DEMOGRAPHIC CHARACTERISTICS, PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF ANTIBIOTIC RESISTANT *KLEBSIELLA PNEUMONIAE* ISOLATED FROM CLINICAL SAMPLES AT THE NAIROBI HOSPITAL, KENYA

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Demographic Characteristics, Phenotypic and Genotypic Characterization of Antibiotic Resistant *Klebsiella pneumoniae* Isolated from Clinical Samples at The Nairobi Hospital, Kenya

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DECLARATION

This thesis is my original work and has not been presented for award of degree in any other University.

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DEDICATION

This thesis is dedicated to my wife and my children who have tirelessly supported me all the way since the beginning of my studies. Their prayers and moral support have been the driving momentum that has enabled me to achieve the targeted goal in my studies. I have to thank my parents, grandmother for their love and support throughout my life, and for the silent prayers you made for me.

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ABREVIATIONS

AmpC	Ampicillinase
ATCC	American Type Culture collection
Bla	Beta lactamase coding gene
CDC	Centers for Disease control and prevention
CLSI	Clinical Laboratory Standards Institute
CMY	Cephamycinase
CPS	Capsular polysaccharides
CTX-M	Active on cefatoxime first isolated in Munich
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ESBL	Extended Spectrum β- Lactamase
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella Pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, Enterobacter*
FOX	Cefoxitinase
GEI	Genomic Islands
ICE	Integrative and conjugative elements
IMP	Imipenemase
Kbp	Kilobase pairs
KPC	*Klebsiella pneumoniae* carbapenemase
LPS	Lipopolysaccharides
MBL	Metallo Beta lactamases
MDR	Multidrug resistant
MIC	Minimal inhibitory concentration
MOX	Moxalactam
MR/K-HA	Mannose Resistant Klebsiella Haemagglutination
MSHA	Mannose sensitive haemagglutination
CLSI	Clinical Laboratory Standards Institute
NDM	New Delhi Metallo Beta Lactamase

OMP	Outer membrane proteins
ORF	Open reading frame
OXA	Oxacillinase
PCR	Polymerase chain reaction
PLA	Pyogenic Liver abscess
RNA	Ribonucleic acid
RTI	Respiratory tract infections
SHV	Sulfhydryl variable
TEM	Named after patient Temoniera from whom it was Isolated
UK	United Kingdom
USA	United States of America
UTI	Urinary tract infection
UV	Ultraviolet
VIM	Verona Integron encoded Metallo β- lactamase
WHO	World Health Organization

ABSTRACT

Antimicrobial resistance is a growing threat worldwide. The increasing number of β-lactamase producing *K. pneumoniae* leaves very few treatment options for clinicians and identification of these enzymes helps clinicians and microbiologists to rationalize the choice of antibiotics. The present study aimed to determine the demographic characteristics of patients from whom were collected *K. pneumoniae* isolates, antimicrobial susceptibility patterns of *K. pneumoniae* isolates, the presence of ESBL, Amp C and carbapenemase producing *K. pneumoniae* isolated from clinical samples at The Nairobi Hospital laboratory. The cross-sectional study was performed from May 2017 to April 2018 and included 272 positive clinical samples for *K. pneumoniae* tested with standard methods. Identification, antibiotic susceptibility pattern and detection of β-lactamases were done by automated VITEK 2 System. ESBL and Carbapenemase genes were detected by using PCR. Out of 272 *K. pneumoniae* isolates from various clinical samples, *K. pneumoniae* isolates was more collected in females as compared to males in almost age sets except at age group of 0-10 years. *K. pneumoniae* isolates were lower resistant to amikacin (18 %), gentamicin (21 %), nitrofurantoin (21 %), meropenem (21.7 %) and ciprofloxacin (25.4 %) compared to commonly used antibiotics. ESBL production was detected in 29.8 % (81 /272). The proportion of carbapenemase production was 7.0 % (19/272). The rate of ESBL and carbapenemase co-production was 5.1 % (14/272); ESBL and Amp C co-production 0.7 % (2/272); Amp C and carbapenemase 1.8 % (5/272) and ESBL/ AmpC/carbapenemase co-occurred in 2.6 % (7/272). In ESBL producing *K. pneumoniae*, the proportion of bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ was 5.77 %, 1.92 % and 2.88 % respectively; bla_{TEM} /bla_{SHV} /$bla_{CTX\text{-}M}$ (52.88 %), bla_{TEM}/bla_{SHV} (13.46 %), bla_{SHV}/$bla_{CTX\text{-}M}$ (8.65 %), bla_{TEM} /$bla_{CTX\text{-}M}$ (4.81 %) and bla_{TEM}/bla_{SHV}/$bla_{CTX\text{-}M}$/$bla_{OXA\text{-}48}$ (9.62 %). In carbapenemase producing *K. pneumoniae*, $bla_{OXA\text{-}48}$ was the most dominant gene (62.2 %) and bla_{KPC} genes (24.4 %) whereas bla_{VIM} and bla_{IMP} were not detected. The presence of β-lactamase enzymes singly or in combination were detected in this study. The findings obtained were essential for performing continuous monitoring of β lactamases, strict antibiotic policy in reducing *K. pneumoniae* resistance in hospital settings. Maintaining proper sanitation, antimicrobial policy and epidemiological surveys will help in controlling and preventing the spread of these resistant bugs in hospital environment.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Klebsiella pneumoniae (*K .pneumoniae*) is a part of *Enterobacteriaceae* family and is described as Gram negative rods shaped, facultative anaerobic, encapsulate, lactose fermenting oxidase negative, catalase positive, and non-motile bacterium (Patel *et al.*, 2017). Hospital acquired infections affect the clinical outcomes in hospitalized patients and represent a serious concern worldwide (Wang *et al.*, 2019). *K. pneumoniae* is a common opportunistic pathogen nosocomial infections. It is associated with pneumonia, urinary tract infection, septicemia, as well as bacterial meningitis and biliary tract infection (Podschun & Ullmann, 1998; Struve & Krogfelt, 2004). *K. pneumoniae* can survive in hospitals, persist on environmental surface and colonize human skin, respiratory tract and bowels (Boll *et al*., 2012; Jikun *et al*., 2014).

