishiri (2017). The DNA concentration and purity were assessed using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The extracted DNA was considered pure when the 260/280 ratio ranged between 1.8-2.0, subsequently leading to storage at -20 °C for further downstream applications. Using the extracted genomic DNA, the polymerase chain reaction (PCR) for the amplification of the the *khe* gene (encoding haemolysin) was used to detect *K. pneumoniae* (Jian-Li *et al*., 2017; Yin-Ching *et al*., 2002). The PCR was carried out using a T100^{TM} Thermal Cycler (Bio-Rad, Hercules, USA) with the conditions specified in Table 2.1. The total reaction volume was 25

µl, and it consisted of 12.5 µl DreamTaq PCR master mix (2X) (Thermo Fisher Scientific, Waltham, USA), 5.5 µl nuclease-free water (Thermo Fisher Scientific, Waltham, USA), 5 µl template DNA and 1 µl of each primer (working stock concentration of 10 µM). All primers were purchased from Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. The resulting PCR products were loaded into 1.5% (w/v) agarose (SeaKem LE Agarose, Lonza Bioscience, Basel, Switzerland) gel stained with ethidium bromide (Thermo Fisher Scientific, Waltham, USA) and subjected to gel electrophoresis (Bio-Rad, Hercules, USA) at 60 V for 90 minutes using 0.5X Tris-borate-EDTA (TBE) buffer (Thermo Fisher Scientific, Waltham, USA). A 100 bp DNA ladder (New England Biolabs, Ipswich, USA) was used as a molecular weight marker. The results were visualised using the ChemiDoc imaging system (Bio-Rad, Hercules, USA). *K. pneumoniae* ATCC BAA-1705 served as a positive control. All the PCR reactions conducted in this study included a negative control using nuclease-free water (Thermo Fisher Scientific, Waltham, USA) instead of template DNA.

2.2.6 Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed on all the PCR confirmed *K. pneumoniae* isolates using the Kirby-Bauer disk diffusion method on Mueller Hinton Agar (Oxoid, Basingstoke, United Kingdom) as per Clinical Laboratory Standards Institute guidelines (CLSI, 2017). A total of 12 antibiotics belonging to seven different classes were screened. These include antibiotics from the β-lactam class: ampicillin (10 µg), cefazolin (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), meropenem (10 µg) and amoxicillin-clavulanate (20/10 µg); the aminoglycosides class: gentamicin (10 µg); the fluoroquinolone class: ciprofloxacin (5 µg); the tetracycline class: tetracycline (30 µg); the folate pathway inhibitors class: trimethoprim-sulfamethoxazole (1.25/23.75 µg); the phenicols class: chloramphenicol (30 µg); and lastly the nitrofurans class: nitrofurantoin (300 µg) (Oxoid, Basingstoke, United Kingdom). *Escherichia coli* ATCC 25922 was used for quality control purposes. The results were interpreted according to the CLSI guidelines (CLSI, 2017). Isolates were classified multidrug-resistant (MDR) when they showed resistance to at least one antibiotic in three or more different antibiotic classes (Magiorakos *et al*., 2012).

2.2.7 Phenotypic detection of extended-spectrum β-lactamase producers

The isolates resistant to ceftriaxone (CLSI, 2017) were subjected to the ESBL test per the CLSI guidelines (CLSI, 2017). The combination disc method was performed on Mueller-Hinton Agar (Oxoid, Basingstoke, United Kingdom) using discs (D67C MAST Discs, Mast Group, Bootle, United Kingdom) of ceftazidime (30 µg) and cefotaxime (30 µg) alone and in conjunction with clavulanate (10 µg). Results were interpreted according to the CLSI guidelines (CLSI, 2017). *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as a positive and negative control, respectively.

2.2.8 Identification of virulence and antibiotic resistance genes

PCR was used to detect β-lactamase (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ group one), aminoglycoside [*aac*(3')-*II*, *aac*(6')-*Ib*] and fluoroquinolone (*qnrB*) associated resistance genes. Virulence genes encoding type three pili (*mrkD*), siderophore (*entB*) and lipopolysaccharide (*wabG*) were also screened using PCR. All the PCR conditions used are outlined in Table 2.1. Singleplex (*entB*, *bla*$_{TEM}$, *bla*$_{SHV}$) reactions were conducted in the volumes previously described above in section 2.2.5. All the multiplex reactions were made to a total volume of 25 µl comprised of 12.5 µl DreamTaq PCR master mix (2X) (Thermo Fisher Scientific, Waltham, USA) and 5 µl of DNA. The primer (working stock concentration of 10 µM) volumes differed in each reaction and they were as follows respectively: *mrkD* and *wabG*, 0.5 µl and 1 µl; *bla*$_{CTX-M}$ group 1 and *aac*(3')-*II*, 1.5 µl and 1 µl; *qnrB* and *aac*(6')-*Ib*, 1.5 µl and 0.5 µl. Nuclease-free water (Thermo Fisher Scientific, Waltham, USA) was added in different volumes to the different reactions to make up the total volume. For quality control purposes the following positive controls were used: *K. pneumoniae* ATCC BAA-1705 for the virulence genes, *K. pneumoniae* strain 3_S2 (accession number: LJDW00000000) for *qnrB* and *aac*(6')-*Ib* and *K. pneumoniae* strain 15_S8 (accession number: LJEC00000000) was used for all the remaining antibiotic resistance genes. The resulting PCR products were analysed using the conditions specified previously in section 2.2.5.

Table 2.1: The expected amplification size, primer sequences and PCR conditions for the identification, virulence and antibiotic resistance genes utilised in this study.

Target gene	Initial denaturation °C/Time	Denaturation °C/Time	Annealing °C/Time	Extension °C/Time	Number Of cycles	Final Extension °C/Time	Amplification Size (bp)	Primer sequence 5′- 3′	Reference
khe	94 °C/ 4 minutes	94 °C/ 30 seconds	58 °C/ 30 seconds	72 °C/ 1 minute	28	72 °C/ 8 minutes	486	F:TGATTGCATTCGCCACTGG R:GGTCAACCCAACGATCCTG	(Jian-Li *et al.*, 2017)
entB	94 °C/ 5 minutes	94 °C/ 30 seconds	60 °C/ 30 seconds	72 °C/ 1:30 minutes	30	72 °C/ 10 minutes	385	F:CTGCTGGGAAAAGCGATTGTC R: AAGGCGACTCAGGAGTGGCTT	(Wasfi *et al.*, 2016)
mrkD **and** ***wabG***	94 °C/ 5 minutes	94 °C/ 30 seconds	53 °C/ 30 seconds	72 °C/ 1:30 minutes	35	72 °C/ 10 minutes	226 683	F:CCACCAACTATTCCCTCGAA R:ATGGAACCCACATCGACATT F:CGGACTGGCAGATCCATATC R:ACCATCGGCCATTTGATAGA	(Wasfi *et al.*, 2016) (Jian-Li *et al.*, 2017)
***bla*$_{TEM}$**	94 °C/ 10 minutes	94 °C/ 40 seconds	58 °C/ 40 seconds	72 °C/ 1 minute	30	72 °C / 7 minutes	800	F:CATTTCCGTGTCGCCCTTATTC R:CGTTCATCCATAGTTGCCTGAC	(Dallenne *et al.*, 2010)
bla*$_{CTX-M}$ group one and *aac*(3')-*II	94 °C/ 10 minutes	94 °C/ 30 seconds	60 °C/ 30 seconds	72 °C/ 1 minute	30	72 °C/ 7 minutes	688 877	F:TTAGGAARTGTGCCGCTGYA[a] R:CGATATCGTTGGTGGTRCCAT[a] F:ATATCGCGATGCATACGCGG R:GACGGCCTCTAACCGGAAGG	(Dallenne *et al.*, 2010) (El-Badawy *et al.*, 2017)
***bla*$_{SHV}$**	94 °C/ 10 minutes	94 °C/ 40 seconds	58 °C/ 40 seconds	72 °C/ 1 minute	30	72 °C / 7 minutes	713	F:AGCCGCTTGAGCAAATTAAAC R:ATCCCGCAGATAAATCACCAC	(Dallenne *et al.*, 2010)
qnrB **and** ***aac*(6')-*Ib***	94 °C/ 10 minutes	94 °C/ 30 seconds	57 °C/ 30 seconds	72 °C/ 1 minute	30	72 °C / 7 minutes	264 472	F:GGMATHGAAATTCGCCACTG[a] R:TTTGCYGYYCGCCAGTCGAA[a] F:TTGCGATGCTCTATGAGTGGCTA R:CTCGAATGCCTGGCGTGTTT	(Cattoir *et al.*, 2007) (El-Badawy *et al.*, 2017)

[a]Y=T or C; R= A or G; M=A or C; H= A or C or T

2.2.9 Investigating genetic diversity using enterobacterial repetitive intergenic consensus polymerase chain reaction

Representative isolates were subjected to enterobacterial repetitive intergenic consensus polymerase chain reaction **(**ERIC-PCR) to assess clonality. Using pure cultures, 48 isolates were subjected to genomic DNA extraction using the Quick DNA Fungal/Bacterial Miniprep kit (Zymo Research, Irvine, USA) following the manufacturer's instructions. The extracted genomic DNA was quantified and assessed for purity using the conditions previously specified in section 2.2.5. The extracted DNA was utilised to conduct ERIC-PCR using a T100^{TM} Thermal Cycler (Bio-Rad, Hercules, USA). The following PCR conditions were applied: 2 minutes of initial denaturation at 95 °C, 34 cycles of 30 seconds of denaturation at 90 °C, 1 minute of annealing at 52 °C, 8 minutes of extension at 65 °C, and a final elongation of 16 minutes at 65 °C (McIver *et al*., 2020). The total volume was 25 µl, and it consisted of 12.5 µl of DreamTaq PCR master mix (2X) (Thermo Fisher Scientific, Waltham, USA), 7.5 µl of nuclease-free water (Thermo Fisher Scientific, Waltham, USA), 3 µl of genomic DNA and 1 µl of each ERIC 1 and ERIC 2 primer (working stock concentration of 10 µM) (Versalovic *et al*., 1991). Only, 7 µl of the PCR products were loaded into a 1% (w/v) agarose (SeaKem LE Agarose, Lonza Bioscience, Basel, Switzerland) gel and subjected to gel electrophoresis (Bio-Rad, Hercules, USA) at 75 V for 180 minutes using 1X Tris-acetate-EDTA (TAE) buffer (Thermo Fisher Scientific, Waltham, USA). A 1 kb DNA ladder (New England Biolabs, Ipswich, USA) was used as a molecular weight marker. Thereafter the gel was stained with ethidium bromide (Thermo Fisher Scientific, Waltham, USA) for 30 minutes and visualised using the ChemidocTM imaging system (Bio-Rad, Hercules, USA). *K. pneumoniae* ATCC BAA-1705 was used for quality control purposes. The resulting banding patterns were analysed using Bionumerics version 6.6.11 (Applied Maths NV, Sint-Martens-Latem, Belgium). The similarity of the banding patterns was assessed using the Dice coefficient. The dendrogram was generated using the unweighted pair group method with arithmetic averages (UPGMA), using 1% tolerance and 0.5% optimisation. The isolates were grouped into clusters based on a similarity of $\geq$ 60%.

2.2.10 Statistical analysis

The data generated was analysed using the IBM Statistical Package for Social Sciences (SPSS) version 25 (IBM Corporation, New York, USA). A chi-square test of homogeneity was used to investigate whether the prevalence differed (non-ESBL and ESBL) between the different

hospitals and between the various wards. Significant differences were further examined by conducting multiple pairwise comparisons via the implementation of a Post-Hoc analysis using the Z test of two proportions with a Bonferroni correction. Chi-square test for association and Fischer's exact test investigated the association between the different antibiotic resistance genes and virulence genes. All results were considered significant when $p<0.05$.

2.3 Results

2.3.1 Prevalence of *K. pneumoniae*

From the 777 samples collected, a total of 75 (10%) *K. pneumoniae* isolates were obtained over a period of three months. The total number of *K. pneumoniae* isolates recovered, was equally high in tertiary hospital B and district hospital D. The total occurrence of *K. pneumoniae* was lowest in central hospital A (Figure 2.1A). The total sum of *K. pneumoniae* isolates detected, was highest in the paediatric ward (Figure 2.1B). From the 11 sites included in this study, *K. pneumoniae* was only recovered from six sites, and the highest total contamination was observed from the occupied bed followed by the sink, mop, unoccupied bed, patient file and BP monitor (Figure 2.1C). Zero (0%) *K. pneumoniae* isolates were recovered from the nurse's table, door handle, drip stand, telephone and ventilator.

Thirty (40%) of the 75 *K. pneumoniae* were phenotypically identified as ESBL producers. The proportion of ESBLs recovered from tertiary hospital B was significantly ($p<0.05$) higher compared to central hospital A and district hospital D. Although more ESBL isolates were detected in tertiary hospital B compared to regional hospital C, this difference was not significant ($p\geq0.05$) (Figure 2.1A). The number of ESBL isolates obtained from the ICU was significantly ($p<0.05$) higher compared to the general and paediatric ward (Figure 2.1B).

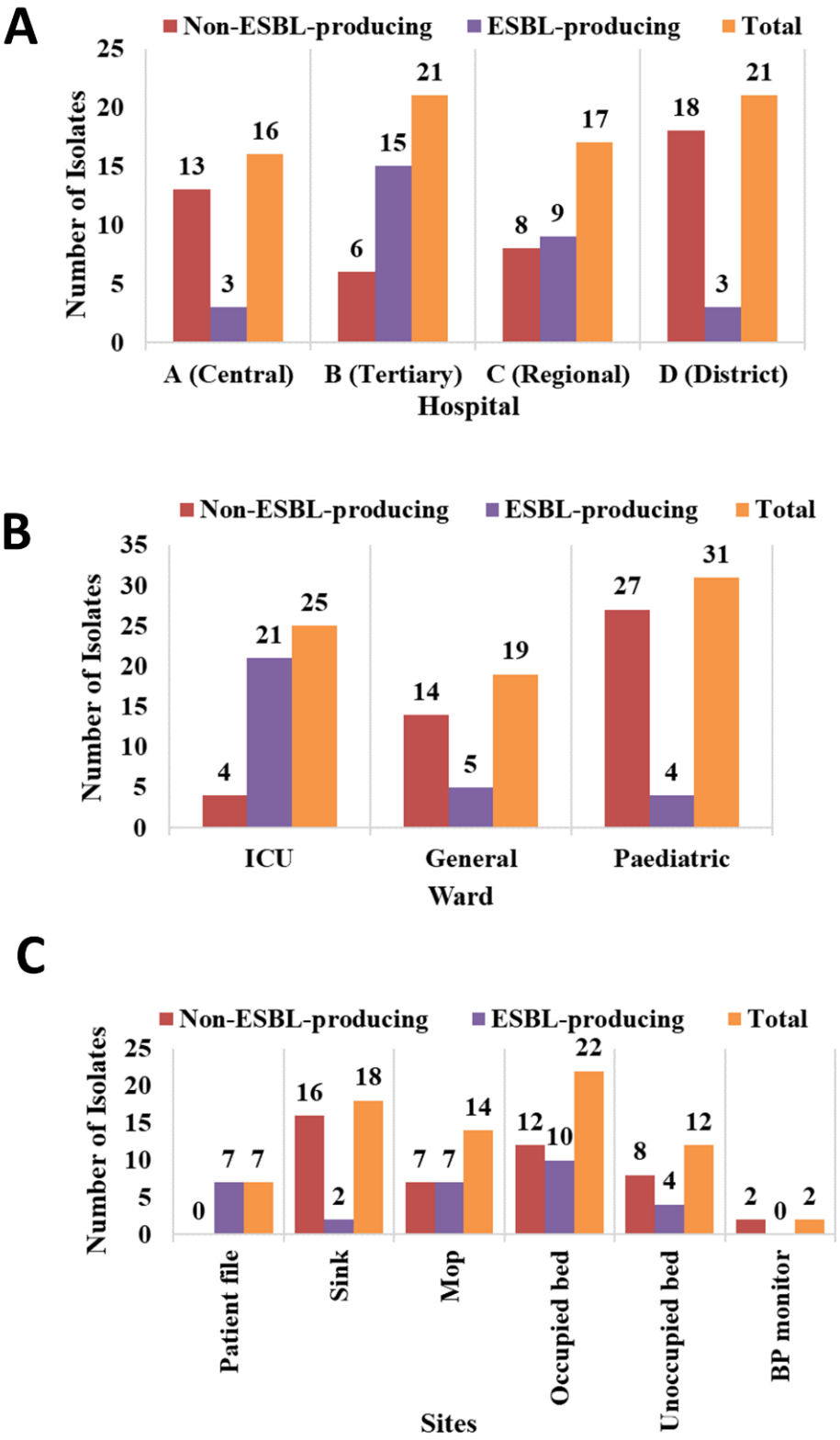


Figure 2.1: The overall proportion of non-ESBL and ESBL-producing *K. pneumoniae* isolates recovered across the different A) Hospitals; B) Wards; C) Sites.

2.3.2 Antibiotic susceptibility testing

The ESBLs displayed higher resistance to the antibiotics screened compared to the non-ESBLs (Table 2.2). A total of 29 ESBL isolates were classified as MDR.

Table 2.2: The antibiotic susceptibility profiles for the non-ESBL and ESBL-producing *K. pneumoniae* isolates collected within the hospital environment.