Nosocomial isolates are often associated with extended spectrum β lactamases, including, recently, carbapenemases such as KPC and OXA-48 making treatment options limited (Turton *et al.,* 2010). During the 1970s, *K. pneumoniae* became an important cause of nosocomial infections, especially urinary tract infections (UTIs), respiratory infections and bloodstream-associated infections. The management of infections due to *K. pneumoniae* has been complicated by the emergence of antimicrobial resistance, especially since the 1980s due to the carbapenemase production (Pitout *et al.*, 2015).

The antimicrobials resistance to *K. pneumoniae* has been linked to an up regulation in efflux pumps, decreased of antibiotic penetration, modification of the antibiotic molecule and the changes in targets sites. Furthermore, the genetic exchange has been implicated in the dissemination of resistance to used antibiotics (Munita & Arias , 2016).

The mechanisms of foreign DNA transfer between or within bacteria alter the antibiotic action following three mechanisms: transduction (Phage mediated),

conjugation (bacterial “sex” and transformation (incorporation of naked DNA) (Gyles & Boerlin, 2014 ;Munita & Arias , 2016).

Antimicrobials have been widely used against to *K. pneumoniae*. However, infections are very refractory to therapeutic interventions and since the overuse of antibiotics and persistent exposure of *K. pneumoniae* strains to a number of antimicrobials, multidrug resistant strains have been selected (Heidary *et al.*, 2018). These strains are resistant to extended-spectrum β-lactam antibiotics aminoglycosides, fluoroquinolones, and even the most effective antimicrobial agent of carbapenems (Jikun *et al*., 2014) . Several global surveillance studies during the 2000s showed that 20 to 80% of *K. pneumoniae* isolates were resistant to first line antibiotics, including the cephalosporins, fluoroquinolones and aminoglycosides of special concern is the emerging resistance to carbapenems; since these agents are often the last line of effective therapy available for the treatment of infections caused by multidrug resistant *K .pneumoniae* (Pitout *et al.,* 2015) **.**

The resistance to β-lactam antibiotics is an increasing problem worldwide and β-lactamases production is the most common mechanism of drug resistance and these β-lactamases are now capable of hydrolyzing penicillins, broad spectrum cephalosporins and monobactams (Sehlawi *et al.*, 2013; Singh Moodley & Perovic, 2016). Antibacterial resistance continues to be a global public health concern, threatening the effectiveness of the therapy, and challenging the efforts for developing novel antibacterial (Srinivasan *et al.,* 2015). *K. pneumoniae* has been identified as a major cause of community-acquired pneumonia and is responsible for approximately 10% of all hospital acquired infections, ranking second among Gram-negative pathogens (Nordmann *et al.,* 2009). Studies performed in Asia have demonstrated that the fatality rate of *K. pneumoniae* induced pneumonia in elderly people was 15% to 40% (Hou *et al.*, 2015). Selective pressure, the extensive use of antibiotics, and the conjugational transmission of antibiotic resistance genes across bacterial species and genera facilitate the emergence of multidrug-resistant (MDR) *K. pneumoniae* (Hou *et al.*, 2015).