Antibiotics	Non-ESBL N=45			ESBL N=30			Total N=75		
	R n (%)	I n (%)	S n (%)	R n (%)	I n (%)	S n (%)	R n (%)	I n (%)	S n (%)
Ampicillin	45 (100)	0 (0)	0 (0)	30 (100)	0 (0)	0 (0)	75 (100)	0 (0)	0 (0)
Cefazolin	10 (22)	14 (31)	21 (47)	30 (100)	0 (0)	0 (0)	40 (53)	14 (19)	21 (28)
Cefoxitin	1 (2)	2 (4)	42 (93)	15 (50)	0 (0)	15 (50)	16 (21)	2 (3)	57 (76)
Ceftriaxone	1 (2)	1 (2)	43 (96)	30 (100)	0 (0)	0 (0)	31 (41)	1 (1)	43 (57)
Meropenem	1 (2)	1 (2)	43 (96)	19 (63)	2 (7)	9 (30)	20 (27)	3 (4)	52 (69)
Amoxicillin-clavulanate	2 (4)	6 (13)	37 (82)	26 (87)	3 (10)	1 (3)	28 (37)	9 (12)	38 (51)
Gentamicin	0 (0)	2 (4)	43 (96)	29 (97)	0 (0)	1 (3)	29 (39)	2 (3)	44 (59)
Ciprofloxacin	0 (0)	2 (4)	43 (96)	21 (70)	9 (30)	0 (0)	21 (28)	11 (15)	43 (57)
Tetracycline	0 (0)	0 (0)	45 (100)	10 (33)	0 (0)	20 (67)	10 (13)	0 (0)	65 (87)
Trimethoprim-sulfamethoxazole	0 (0)	0 (0)	45 (100)	29 (97)	0 (0)	1 (3)	29 (39)	0 (0)	46 (61)
Chloramphenicol	0 (0)	0 (0)	45 (100)	11 (37)	0 (0)	19 (63)	11 (15)	0 (0)	64 (85)
Nitrofurantoin	4 (9)	10 (22)	31 (69)	18 (60)	6 (20)	6 (20)	22 (29)	16 (21)	37 (49)

Abbreviations: R: Resistant; I: Intermediate; S: Susceptible

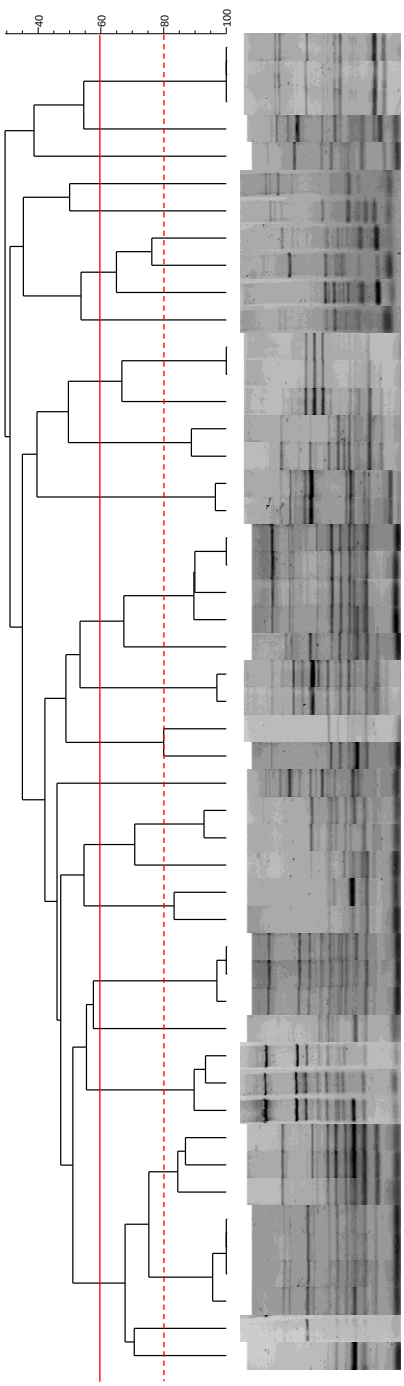
2.3.3 Detection of virulence and antibiotic resistance genes

The *bla*$_{\text{CTX-M}}$ group one and *aac*(3')-*II* resistance genes, were equally abundant; thus, they were detected in 30 (40%) isolates. The prevalence of the other resistance genes was 28 (37%) for *bla*$_{\text{TEM}}$, 17 (23%) for *qnrB* and 14 (19%) for *aac*(6')-*Ib*. A significant ($p<0.05$) association between each of these aforementioned resistance genes, was detected. The *bla*$_{\text{SHV}}$ resistance gene was the most prominent; hence it was observed in 69 (92%) isolates; however, it showed no significant ($p\geq0.05$) association with the other screened resistance genes.

The prevalence of the virulence genes was 71 (95%) for *mrkD*, 70 (93%) for *wabG* and 69 (92%) for *entB*. All the virulence genes screened were significantly ($p<0.05$) associated with one another.

2.3.4 Investigating clonality using enterobacterial repetitive intergenic consensus polymerase chain reaction

The genetic diversity amongst 48 *K. pneumoniae* isolates was investigated using ERIC-PCR. At each hospital, ward and site, these selected isolates had a unique antibiogram and resistome. The isolates revealed 20 different ERIC-types (A to T) based on a similarity of ≥ 60%. A total of 13 major ERIC-types were further defined for isolates displaying similarity of ≥ 80% (Figure 2.2). In contrast to all the other ERIC-types which were hospital-specific, ERIC-type T consisted of isolates from tertiary hospital B and regional hospital C (Figure 2.2). Within the major ERIC-types, none of the ESBL and non-ESBL isolates clustered together. Major ERIC-type K consisted of isolates regarded as 100% identical and were recovered from the sheets of an occupied and an unoccupied bed in the ICU of tertiary hospital B (Figure 2.2). Major ERIC-type M consisted of isolates from the sink and sheet of an occupied bed in the ICU of tertiary hospital B. These isolates shared a similarity of ≥80% (Figure 2.2). Within their respective clusters, isolates from ERIC-type F and ERIC-type H shared a similarity of ≥60%. ERIC-type F consisted of genetically similar isolates obtained from the sink and occupied bed in the general ward of district hospital D. ERIC-type H consisted of genetically similar isolates from the sheet of occupied and unoccupied beds in the general ward of central hospital A (see Figure 2.2).



Type	Key	Hosp.	Ward	Site	ESBL	Antibiogram
A	1MGF1	A	GEN	Mop	+	SXT_KZ_CN_CIP_CRO_AMP_AMC
A	1MGF3	A	GEN	Mop	+	SXT_KZ_CN_CRO_AMP_AMC
A	1MGF4	A	GEN	Mop	+	SXT_KZ_CN_C_FOX_CIP_CRO_MEM_AMP_AMC
B	1CPH2	C	PAED	Sink	-	AMP
C	1UPF3	B	PAED	Mop	-	AMP
D	1SPF2	D	PAED	Mop	-	AMP
E	1SPJ1_1C	D	PAED	Occ. Bed	-	AMP
F	1SGJ1_3C	D	GEN	Occ. Bed	-	AMP
F1	1SPF1	D	PAED	Mop	-	KZ_AMP
F2	1SGH3	D	GEN	Sink	-	AMP
G	1SPH1	D	PAED	Sink	-	AMP
H	1MGJ1_1	A	GEN	Occ. Bed	-	KZ_AMP
H	1MGJ1_2	A	GEN	Occ. Bed	-	AMP_F
H1	1MGJ2_1	A	GEN	Unocc. Bed	-	AMP
I	1MPJ1_1	A	PAED	Occ. Bed	-	KZ_AMP
I	1MPJ1_2	A	PAED	Occ. Bed	-	AMP
J	1MPF1	A	PAED	Mop	-	KZ_AMP
J	1MPF3	A	PAED	Mop	-	KZ_AMP
K	3UIJ1_2	B	ICU	Occ. Bed	+	SXT_KZ_CN_C_FOX_CIP_CRO_MEM_AMP_F_AMC
K	3UIJ2_2	B	ICU	Unocc. Bed	+	SXT_KZ_CN_C_FOX_CIP_CRO_MEM_AMP_AMC
K	3UIJ2_3	B	ICU	Unocc. Bed	+	SXT_KZ_CN_C_FOX_CIP_CRO_MEM_AMP_F_AMC
K	3UIJ2_1	B	ICU	Unocc. Bed	+	SXT_KZ_CN_C_FOX_CIP_CRO_MEM_AMP_F_AMC
K1	3UID1	B	ICU	P. File	+	KZ_FOX_CRO_AMP_F
L	2MPC1	A	PAED	BP monitor	-	AMP
L	2MPC2	A	PAED	BP monitor	-	KZ_MEM_AMP_F_AMC
M	3UIH3	B	ICU	Sink	+	SXT_KZ_CN_C_FOX_CIP_CRO_MEM_AMP_F_AMC
M	3UIJ1_1	B	ICU	Occ. Bed	+	SXT_KZ_CN_C_FOX_CIP_CRO_MEM_AMP_F_AMC
N	2CPH2	C	PAED	Sink	-	AMP
O	1CGJ1_1	C	GEN	Occ. Bed	+	SXT_KZ_CN_CIP_CRO_MEM_AMP
O	1CGJ1_3	C	GEN	Occ. Bed	+	SXT_KZ_CN_CRO_AMP_AMC
O1	1CPJ1_2	C	PAED	Occ. Bed	+	SXT_KZ_CN_CIP_CRO_AMP_F
P	1CPJ1_6	C	PAED	Occ. Bed	+	SXT_KZ_CN_FOX_CRO_AMP_F_AMC
P	1CPJ1_7	C	PAED	Occ. Bed	+	SXT_KZ_CN_FOX_CIP_CRO_MEM_AMP_F_AMC
Q	3UIF3	B	ICU	Mop	+	SXT_KZ_CN_CIP_CRO_AMP_TE_F_AMC
Q	3UIF4	B	ICU	Mop	+	SXT_KZ_CN_CIP_CRO_MEM_AMP_TE_F_AMC
Q	3UIF1	B	ICU	Mop	+	SXT_KZ_CN_CRO_AMP_TE_F_AMC
R	1SPJ2_1D	D	PAED	Unocc. Bed	-	AMP
S	1SID1	D	ICU	P. File	+	SXT_KZ_CN_CIP_CRO_AMP_TE_AMC
S	1SID3	D	ICU	P. File	+	SXT_KZ_CN_CIP_CRO_MEM_AMP_TE_F
S	1SID2	D	ICU	P. File	+	SXT_KZ_CN_CRO_AMP_TE_AMC
T	3CID1	C	ICU	P. File	+	SXT_KZ_CN_CRO_MEM_AMP_TE_AMC
T	3CID2	C	ICU	P. File	+	SXT_KZ_CN_CIP_CRO_MEM_AMP_TE_AMC
T	3CID3	C	ICU	P. File	+	SXT_KZ_CN_CRO_MEM_AMP_TE_AMC
T1	2UIH6	B	ICU	Sink	-	AMP_F
T1	2UIH7	B	ICU	Sink	-	AMP
T1	2UPJ1_1	B	PAED	Occ. Bed	-	AMP_F
T1	2UIH4	B	ICU	Sink	-	KZ_FOX_CRO_AMP_AMC
ATCC 1705						
T2	3CGJ2_1	C	GEN	Unocc. Bed	-	AMP

Figure 2.2: Dendrogram depicting the ERIC-PCR profiles from 48 *K. pneumoniae* isolates. *K. pneumoniae* ATCC BAA 1705 was used as a positive control. The solid red line at 60% specifies the ERIC-type cut off whereas the dashed line at 80% specifies the major ERIC-type cut-off. Abbreviations: Hosp.: Hospital; A: Central Hospital; B: Tertiary Hospital; C: Regional Hospital; D: District Hospital; PAED: Paediatric; ICU: Intensive Care Unit; GEN: General; Occ. Bed: Occupied Bed; Unocc. Bed: Unoccupied Bed; P. File: Patient File; + indicates an ESBL-producing isolate and – indicates a non-ESBL-producing isolate; AMP: Ampicillin; KZ:

Cefazolin; FOX: Cefoxitin; CRO: Ceftriaxone; MEM: Meropenem; CIP: Ciprofloxacin; CN: Gentamicin; AMC: Amoxicillin-clavulanate; F: Nitrofurantoin; SXT: Trimethoprim-sulfamethoxazole; TE: Tetracycline; C: Chloramphenicol.

2.4 Discussion

A study involving public hospitals situated in KwaZulu-Natal, South Africa was conducted to determine the prevalence, clonality, virulence and antibiotic resistance profile of *K. pneumoniae* recovered on frequently encountered surfaces in the hospital environment. The overall prevalence was 10%, and this low prevalence is relatively similar to the 11.5% reported in Uganda (Sserwadda *et al*., 2018) and the 11.6% reported in Ethiopia (Getachew *et al*., 2018), involving the recovery of *Klebsiella* from the hospital environment.

Tertiary hospital B had an overall higher prevalence of *K. pneumoniae* compared to central hospital A (Figure 2.1A). Although both these hospitals can receive referral patients from other provinces, the former hospital contains more beds; thus, it can accommodate more patients. The higher influx of patients may influence environmental contamination leading to a higher prevalence. Similarly, this can possibly explain the higher prevalence of *K. pneumoniae* in district hospital D compared to regional hospital C (Figure 2.1A), as the former hospital contains more beds. In addition, district hospitals support primary healthcare facilities as they provide services to patients referred from community healthcare centres or clinics. Furthermore, they also serve as a gateway for patients to access specialised care (Pillay & Mahomed, 2019). This dual function may also contribute to a higher inflow of patients. The number of people present in the hospital environment is one factor that can influence the quantity and types of microorganisms detected (Suleyman *et al*., 2018).

Healthcare textiles such as bedsheets and staff uniforms may play a role in disseminating bacteria (Fijan & Turk, 2012). In this study, *K. pneumoniae* was recovered from the bedsheets of occupied and unoccupied beds (Figure 2.1C). The high prevalence on occupied beds is expected as bedsheets used by patients can be contaminated with bodily substances (blood, skin, urine and stool) containing a high number of microorganisms (Fijan & Turk, 2012). Carbapenem-resistant *K. pneumoniae* has also been detected in hospital bed linen surrounding the pillow in a study conducted in China (Yan *et al*., 2019). Healthcare workers can touch these surfaces during patient care, and in the absence of hand hygiene, be disseminated throughout the hospital. The identification of *K. pneumoniae* on the linen of unoccupied beds is

problematic because a patient admitted and assigned to that unoccupied bed is at risk of developing an HAI caused by *K. pneumoniae.* The information available regarding the survival of microorganisms on hospital textiles after laundering differs amongst researchers due to the different temperatures and conditions investigated (Fijan & Turk, 2012). Furthermore, even if laundry processes are efficient at removing microorganisms, recontamination can occur if there is negligence at various post-handling processes such as sorting, ironing, folding and packing (Mitchell *et al*., 2015).

Handwashing sinks were the second most abundant site where *K. pneumoniae* was recovered (Figure 2.1C). Moist environments such as sinks favour the survival of Gram-negative bacteria (Muzslay *et al*., 2017). Sinks also accommodate the formation of bacterial biofilms and are an ideal environment where bacteria can exchange resistance genes through horizontal gene transfer (HGT) (Grabowski *et al*., 2018; Kotay *et al*., 2017). It has been suggested that when hand washing occurs in a pathogen contaminated sink or sink drain, the hands of healthcare workers can be colonised with bacteria due to the backsplash. Contaminated hands can contact patients during routine care activities, thus demonstrating a possible route of transmission (Tofteland *et al*., 2013). A *K. pneumoniae* outbreak linked to a contaminated handwashing sink occurred between 2009 and 2010 in Sweden (Starlander & Melhus, 2012). This suggests that the effective and efficient cleaning of sinks in hospitals should be emphasised.

Healthcare workers frequently handle patient files to write daily notes after attending to a patient during routine checks and after performing medical procedures (Panhotra *et al*., 2005). In this study, *K. pneumoniae* was also detected on patient files (Figure 2.1C). Patient files are vulnerable to contamination because they are often placed on multiple surfaces, such as nursing stations and patient beds (Chen *et al*., 2014). Ineffective hand hygiene between handling these contaminated files and touching patients increases the probability of transmission (Panhotra *et al*., 2005). Previous studies conducted in Saudi Arabia (Panhotra *et al*., 2005) and Taiwan (Chen *et al*., 2014), have also reported the recovery of *K. pneumoniae* from patient files.

ESBL production was observed in 30 of the 75 (40%) *K. pneumoniae* isolates recovered, thus correlating with the resistance to ceftriaxone detected (Table 2.2). The ESBL percentage reported in this study is lower than previous studies conducted in Egypt (Afifi, 2013) and Ethiopia (Engda *et al*., 2018), where *K. pneumoniae* accounted for 56.25% and 42.10% of the ESBL isolates recovered from hospital surfaces and equipment, respectively. These differences amongst the various countries may be due to several factors. Firstly, the higher or lower usage

of ESBL selecting β-lactam antibiotics. Secondly, the implementation of IPC measures may vary from optimal to suboptimal. Antibiotic-resistant bacteria emerge due to selection pressure; however, these microorganisms are maintained within the hospital environment due to inadequate IPC measures (Essack, 2006).

Tertiary hospital B is classified as a level three healthcare facility and it had a high quantity of ESBLs (Figure 2.1A). The referral system suggests that medical conditions presented at healthcare facilities become increasingly complex and severe as the hospital classification levels progress, leading to the higher usage of antibiotics, especially broad-spectrum antibiotics (Essack *et al*., 2005). When third-generation cephalosporins are used, the outcome is the emergence of ESBLs (Pereira *et al*., 2004). These results are consistent with a study conducted on clinical isolates obtained from 16 different public hospitals situated in KwaZulu-Natal which found that the number of ESBL-producing bacteria was highest in tertiary hospitals followed by regional then district hospitals (Essack *et al*., 2005). Central hospital A also deals with complex medical cases, but it had fewer ESBLs (Figure 2.1A). Compared to other hospitals included in this study, central hospital A has more financial and medical resources that can be directed at improving IPC measures. Several factors influence the implementation of IPC measures. These factors include limited financial resources, inadequate infrastructure and poorly implemented systems. The latter consists of the unsatisfactory purchase and distribution of supplies such as hand disinfectant (Damani, 2007; Sastry *et al*., 2017).