Carbapenem resistant *Enterobacteriaceae* have been frequently reported worldwide. They represent a serious concern because of the limited therapeutic options (Ribeiro *et al.*, 2013). Carbapenemase producers are usually resistant to almost all of the effective antibiotics (Candan & Aksöz, 2015). Carbapenemases producing *K. pneumoniae* emerged in the late 1990s and has become a serious health problem in the world (Fatma *et al.*, 2016). The misuse and overuse of antibiotics in medicine and agriculture has led to the development of multidrug resistant bacteria .In addition to higher risk of poor clinical outcomes and death, multidrug resistant bacterial infections are associated with increased economic burden of patients (Wang *et al.*, 2019)

In Gram-negative pathogens, β-lactamase production is the most important factor complicating the treatment of nosocomial infections. β- lactamases are bacterial enzymes that inactivates β-lactam antibiotics by hydrolysis, resulting in ineffective compounds even for drugs of choice in clinical antibiotic therapy (Njage *et al.*, 2012). Among them, extended-spectrum β-lactamases (ESBL) are of great concern. ESBLs have been reported worldwide, most frequently in *Enterobacteriaceae*; TEM-type ESBLs and SHV-type ESBLs and CTX-M-type ESBLs has been described (Dallenne *et al.*, 2010). Infection with either ESBL producing *Enterobacteriaceae* or carbapenemase producing *Enterobacteriaceae* is associated with increased mortality rates, time to effective therapy, length of stay of stay and overall healthcare costs (Wilson & Torok , 2018).

1.2 Statement of the problem

Klebsiella pneumoniae (K. pneumoniae) has been identified as a major cause of community-acquired pneumonia (CAP) and is responsible for approximately 10 % of all hospital acquired infections, ranking second among Gram-negative pathogens. Studies performed in Asia have demonstrated that the fatality rate of *K. pneumoniae* induced pneumonia in elderly people was 15% to 40% (Hou *et al.*, 2015). *K. pneumoniae* are often the cause of nosocomial pneumonia (7 % to 14%), septicaemia (4 % to 15 %), urinary infections (6 % - 17 %), infections of wounds (2 % - 4 %),

infections in the intensive care unit (4 % to 17 %) and neonatal septicemia (3 % to 20 %) (Mousse *et al.*, 2016).

K. pneumoniae caused 20% of total nosocomial bacteremia episodes between 2002 and 2009 in Kilifi county hospital (Henson *et al.*, 2017). A retrospective study conducted of all blood culture by Apondi *et al*.(2016) in Moi Teaching and Referral Hospital, Eldoret reported 23% of the hospital *K. pneumoniae* isolates (Apondi *et al.*, 2016). Previous studies based have suggested several comorbidies as risk factors for the development of *K. pneumoniae* bacteremia, including diabetes, cancer, chronic liver disease and biliary disease (Kang *et al.,* 2006; Tsay *et al.,* 2002). In Zambia, Nagelkerke *et al*. (2017) reported a significant difference in colonization rate with resistant *Enterobacteriaceae* between in-and outpatients, with a higher proportion of inpatients carrying resistant strains.

During the time inpatients were hospitalized their intestinal microbiome could have changed. Several factors such as the underlying disease, antibiotic treatment and acquisition of (multi-resistant) from environment during the stay, have been reported to play a role in the change in the gastrointestinal flora during hospital admission (Ramirez,2006). Antibiotic exposure for ≥ 14 days was the risk factors associated with carbapenemase resistance *K. pneumoniae* bacteremia (Hussein *et al.,* 2013).

The recognized risk factors for *K. pneumoniae* community acquired pneumonia are sex (female), diabetes, mellitus and alcoholism as it was reported by other researchers (Inghammar *et al.,* 2018; Rammaert *et al.,* 2012). Gorrie *et al.* (2017) reported a positive association between *K. pneumoniae* and age among patients in an intensive care unit (Gorrie *et al.,* 2017)

The World Health Organization (WHO) has published a global priority pathogens list (included *K. pneumoniae*) to focus attention on most significant resistant pathogens .*Enterobacteriaceae* resistant to 3rd generation cephalosporins and *Enterobacteriaceae* resistant to carbapenem are included in the list.(WHO, 2017). In New York, USA, half of infections caused by carbapenem resistant E. coli and *K. pneumoniae* in 2014 were hospital acquired. It has been previously reported that *K. pneumoniae* is the second most common cause Gram negative bloodstream infections, after *E.coli*, in adult population (Nielsen *et al.*, 2014).

A systematic review done by World Health Organization on healthcare associated infection showed, by order of decreasing frequency *P. aeruginosa, E.coli, K. pneumoniae* and *Enterobacter spp.* (Nejad *et al.*, 2011). Studies conducted in Malaysia and Japan estimate the incidence rate in elderly persons to be 15-40% and the Centers for Disease Control and prevention report that *Klebsiella* strains were responsible for 3% of all pathogenic epidemic outbreaks (Shahab , 2018). The prevalence of *K. pneumoniae* in the community is likely fueled by unrestricted use antimicrobials that may be purchased without prescription (Muzaheed *et al.,* 2008). They are many possible contributing factors to the emergence rise and spread of antibiotic resistance ,including the new acquisition of resistance genes; transfer of resistance genes ;healthcare exposure; use of indwelling medical devices; limited diagnostic facilities; lack of effective and reliable surveillance systems; immunosuppressed states; travel to areas with a high endemicity of multidrug resistant bacteria; lack of new antimicrobial therapeutic; and inappropriate and excessive antibiotic use in health care ,food producing animals and agriculture (Ferreira *et al.,* 2019).

In healthcare services, patient deaths resulting from antimicrobial resistance are projected to reach 10 million annually by 2050 and a cumulative 100 trillion USD of economic outputs are at risk to the rise of drug resistant infection.Today,700,000 people die of resistant infections every year (O'Neill, 2014). The resistance mechanisms developed by *K. pneumoniae* is the production of β-lactamase which can destroy the β-lactam antibiotics. Extended spectrum β-lactamases (ESBLs) are most prevalent in *K. pneumoniae,* which has resulted in their resistance to third-generation cephalosporins and cephamycin (Sehlawi *et al.*, 2013).

The worldwide emergence and spread of carbapenem resistant *Enterobacteriaceae* isolates is a challenge for physicians and clinical microbiologists. (Çakar *et al*., 2016; (Majid *et al*., 2016). The presence of carbapenemase increases mortality rates. (Clarivet *et al.*, 2016). Due to the alarming increase in carbapenem resistant *Enterobacteriaceae*, of which *K. pneumoniae* comprises the majority of infections, the Centers for Disease Control (CDC) designated these bacteria as an urgent threat to public health (Bachman *et al*., 2015).

1.3 Justification

K. pneumoniae has become a leading cause of acquired community and nosocomial infections. Antibiotic resistance continues to plague antimicrobial chemotherapy of infectious disease (Poole, 2005)**.** The problem of antimicrobial resistance is highlighted by a recent increase of carbapenem resistant *K. pneumoniae*, which has largely been driven by the emergence and spread of mobile genetic elements carrying carbapenemase resistance genes (Chung *et al*., 2015).

The epidemiology of bacteria producing carbapenemases has been described in Europe, North America and Asia; however, little is known about their spread and clinical relevance in Africa. Antibiotic stewardship and surveillance systems should be implemented to monitor and reduce the spread of carbapenemase producing bacteria (Rendani *et al.,* 2015). *K. pneumoniae* producing carbapenemase are among the highly antimicrobial resistant Gram negative and infections due them are an increasingly major public health problem worldwide (Firoozeh *et al.,* 2016) associated with higher mortality rates, longer hospitalization and increased healthcare costs (Villegas *et al.,* 2016).

This has led to multidrug- resistant (MDR) *K. pneumoniae* being recently singled out as an "urgent threat to human health" by the U.S. Center for Disease Control and Prevention (CDC) and the World Health Organization (Chung *et al.*, 2015).