A significant ($p<0.05$) proportion of the ESBLs recovered were from ICU (Figure 2.1B). This finding is possibly attributable to the high intake of antibiotics in this ward as it is specifically designed for critically ill patients (Mora *et al*., 2016; Muzslay *et al*., 2017). Frequently administered antibiotics are often broad-spectrum and constant exposure can facilitate the emergence of MDR bacteria (Curcio, 2013).

Majority of the ESBLs were MDR, and they exhibited higher levels of resistance compared to non-ESBLs (Table 2.2). A similar finding was also reported in a previous study conducted in Egypt (Afifi, 2013), involving isolates recovered from hospital surfaces. The resistance to gentamicin, ciprofloxacin and all the β-lactams screened in this study (Table 2.2), is of great concern as antibiotics from the aminoglycoside, fluoroquinolone and β-lactam class are often used to treat infections caused by bacteria within *Enterobacteriaceae* family such as *K. pneumoniae* (Teklu *et al*., 2019).

Most isolates showing phenotypic resistance to gentamicin (Table 2.2) harboured the *aac*(3')-*II* gene. This gene was detected simultaneously with *aac*(6')-*Ib* in some isolates but at a lower prevalence. Not all the isolates exhibiting phenotypic resistance to ciprofloxacin (Table 2.2) tested positive for *qnrB*. This suggests that other mechanisms were involved in this resistance. The high prevalence of the bla_{SHV} gene is possibly due to the presence of the $bla_{SHV\text{-}1}$ gene on the chromosome, resulting in the intrinsic resistance to ampicillin (Wyres & Holt, 2018).

A great number of the ESBLs harboured the $bla_{CTX\text{-}M}$ resistance gene. Only variants belonging to group one were screened and detected. The group one primer set collectively targeted group one variants such as CTX-M-1, CTX-M-3 and CTX-M-15 (Dallenne *et al*., 2010). The latter is the most commonly detected in Africa (Zeynudin *et al*., 2018). This study found that the $bla_{CTX\text{-}M}$ gene was significantly ($p<0.05$) associated with the aminoglycoside and fluoroquinolone encoding genes screened and detected in most ESBL isolates. Co-resistance phenotypes to fluoroquinolones and aminoglycosides have been linked to the CTX-M type ESBL (Zeynudin *et al*., 2018). These results coincide with a study conducted in Algeria reporting the detection of group one variants (CTX-M-15 and CTX-M-3) in ESBL-producing *K. pneumoniae* isolates recovered from hospital surfaces. Furthermore, in most isolates the study also showed the association of the $bla_{CTX\text{-}M}$ gene with *qnrB* and *aac*(6')-*Ib*; however, most of the isolates carried the *aac*(6')-*Ib-cr* variant (Zenati *et al*., 2017).

Type three pili play a significant role in biofilm formation on abiotic surfaces. In contrast, enterobactin is a common siderophore secreted by *K. pneumoniae* to acquire bound and unbound iron in the host environment. Iron is an essential element needed by the bacterium to thrive during an infection (Bellifa *et al*., 2013; Martin & Bachman, 2018; Paczosa & Mecsas, 2016). These virulence factors are encoded by the *mrkD* and *entB* genes, respectively. In this study, the overall prevalence of the *mrkD* gene was 95%. This high prevalence is relatively similar to the 92% reported in Algeria (Bellifa *et al*., 2013) and the 96% reported in Brazil (Ferreira *et al*., 2019), involving the recovery of *K. pneumoniae* from medical devices and clinical isolates. However, the frequency of the *entB* gene observed in this study (92%), is slightly lower than the 100% reported in Brazil (Ferreira *et al*., 2019).

There is a clustering of genetically similar isolates (ERIC-type T) between tertiary hospital B and regional hospital C (Figure 2.2). This is most likely possible because patients in hospital C can get referred to hospital B when they require services not offered by regional hospitals but present in tertiary hospitals.

Genetically related isolates within major ERIC-type M were isolated from the sheet of an occupied bed and sink in the ICU of tertiary hospital B. Similarly, related clones of ERIC-type F were noted from different sites (the occupied bed and sink) in the general ward of district hospital D (Figure 2.2). By inserting a fluorescent marker into a sink drain cover, Hota *et al*. 2009 showed that during a 15-second handwash, fluorescent residues were transmitted and detected in the immediate vicinity as well as one metre from the sink. Researchers further suggested the probability of undetected microparticles travelling further than one metre (Hota *et al*., 2009). Kotay *et al*. (2017) demonstrated that when a sink strainer or sink bowl was colonised with green fluorescent protein (GFP)-expressing *Escherichia coli*, dispersion of the microorganism to surrounding surfaces occurred when the faucet was turned on (Kotay *et al*., 2017). Furthermore, when water tap handles are contaminated with bacteria, there is a chance of hand recontamination occurring upon contact, which can nullify any handwashing that has happened (Bhatta *et al*., 2018). The recontamination of hands can result in the dissemination of bacteria if contact is made with other sites.

Major ERIC-type K consisted of genetically similar isolates originating from the sheet of an occupied and unoccupied ICU bed in tertiary hospital B. Genetically related isolates within ERIC-type H were derived from a sheet of an occupied and unoccupied bed in the general ward of hospital A (Figure 2.2). The failure to adhere to IPC measures is a likely cause. However, due to the detection of *K. pneumoniae* on the sheets of unoccupied beds, hospital textiles including laundry and linen services should not be excluded when investigating possible routes of transmission.

2.5 Conclusion

This study is one of the very few hospital environment-based studies conducted in South Africa. It highlighted the prevalence of *K. pneumoniae* on various frequently touched hospital surfaces and equipment, which can be further disseminated to other sites if IPC measures are suboptimal. This was further supported by the recovery of genetically similar isolates from different surfaces within the same hospital. Some of the isolates recovered were characterised as ESBLs and showed high resistance to clinically significant antibiotics. Overall, the study emphasised the importance of regular monitoring of the hospital environment. The findings of this study can assist IPC staff in identifying the possible sources of transmission and assessing and improving current IPC interventions in place.

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2.7 Authors' contributions

Co-conceptualisation of study: NM, CS, DA, OT and LB. Performed laboratory work: NM. Analysed the data: NM, CM, DA and LB. Wrote the paper: NM. Critically reviewed the paper: All.

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2.9 Conflict of interest statement

None.

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CHAPTER THREE:

OVERVIEW OF MAIN FINDINGS, CONCLUSION AND SIGNIFICANCE OF STUDY, LIMITATIONS AND FUTURE RECOMMENDATIONS

3.1 Main findings

A total of 75 (10%) *Klebsiella pneumoniae* isolates were successfully isolated and identified using phenotypic and genotypic methods. The former incorporated the usage of laboratory media and a series of biochemical tests. Compared to the latter, which relied on conducting polymerase chain reaction (PCR) targeting the *khe* gene (Jian-Li *et al*., 2017).

The overall prevalence of *K. pneumoniae* differed across the selected public hospitals, with the highest occurrence being observed in tertiary hospital B and district hospital D. These two hospitals experience an increased inflow of patients leading to higher levels of environmental contamination. Only six sites harboured the bacterium of interest, and the highest level of contamination was detected on the occupied bed and sink. The former is bound to be contaminated as patients' beds are often covered in hospital textiles such as bedsheets or blankets. These textiles can be contaminated with bodily fluids which contain many microorganisms (Fijan & Turk, 2012). The high quantity of *K. pneumoniae* in sinks can be attributed to the moist environment favourable for Gram-negative bacteria (Muzslay *et al*., 2017). The moderate contamination of unoccupied beds is a significant concern as it poses a threat to the next patient admitted and allocated to the contaminated bed.

Only 30 of the 75 (40%) *K. pneumoniae* isolates were phenotypically characterised as extended-spectrum β-lactamases (ESBLs) and most were recovered from tertiary hospital B and the intensive care unit (ICU). The high prevalence in the areas mentioned above is probably due to the increased utilisation of β-lactam antibiotics, promoting the emergence of ESBLs. Moreover, the ESBL isolates, which mostly harboured the $bla_{\text{CTX-M}}$ group one gene, showed higher resistance levels than the non-ESBLs. The CTX-M type ESBL has been linked with co-resistance to fluoroquinolones and aminoglycosides (Zeynudin *et al*., 2018). This can possibly explain the enhanced resistance observed in ESBL isolates and the corresponding detection of the different fluoroquinolone (*qnrB*) and aminoglycoside [*aac*(3')-*II and aac*(6')-*Ib*] associated genes.

The different virulence genes were detected in high frequencies. These genes encode different virulence factors that have various functions during infections (Martin & Bachman, 2018). Lastly, some isolates collected from different sites (occupied bed, sink, unoccupied bed) within the same ward and hospital shared a genetic similarity ranging between 60-100%, suggesting that the dissemination of bacteria between sites occurs within the hospital environment. Moreover, various isolates from two different hospitals shared a genetic similarity of ≥60%, suggesting that bacteria can be circulated between hospitals through referral patients.

3.2 Conclusions and significance of study

The study's findings suggest that a contaminated hospital environment can play a role in disseminating bacteria when infection prevention and control (IPC) measures are substandard. This indicates that current IPC measures in the studied hospitals may not be adequate or strictly adhered to. The hospitals that had a high recovery of *K. pneumoniae* are high-risk; thus, IPC measures need to be emphasised to reduce bacterial transmission chances. The various surfaces found to be contaminated with *K. pneumoniae* can indicate to hospital staff which areas require evaluation so that current cleaning protocols in place can improve. Such action is essential because many South Africans rely on the overburdened public hospital for healthcare services. Thus, focusing on the high-risk areas can reduce contamination levels, potentially reducing the chances of bacterial transmission consequently preventing HAIs. Furthermore, the study identifies the need to regularly monitor support services linked to the hospital, such as laundry and linen.

The information regarding the antibiotic resistance profiles and prevalence of ESBLs can assist in encouraging hospital staff to use antibiotics appropriately as some need to be preserved for more severe infections whereas the usage of others needs to be reduced to limit the emergence of specific strains. The study also highlights and emphasises the importance of IPC adherence as it plays a crucial role in reducing the transmission of resistant bacteria. This is vital as the hierarchical referral system in South African public hospitals has the potential to facilitate the dissemination of resistant bacteria between districts or provinces through patients. This can further intensify the existing problem of antibiotic resistance experienced globally. The detection of resistance genes can help identify and understand the different mechanisms currently expressed by *K. pneumoniae* to confer resistance to antibiotics. Overall, this study

was able to broaden the available data regarding *K. pneumoniae* in KwaZulu-Natal public hospitals.

3.3 Limitations

There were several limitations associated with this study. The most prominent is that hospital staff were informed about each sampling visit beforehand; thus, this anticipated visit could have increased cleaning efforts and encouraged strict adherence to IPC measures, consequently underestimating the actual prevalence. Secondly, only hospitals from one health district were included in this study. Thus, the results obtained do not represent all the public hospitals situated in KwaZulu-Natal as the other 10 health districts were not included in the study. Likewise, only three wards per hospital were selected. Therefore, the prevalence of *K. pneumoniae* reported does not represent the entire hospital as other wards excluded from the study could be less or more contaminated with *K. pneumoniae*. Similarly, only 11 sites were included in this study. Although these surfaces were carefully selected based on literature and oral communication with healthcare workers, other surfaces that were not considered in this study could also potentially serve as reservoirs. Lastly, some of the resistance genes could not be sequenced to determine the exact variant involved due to cost constraints.

3.4 Future recommendations

Samples should be taken from inanimate surfaces as well as healthcare workers, janitor staff, patients and visitors to determine the extent of their role in disseminating bacteria. In addition, whole-genome sequencing (WGS) should be incorporated within these environmental studies as this can provide the full genetic profile of bacterial isolates in one step since some essential resistance and virulence genes may have been overlooked, especially those that are not readily associated with *Klebsiella*. Hypervirulent *K. pneumoniae* strains are slowly gaining attention as they acquire resistance. Therefore, future studies should focus on detecting these strains as they possess additional virulence factors. Phenotypic methods to investigate the biofilm-forming ability of *K. pneumoniae* should be conducted. The ability to form biofilms is an advantage to the survival and persistence of bacteria on surfaces.

3.5 References:

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Zeynudin, A., Pritsch, M., Schubert, S., Messerer, M., Liegl, G., Hoelscher, M., Belachew, T., & Wieser, A. (2018). Prevalence and antibiotic susceptibility pattern of CTX-M type extended-spectrum β-lactamases among clinical isolates of Gram-negative bacilli in Jimma, Ethiopia. *BMC Infectious Diseases*, *18*, 524.

APPENDICES

Appendix 1: The collection data and results for antibiotic susceptibility testing and molecular detection of resistance and virulence genes for each *K. pneumoniae* isolate included in the study.

Isolate	Collection data			Antibiotics												Antibiotic resistance genes						Virulence genes		
	Hosp.	Ward	Site	SXT	KZ	CN	C	FOX	CIP	CRO	MEM	AMP	TE	F	AMC	SHV	TEM	CTX-M	Aac(3')-II	QnrB	Aac(6')-Ib	entB	mrkD	wabG
1SID1*	D	ICU	P.File	R	R	R	S	S	R	R	S	R	R	I	R	+	+	+	+	+	-	+	+	+
1SID2*	D	ICU	P.File	R	R	R	S	S	I	R	S	R	R	I	R	+	+	+	+	+	+	+	+	+
1SID3*	D	ICU	P.File	R	R	R	S	S	R	R	R	R	R	R	I	+	+	+	+	+	+	+	+	+
1SGH1C	D	GEN	Sink	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1SGH1D	D	GEN	Sink	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1SGH3	D	GEN	Sink	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1SGH4	D	GEN	Sink	S	I	S	S	S	S	S	S	R	S	I	S	+	-	-	-	-	-	+	+	+
1SGJ12	D	GEN	Occ. bed	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1SGJ13C	D	GEN	Occ. bed	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1SGJ14	D	GEN	Occ. bed	S	I	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+

Abbreviations: Hosp.: Hospital; P.File: Patient File; Occ.bed: Occupied Bed; Unocc.bed: Unoccupied bed; AMP: Ampicillin; KZ: Cefazolin; FOX: Cefoxitin; CRO: Ceftriaxone; MEM: Meropenem; CIP: Ciprofloxacin; CN: Gentamicin; AMC: Amoxicillin-clavulanate; F: Nitrofurantoin; SXT: Trimethoprim-sulfamethoxazole; TE: Tetracycline; C: Chloramphenicol; S: Sensitive; I: Intermediate; R: Resistance; * indicates an ESBL-producing isolate.

Continued Appendix 1: The collection data and results for antibiotic susceptibility testing and molecular detection of resistance and virulence genes for each *K. pneumoniae* isolate included in the study.

Isolate	Collection data			Antibiotics												Antibiotic resistance genes						Virulence genes		
	Hosp.	Ward	Site	SXT	KZ	CN	C	FOX	CIP	CRO	MEM	AMP	TE	F	AMC	SHV	TEM	CTX-M	Aac(3')-II	Qnr B	Aac(6')-Ib	entB	mrkD	wabG
1SPF1	D	PEAD	Mop	S	R	S	S	S	S	S	S	R	S	I	S	-	-	-	-	-	-	-	-	-
1SPF2	D	PAED	Mop	S	S	S	S	S	S	S	S	R	S	I	S	+	-	-	-	-	-	+	+	+
1SPH1	D	PAED	Mop	S	S	S	S	S	S	S	S	R	S	I	S	+	-	-	-	-	-	+	+	+
1SPH2	D	PAED	Mop	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1SPJ11	D	PAED	Occ. bed	S	I	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1SPJ11C	D	PAED	Occ. bed	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1SPJ12	D	PAED	Occ. bed	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1SPJ21C	D	PAED	Unocc. bed	S	I	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1SPJ21D	D	PAED	Unocc. bed	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1SPJ22C	D	PAED	Unocc. bed	S	I	S	S	S	S	S	S	R	S	S	I	+	-	-	-	-	-	+	+	+
1SPJ21E	D	PAED	Unocc. bed	S	I	S	S	S	S	S	S	R	S	S	I	+	-	-	-	-	-	+	+	+
1CGJ1 1*	C	GEN	Occ. bed	R	R	R	S	S	R	R	R	R	S	I	I	+	+	+	+	+	+	+	+	+
1CGJ1 3*	C	GEN	Occ. bed	R	R	R	S	S	I	R	S	R	S	S	R	+	+	+	+	-	-	+	+	+
1CPH1	C	PAED	Sink	S	I	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1CPH2	C	PAED	Sink	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1CPJ11*	C	PAED	Occ. bed	R	R	R	S	R	R	R	R	R	S	R	R	+	+	+	+	+	+	+	+	+
1CPJ12*	C	PAED	Occ. bed	R	R	R	S	S	R	R	S	R	S	R	I	+	+	+	+	+	+	+	+	+

Continued Appendix 1: The collection data and results for antibiotic susceptibility testing and molecular detection of resistance and virulence genes for each *K. pneumoniae* isolate included in the study.