WHO has identified *K. pneumoniae* as one of a group of pathogens named ESKAPE and these pathogens have become today resistant or persistant to antibiotic treatment in clinical practice (Santajit & Indrawattana, 2016). The occurrence of multiple beta lactamases among bacteria not only limits the therapeutic options but also poses diagnostic challenge to the microbiologist (Sheemar *et al.,* 2016). The genes responsible for the transfer of these enzymes may become endemic in hospitals, with eradication becoming unattainable despite intensive infection control measures. It is therefore necessary to contribute to reliable detection of β-lactamase producing *K. pneumoniae* for the control of *K. pneumoniae.*

1.4 Research questions

1. What are the characteristics of patients of *K. pneumoniae* isolates from clinical samples at The Nairobi Hospital, Kenya?
2. What are the antimicrobial susceptibility patterns of *K. pneumoniae* isolates to commonly used antibiotics at The Nairobi Hospital, Kenya?
3. What is the proportion of ESBL, AmpC and carbapenemase producing *K. pneumoniae* from clinical samples at The Nairobi, Hospital, Kenya?

1.5 Objectives

I.5.1 General Objective

To determine the characteristics of patients with *K. pneumoniae* infections, antimicrobial susceptibility patterns, the proportion of ESBL, Amp C and carbapenemase producing *K. pneumoniae* isolates from clinical samples at The Nairobi Hospital, Kenya.

I.5.2 Specific objectives

1. To determine the characteristics of patients with *K. pneumoniae* infections at The Nairobi Hospital, Kenya.
2. To determine antimicrobial susceptibility patterns of *K. pneumoniae* isolates from clinical samples at The Nairobi Hospital, Kenya.
3. To determine the proportion of ESBL, Amp C and carbapenemase resistance genes in ESBL and carbapenemase producing *K. pneumoniae* from clinical samples at The Nairobi Hospital, Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1. Taxonomic history and general characteristics of *K. pneumoniae*

The bacterial genus *Klebsiella*, in the family *Enterobacteriaceae*, was named by Trevisan (1885) to honor the German microbiologist Edwin Klebs (1834–1913). The first *Klebsiella* species ever described was a capsulated bacillus from patients with rhinoscleroma. The organism was named "*K. rhinoscleromatis*"by Trevisan (1887) (Brisse *et al.,* 2006) . Abel (1893) observed a capsulated bacillus, "*Bacillus mucosus ozaenae*" from the nasal secretion of patients with ozaena. Friedländer (1883) described a bacterium from the lungs of a patient who had died of pneumonia and the organism was named "*Hyalococcus pneumoniae* "and *K. pneumoniae* (Asati , 2013 ;Brisse *et al.,* 2006).

K. pneumoniae is a part of *Enterobacteriaceae* family and is described as Gram negative rods shaped, facultative anaerobic, encapsulate, lactose fermenting oxidase negative, catalase positive, and non-motile bacterium (Patel *et al.,* 2017). In addition to the ability to colonize gastrointestinal tract, nasopharynx and skin, *K. pneumoniae* could cause a various infection syndromes, including urinary tract infection, intra-abdominal infection, skin and soft tissue infection and pneumonia in both community and healthcare associated settings (Lin *et al.,* 2016)

The medical importance of the genus *Klebsiella* led do its being subdivided into three species corresponding to the diseases they caused: *K. pneumoniae, K. ozaenae* and *K. rhinoscleromatis* (Podschun & Ullmann, 1998). The name "*K. ornithinoltica*" was proposed for ornithine positive, indole positive strains of *Klebsiella*. With the recent additions of *K. michiganensis* and *K. quasipneumoniae* (with two subspecies) to the genus, the number of validly published species is now at 15 (Janda, 2015). The genus *Klebsiella* is classified into four species such as *K. pneumoniae, K. oxytoca, K. terrigena* and *K. planticola*, with *K. pneumoniae* consisting of three species: *K. pneumoniae* subsp. *pneumoniae, K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis* (Vasaikar *et al.,* 2017).

The vast majority of human *Klebsiella* infections are still attributed to *K. pneumoniae* and *K. oxytoca* sensu stricto (Janda, 2015). The species classification in the *Klebsiella* genus was continually revised due to the development of new methods. Table 2.1 shows the three main classification

Table 2.1: Species classification of the genus *Klebsiella* (Podschum & Ullman, 1998)

	Classification by	
Cowan	**Bascomb**	**φrskov**
K.aerogenes/K. edwardsii subsp.*edwardsii* subsp.*atlantae*	*K.aerogenes/oxytoca /edwardsii* *K. pneumoniae* sensu stricto	*K. pneumoniae* subsp. *pneumoniae* subsp. ozaenae subsp. *rhinoscleromatis*
K. pneumoniae *K. ozaenae*	sensu lato *K.ozaenae*	*K. oxytoca* *K. terrigena* *K.planticola(Syn.K.trevisanii)*
K. rhinoscleromatis	*K. rhinoscleromatis* K."unnamed group" Enterobacter aerogenes	*K. ornithinolytica*