Isolate	Collection data			Antibiotics												Antibiotic resistance genes						Virulence genes		
	Hosp.	Ward	Site	SXT	KZ	CN	C	FOX	CIP	CRO	MEM	AMP	TE	F	AMC	SHV	TEM	CTX-M	Aac(3')-II	Qnr B	Aac(6')-Ib	entB	mrkD	wabG
1CPJ16*	C	PAED	Occ. bed	R	R	R	S	R	I	R	I	R	S	R	R	+	-	+	+	-	-	+	+	+
1CPJ17*	C	PAED	Occ. bed	R	R	R	S	R	R	R	R	R	S	R	R	+	+	+	+	+	+	+	-	-
2CPH1	C	PAED	Sink	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
2CPH2	C	PAED	Sink	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
2CPH3	C	PAED	Sink	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
2CPH4	C	PAED	Sink	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
3CID1*	C	ICU	P.File	R	R	R	S	S	I	R	R	R	R	S	R	+	+	+	+	+	-	+	+	+
3CID2*	C	ICU	P.File	R	R	R	S	S	R	R	R	R	R	I	R	+	+	+	+	+	-	+	+	+
3CID3*	C	ICU	P.File	R	R	R	S	S	I	R	R	R	R	S	R	+	+	+	+	+	+	+	+	+
3CGJ21	C	GEN	Unocc. bed	S	S	S	S	S	S	S	S	R	S	I	S	+	-	-	-	-	-	+	+	+
3CGJ21	C	GEN	Unocc. bed	S	I	S	S	S	S	S	S	R	S	S	I	+	-	-	-	-	-	+	+	+
1UPF3	B	PAED	Mop	S	I	S	S	S	S	S	S	R	S	S	S	-	-	-	-	-	-	-	-	-
2UIH3	B	ICU	Sink	S	S	S	S	S	S	S	S	R	S	I	S	+	-	-	-	-	-	+	+	+
2UIH4	B	ICU	Sink	S	R	S	S	R	I	R	I	R	S	I	R	+	-	-	-	-	-	+	+	+
2UIH6	B	ICU	Sink	S	I	S	S	S	S	S	S	R	S	R	S	+	-	-	-	-	-	+	+	+
2UIH7	B	ICU	Sink	S	S	S	S	S	S	S	S	R	S	I	S	+	-	-	-	-	-	+	+	+

Continued Appendix 1: The collection data and results for antibiotic susceptibility testing and molecular detection of resistance and virulence genes for each *K. pneumoniae* isolate included in the study.

Isolate	Collection data			Antibiotics												Antibiotic resistance genes						Virulence genes		
	Hosp.	Ward	Site	SXT	KZ	CN	C	FOX	CIP	CRO	MEM	AMP	TE	F	AMC	SHV	TEM	CTX-M	Aac(3')-II	Qnr B	Aac(6')-Ib	entB	mrkD	wabG
2UPJ11	B	PAED	Occ. bed	S	I	S	S	S	S	S	S	R	S	R	S	+	-	-	-	-	-	+	+	+
3UIF1*	B	ICU	Mop	R	R	R	S	S	I	R	S	R	R	R	R	+	+	+	+	-	-	+	+	+
3UIF2*	B	ICU	Mop	R	R	R	S	S	I	R	S	R	R	R	R	+	+	+	+	-	-	+	+	+
3UIF3*	B	ICU	Mop	R	R	R	S	S	R	R	S	R	R	R	R	+	+	+	+	-	-	+	+	+
3UIF4*	B	ICU	Mop	R	R	R	S	S	R	R	R	R	R	R	R	+	-	+	+	+	-	+	+	+
3UID1*	B	ICU	P.File	S	R	S	S	R	I	R	I	R	S	R	S	+	-	-	-	-	-	+	+	+
3UIJ11*	B	ICU	Occ. bed	R	R	R	R	R	R	R	R	R	S	R	R	+	+	+	+	-	+	+	+	+
3UIJ12*	B	ICU	Occ. bed	R	R	R	R	R	R	R	R	R	S	R	R	+	+	+	+	+	+	+	+	+
3UIJ13*	B	ICU	Occ. bed	R	R	R	R	R	R	R	R	R	S	R	R	+	+	+	+	+	+	+	+	+
3UIJ14*	B	ICU	Occ. bed	R	R	R	R	R	R	R	R	R	S	R	R	+	+	+	+	+	+	+	+	+
3UIJ21*	B	ICU	Unocc. bed	R	R	R	R	R	R	R	R	R	S	R	R	+	-	+	+	-	-	+	+	+
3UIJ22*	B	ICU	Unocc. bed	R	R	R	R	R	R	R	R	R	S	I	R	+	+	+	+	+	+	+	+	+
3UIJ23*	B	ICU	Unocc. bed	R	R	R	R	R	R	R	R	R	S	R	R	+	+	+	+	-	-	+	+	+
3UIJ25*	B	ICU	Unocc. bed	R	R	R	R	R	R	R	R	R	S	S	R	+	+	+	+	-	-	+	+	+
3UIH3*	B	ICU	Sink	R	R	R	R	R	R	R	R	R	S	R	R	+	+	+	+	-	-	+	+	+
3UIH4*	B	ICU	Sink	R	R	R	R	R	R	R	R	R	S	R	R	+	+	+	+	-	-	+	+	+
1MGF1*	A	GEN	Mop	R	R	R	S	S	R	R	S	R	S	S	R	+	+	+	+	+	+	-	+	+

Continued Appendix 1: The collection data and results for antibiotic susceptibility testing and molecular detection of resistance and virulence genes for each *K. pneumoniae* isolate included in the study.

Isolate	Collection data			Antibiotics												Antibiotic resistance genes						Virulence genes		
	Hosp.	Ward	Site	SXT	KZ	CN	C	FOX	CIP	CRO	MEM	AMP	TE	F	AMC	SHV	TEM	CTX-M	Aac(3')-II	QnrB	Aac(6')-Ib	entB	mrkD	wabG
1MGF4*	A	GEN	Mop	R	R	R	R	R	R	R	R	R	S	I	R	+	+	+	+	-	-	+	+	+
1MGF3*	A	GEN	Mop	R	R	R	S	S	I	R	S	R	S	S	R	+	+	+	+	+	+	+	+	+
1MGJ1 1	A	GEN	Occ. bed	S	R	S	S	S	S	S	S	R	S	I	I	+	-	-	-	-	-	+	+	+
1MGJ1 2	A	GEN	Occ. bed	S	I	S	S	S	S	S	S	R	S	R	S	+	-	-	-	-	-	+	+	+
1MGJ1 4	A	GEN	Occ. bed	S	R	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1MGJ2 1	A	GEN	Unocc. bed	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1MGJ2 2	A	GEN	Unocc. bed	S	S	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	+	+	+
1MPF2	A	PAED	Mop	S	R	I	S	I	S	S	S	R	S	S	I	-	-	-	-	-	-	-	+	+
1MPF1	A	PAED	Mop	S	R	S	S	S	S	S	S	R	S	S	S	-	+	-	-	-	-	+	+	+
1MPF3	A	PAED	Mop	S	R	S	S	S	S	S	S	R	S	S	S	-	-	-	-	-	-	-	+	-
1MPF4	A	PAED	Mop	S	R	S	S	S	S	S	S	R	S	S	S	-	-	-	-	-	-	-	-	-
1MPJ1 1	A	PAED	Occ. bed	S	R	I	S	S	S	S	S	R	S	S	I	+	-	-	-	-	-	+	+	+
1MPJ1 2	A	PAED	Occ. bed	S	I	S	S	S	S	S	S	R	S	S	S	+	+	+	+	-	-	+	+	+
2MPC1	A	PAED	BP monitor	S	I	S	S	S	S	S	S	R	S	I	S	+	-	-	-	-	-	+	+	+
2MPC2	A	PAED	BP monitor	S	R	S	S	I	I	I	R	R	S	R	R	+	-	-	-	-	-	+	+	+

Appendix 2: Ethical approval letter from the Biomedical Research Ethics Committee (BREC) at the University of KwaZulu-Natal (Reference number: BE606/16).

UNIVERSITY OF
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21 May 2018

Dr LA Bester
Biomedical Resource Unit
School of Laboratory Medicine and Medical Sciences
besterl@ukzn.ac.za

Dear Dr Bester

Protocol: To ascertain the nature and extent of infection, prevention and control (IPC) programs at different levels of care in eThekwini district, KwaZulu-Natal.
Degree: Non-degree
BREC reference number: BE606/16

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	30 May 2018
Expiration of Ethical Approval:	29 May 2019

I wish to advise you that your application for Recertification received on 03 May 2018 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

This approval will be ratified by a full Committee at its meeting taking place on 12 June 2018.

Yours sincerely

[redacted]

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

cc postgraduate administrator: dudhrajhp@ukzn.ac.za

Appendix 3: Approval letter from the Health Research and Knowledge Management (Reference number: KZ2017RP24 630).

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

DIRECTORATE:
Health Research & Knowledge Management (HKRM)

330 Langalibalele street,
Private Bag X9051 PMB, 3200
Tel: 033 395 2805/3189/3123 Fax: 033 394 3782
Email: hrkm@kznhealth.gov.za
www.kznhealth.gov.za

Reference: HRKM098/17
KZ_2017RP24_630

23 March 2017

Dear Dr L A Bester
(University of KwaZulu-Natal)

Subject: Approval of a Research Proposal

1. The research proposal titled '**To ascertain the nature and extent of Infection, Prevention and Control (IPC) programs at different levels of care in hospitals in eThekwini district, KwaZulu-Natal**' was reviewed by the KwaZulu-Natal Department of Health (KZN-DoH).