2.2 Epidemiology of ESBL and carbapenemase producing *K. pneumoniae*

K. pneumoniae is ubiquitous in nature and general habitat associations of *K. pneumonia* are humans, animals, sewage polluted water, soils and vegetation (Bagley , 2015 ;Struve & Krogfelt, 2004). *K. pneumoniae* may be regarded as normal flora in many parts of the colon and intestinal tract (Shahab , 2018). *K. pneumoniae* is an important opportunistic pathogen, a leading cause of community acquired and a frequent cause of nosocomial infections (Struve & Krogfelt, 2004). *K. pneumoniae* cause a range of infections in hospitalized patients most commonly pneumonia, wound, soft tissue, urinary tract infections (Holt *et al.*, 2015).

K. pneumoniae cause community acquired meningitidis, brain abscesses and bloodstream infections and new type of community acquired (Yu *et al*., 2007). *K. pneumoniae* associated with pyogenic liver abscess (PLA) and metastatic complications has emerged globally especially in Asia (Hsu *et al.,* 2011).

K. pneumoniae has been identified as one of the most frequent causes of outbreak in neonatal intensive care units (Fatima *et al*., 2015) and is a principal cause of outbreak infection in hospital. Epidemiological investigation revealed that in the most common outbreaks are caused by the acquisition of the multiresistant *K. pneumoniae* strain (Hellen *et al.,* 2015). In healthcare settings, *K. pneumoniae* can spread through person-to-person contact (from patient to patient via the contaminated hands of healthcare personnel, or other persons) (CDC, 2012). *K. pneumoniae* is frequently prevalent as an infectious agent of patients with indwelling urinary catheters, ventilators (breathing machines) or wounds (CDC, 2012; Clegg &Murphy, 2016). *K. pneumoniae* is one of the top three bacteria of international concern according to World Health Organization report on the global status of antibacterial resistance (WHO, 2014).

The epidemiology of *K. pneumoniae* producing KPCs varies geographically. The endemic spread of these bacteria has been reported in the USA, China, Italy, Poland, Greece, Israel, Brazil, Argentina, Colombia, and Taiwan (Munoz-Price *et al.,* 2013.). Sporadic spread of KPC-producing *K. pneumoniae* has also been observed in many European countries including Spain, France, Germany, the Netherlands, the United Kingdom, Ireland, Belgium, Sweden, and Finland, and in several countries in the Asia-Pacific region, including India, South Korea, and Australia (Munoz-Price *et al.,* 2013; Nordmann & Poirel, 2014). In the USA, the transmission of CR *K. pneumoniae* is primarily driven by the spread of organisms carrying KPC enzymes (Kaiser *et al.,* 2013), but other carbapenemase enzymes, such as the New-Delhi metallo-β-lactamase (NDM), have also emerged (Lascols *et al.,* 2013).

In the Asia-Pacific region, the endemic dissemination of KPC-producing *K. pneumoniae* has been reported in China (Li *et al.,* 2014) and Taiwan (Tseng *et al.,* 2015), and the sporadic spread has been reported in India (Shanmugam *et al.,* 2013), South Korea (Yoo *et al.,* 2013), and Australia (Partridge *et al.,* 2015).

Very little data on carbapenem resistant *Enterobacteriaceae* are available from low income countries, especially in sub-Saharan Africa (WHO, 2014).

There is scanty information about *K. pneumoniae* carbapenem resistant prevalence in East African region. The prevalence has been reported in previous studies in Tanzanie, Uganda and Kenya (Ampaire *et al.*, 2014; Apondi *et al.*, 2016; Henson *et al.*,2017;Mushi *et al.*, 2014; Okoche *et al.*, 2015;Poirel *et al.*,2011). Acquired resistance to third generation cephalosporins is mainly due to extended spectrum β-lactamases (Bradford, 2001), which have spread globally since the first identification of an ESBL gene in Germany in 1982 (Knothe *et al.*, 1983). In USA, of 820,017 isolates tested, 56,718(6.9 %) were ESBL positive (Hoffman-Roberts *et al.*, (2016). In Europe, the rates of ESBL producers differ significantly for both *E. coli* and *K. pneumoniae* depending on the regions, with very low rates observed in Northern European countries and much higher rates seen in Eastern and Southern European countries (ECDC,2014; Jones *et al.*, 2014).

In Africa, ESBL rate in different countries have increased from 11.7 to 77.8% among *K. pneumoniae* (Storberg, 2014; Tansarli *et al.*, 2014). In Algeria, Messai reported 19.9 % of *K. pneumoniae* isolates were ESBL positive (Messai et al., 2008). In South Africa, of the 159 resistant Gram negative, 31 (19.5 %) were ESBL producers, of which 9 (29.3 %) were ESBL *K. pneumoniae* (Founou *et al.*, 2019). In Uganda, Kateregga reported *et al*. (2015) reported 28.7 % of ESBL in *K*. pneumoniae from clinical samples. In Kenya, Juma *et al.* (2016) reported 6% of isolates (*Klebsiella* spp and *E. coli*) were ESBL positive. At Kilifi county hospital, ESBL carrying isolates were observed in 79 % of hospital acquired infections and 23 % of community acquired infections and 10 % among Neonates (Henson *et al*., 2017; Kagia *et al*., 2019).

2.3. Virulence determinants of *Klebsiella pneumoniae*

K. pneumoniae utilizes a variety of virulence factors, especially capsule polysaccharide, lipopolysaccharide, fimbriae, siderophores and nitrogen source utilization for survival and immune evasion during infection (Aljanaby & Alhasani, 2016; Bei *et al*., 2014).

2.3.1 Capsular polysaccharides

Capsule, a polysaccharide matrix that coats the cell, is necessary for *K. pneumoniae* virulence (Paczosa & mecsas, 2016). The capsule is the most important virulence factors and it is used in evading the immune system during infection, by protecting bacteria from opsonophagocytosis (Rebekah & Bachman, 2018). Bacterial capsular polysaccharides are major virulence factors that confer protective effects to their bearers against a wide range of environmental pressures, most notably against the immune system during infection of their animal host. Capsular polysaccharides are attached to the outer membrane (Cress *et al.,* 2014).

Bacterial capsules may mimic human cell surfaces and thus evade the immune response of the host immune system (Cress *et al.,* 2014). The capsular repeating subunits, consisting of four to six sugars and, very often, uronic acids (as negatively charged components), can be classified into 78 capsule types (Ho *et al.,* 2011 ; Rebekah & Bachman, 2018). The presence of a thick capsule at cell surface protects *K. pneumoniae* from opsonization and phagocytosis by macrophages, neutrophils epithelial cells and dendritic cells by blocking binding and internalization and prevents killing of the bacteria by bactericidal serum factors (Bei *et al*., 2014).

While *Klebsiella* CPS were generally considered to mediate virulence properties; this consideration has recently been abandoned because of the great differences in virulence observed among different capsular types: strains expressing the capsule antigens K1 and K2 were found to be especially virulent of *K. pneumoniae* (Feizabadi *et al.,* 2013). The K2 serotype is among the most common capsule types isolated from patients with UTI, pneumonia, or bacteremia (Podschun & Ullman, 1998).

2. 3.2 Adhesins or colonization factors

Adhesins are bacterial surface structure structures that enable attachment to host membrane (Paczosa & mecsas, 2016). The ability of bacterial species to colonize and infect host organisms is dependent on their capacity to adhere to cellular surface.

Adhesion is required for the release of toxins and virulence factors that drive infection (Stones & Krachler, 2015).

They are demonstrated mainly on the basis of their ability to agglutinate erythrocytes of different animal species (Nakkash & Al-Husseiny, 2008). At least four types of fimbriae, namely type 1 fimbriae, type 3 fimbriae, Kpc fimbriae and KPF-28 adhesin have been characterized experimentally for *K. pneumoniae*. (Bei *et al*., 2014). Once bacteria enter the body of the host, they must adhere to cells of tissue surface, adherence which is only step in the infections process is followed by development of micro colonies and subsequent steps in the pathogenesis of infection; the adhesive properties in the *Enterobacteriaceae* are generally mediated by different types of pili (Podschum & Ullman, 1998)**.**

2.3.2.1 Type 1 fimbriae or mannose-sensitive hemagglutination

Type 1 pili are thought to aid virulence through their ability to adhere to human mucosal or epithelial cell surfaces (Rebekah & Bachman, 2018). Type 1 fimbriae are one of the best characterized fimbrial adhesins. These fimbriae are encoded on a gene cluste*r (fim*) containing all the genes required for the fimbrial structure and assembly Fim A and Fim H confers adherence to mannose containing glycoconjugates on host cells (Murphy *et al*., 2013).