   The proposal is hereby **approved** for research to be undertaken at

   Hospitals.

2. You are requested to take note of the following:
   a. Make the necessary arrangement with the identified facility before commencing with your research project.
   b. Provide an interim progress report and final report (electronic and hard copies) when your research is complete.

3. Your final report must be posted to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Ms G Khumalo on 033-395 3189.

Yours Sincerely

[redacted]

Dr E Lutge
Chairperson, Health Research Committee
Date: 07/03/17

Appendix 4: Submission confirmation **e**mail from Microbial Drug Resistance

Microbial Drug Resistance <onbehalfof@manuscriptcentral.com> 15 Nov 2020, 17:15 (10 days ago)
to me

15-Nov-2020

Dear Miss Malinga:

A manuscript titled Molecular surveillance and dissemination of Klebsiella pneumoniae on frequently encountered surfaces in South African public hospitals (MDR-2020-0546) has been submitted by Dr. Daniel Amoako to Microbial Drug Resistance.

You are listed as a co-author for this manuscript. The online peer-review system, Manuscript Central, automatically creates a user account for you. Your USER ID and PASSWORD for your account is as follows:

You can use the above USER ID and PASSWORD to log in to the site and check the status of papers you have authored/co-authored. This password is case-sensitive and temporary. Please log in to https://mc.manuscriptcentral.com/mdr to update your account information and change your password.

Thank you for your participation.

Sincerely,
Microbial Drug Resistance Editorial Office
Log in to Remove This Account

Appendix 5: Introduction to Research Ethics (TRREE Certificate)

TRREE

Zertifikat
Certificat
Certificado
Certificate

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

Nongcebo Malinga

a complété avec succès - has successfully completed

Introduction to Research Ethics

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

May 9, 2017

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

FMH Continuing Education Program (5 Credits)
Programme de Formation continue (5 Crédits)

Foederatio Pharmaceutica Helvetiae FPH Programmes de formation continue
Continuing Education Programme
Programmes de formation continue

Ce programme est soutenu par - This program is supported by :

Appendix 6: Research Ethics Evaluation (TRREE Certificate)

TRREE

Zertifikat

Certificat

Certificado

Certificate

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

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a complété avec succès - has successfully completed

Research Ethics Evaluation

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

May 12, 2017

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

FMH Continuing Education Program (5 Credits)

Foederatio Pharmaceutica Helvetiae FPH Programmes de formation continue Continuing Education Programme

Ce programme est soutenu par - This program is supported by :

Appendix 7: Informed Consent (TRREE Certificate)

TRREE

Zertifikat
Certificat
Certificado
Certificate

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

Nongcebo Malinga

a complété avec succès - has successfully completed

Informed Consent

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

[redacted]

May 13, 2017
CID : rfBtQhaHkMq

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

FMH Continuing Education Program (5 Credits)
Programme de Formation continue (5 Crédits)

Foederatio Pharmaceutica Helvetiae FPH Programmes de formation continue
Continuing Education Programme
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European and Developing Countries Clinical Trials Partnership (EDCTP) (www.edctp.org) - Swiss National Science Foundation (www.snf.ch) - Canadian Institutes of Health Research (http://www.cihr-irsc.gc.ca/e/2891.html) - Swiss Academy of Medical Science (SAMS/ASSM/SAMW) (www.samw.ch) - Commission for Research Partnerships with Developing Countries (www.kfpe.ch)

[REV : 20170310]

Appendix 8: Good Clinical Practice (TRREE Certificate)

TRREE

Zertifikat
Certificat
Certificado
Certificate

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Clinical Trials Centre
The University of Hong Kong

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

Nongcebo Malinga

a complété avec succès - has successfully completed

Good Clinical Practice (GCP)

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

May 15th, 2017

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

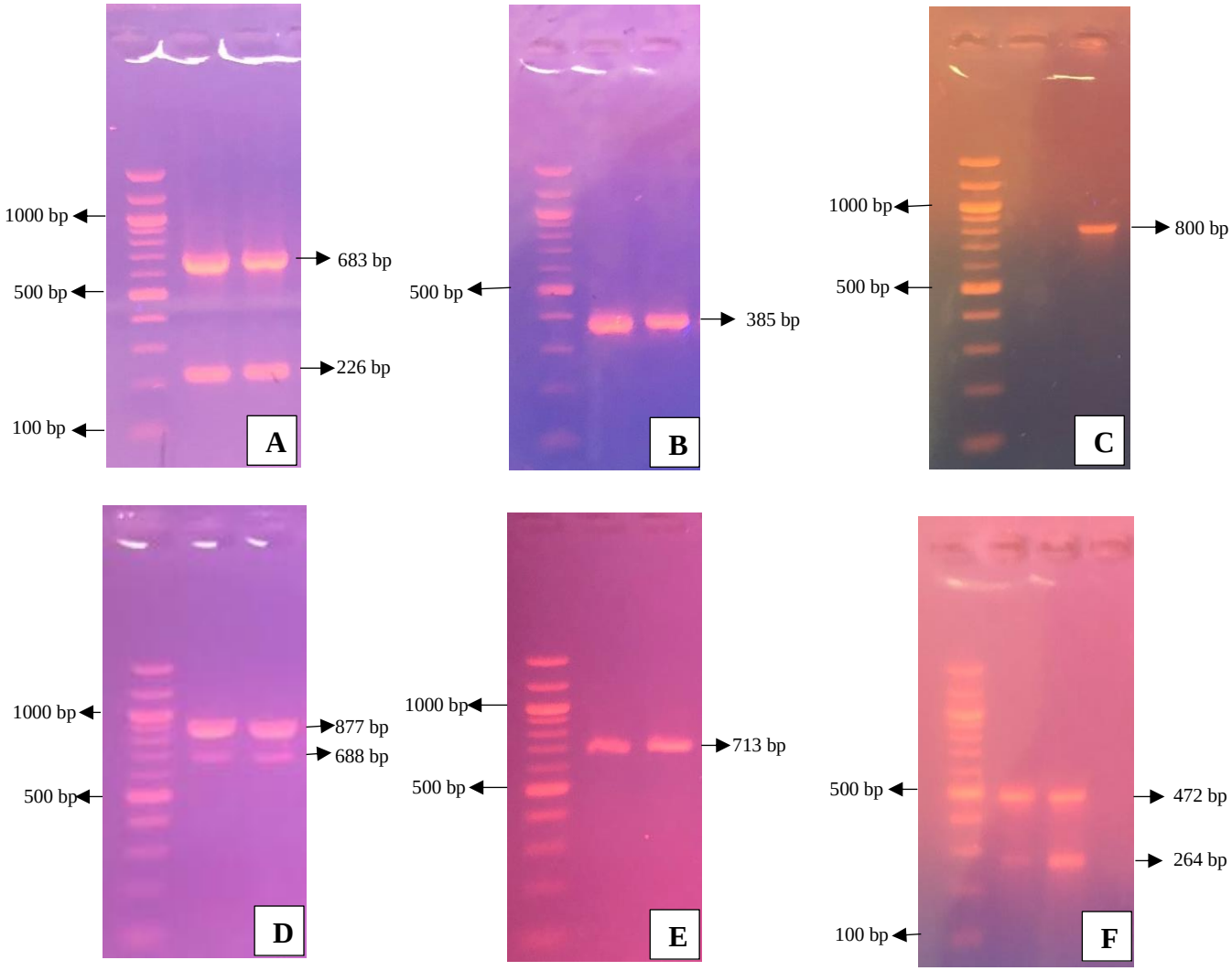
FMH Continuing Education Program (5 Credits)
Programme de Formation continue (5 Crédits)

Foederatio Pharmaceutica Helvetiae FPH Programmes de formation continue
Continuing Education Programme
Programmes de formation continue

Ce programme est soutenu par - This program is supported by :

[RIV : 20170310]

Appendix 9: Agarose gel electrophoresis images showing the molecular amplification of the different virulence and antibiotic resistance genes



A: *mrkD* (226 bp) and *wabG* (683 bp) amplified from *K. pneumoniae*; **B**: *entB* (385 bp) amplified from *K. pneumoniae*; **C**: *bla*$_{TEM}$ (800 bp) amplified from *K. pneumoniae*; **D**: *bla*$_{CTX-M}$ group one (688 bp) and *aac*(3')-*II* (877 bp) amplified from *K. pneumoniae*; **E**: *bla*$_{SHV}$ (713 bp) amplified from *K. pneumoniae*; **F**: *qnrB* (264 bp) and *aac*(6')-*Ib* (472 bp) amplified from *K. pneumoniae*. PCR products were run alongside a 100 bp molecular weight marker.