K. pneumoniae type 1 fimbriae are essential for the initial establishment of urinary tract infection, but have no effect on the ability of *K. pneumoniae* to colonize the intestine or to infect the lung (Bei *et al*., 2014). The mannose sensitive type 1 pili is composed by the major fimbriae FimA subunit and the minor tip adhesion FimH (Villegas *et al.*, 2016)

2.3.2.2 Type 3 fimbriae or mannose-resistant *Klebsiella* hemagglutination

Strains of *K. pneumoniae* expressing type 3 pili adhere to endothelial cells, epithelia of the respiratory tract, and uroepithelial cells (Podschun & Ullman, 1998) but importantly have identified as strong promotors of biofilm formation (Rebekah & Bachman, 2018).

Type 3 pili constitute expression the main *K. pneumoniae* adhesive factor, facilitating adherence and biofilm formation on abiotic surfaces strains of different origin (Di Martino *et al.,* 2003). The nucleotide sequences of six genes involved in the expression of type 3 pili of *K. pneumoniae* have been identified: mrkA, mrkB, mrkC, mrkD, mrk E and mrkF (Clegg & Murphy, 2016) . MrkA gene encodes the major fimbrial subunit; mrkB and mrkC are membrane-associated genes; mrkD gene encodes *Klebsiella*-like hemagglutinin-specific adhesin activity while mrkE genes appear involved in the regulation of type 3 fimbrial expression. MrkF gene product is required to maintain the stability of type 3 pili on the bacterial surface via qualitative measurements (Chen *et al.,* 2007).

The mannose resistant type 3 pili is composed by the major pilus subunit MrkA and the minor tip adhesion MrkD (Alcántar-Curiel *et al.,* 2013). Type 3 fimbriae, encoded by the mrk gene cluster, are produced by the majority of *K. pneumoniae* strains and are involved in biofilm-associated infections (Wu *et al*., 2010). The type 3 fimbriae are characterized by their ability to agglutinate erythrocytes treated by tannic acid in vitro, and this phenotype has been referred to as the mannose -resistant Klebsiella-like hemmagglutination (mR/K) reaction (Murphy *et al.*, 2013)

2.3.2.3 Kpc fimbriae and KPF-28 adhesin

The Kpc fimbriae may contribute to *K. pneumoniae* biofilm formation (Bei *et al*., 2014). KPF-28 is a fimbrial adhesive factor that is found in most of the *K. pneumoniae* strains producing the Sulfhydryl variable-4 (SHV-4) (Di Martino *et al.,* 1997). KPF-28 major subunit structural gene has been located on a 185-kb R plasmid encoding the SHV-4 β-lactamase; however, the plasmid has been shown to be not sufficient for expression of the KPF-28 fimbrial structure (Di Martino *et al.*, 1997). The KPF-28 fimbriae contribute to the adhesion of *K. pneumoniae* to human intestinal cell lines, indicating that the fimbriae may be a colonization factor within the mammalian intestine (Bei *et al.*, 2014).

2.3.3 Lipopolysaccharides

Bacterial lipopolysaccharides (LPS) are the major outer surface membrane components in *K. pneumoniae* (Alexander & Rietschel, 2001*;* Paczosa & mecsas, 2016). The lipopolysaccharides, also called endotoxins, is recognized as the most powerful mediator of septic shock caused by bacteria host sensing of lipopolysaccharides Via Toll-Like receptor 4(TLR4) leads to an inflammatory (Rebekah & Bachman, 2018). Due to their endotoxic properties, LPS are considered important in the pathology of septicemia (Bei *et al*., 2014). They are able to activate complement, which causes selective deposition of C3b onto lipopolysaccharides molecules at sites distant from the bacterial cell membrane. This inhibits the formation of the membrane damage and bacterial cell death (Shahab , 2018). The lipopolysaccharides of *K. pneumoniae* are composed of three structural domains: (i) the lipid A, a major component of the outer leaflet; (ii) the core oligosaccharide, which is linked to lipid A and to O antigen; and (iii) the hydrophilic O antigen polysaccharide chain (O chain) (Hsieh *et al.*, 2012)

2.3.3.1 O- antigens

The O antigen, consisting of many repeats of an oligosaccharide unit, is part of the lipopolysaccharide in the outer membrane of Gram negative bacteria (Wang *et al.*, 2010). At least nine O antigen groups (O1, O2, O2ac, O3, O4, O5, O7, O8 and O12) have been recognized in *K. pneumoniae* and O1 is the most common serotype among clinical *K. pneumoniae* isolates (Hsieh *et al.*, 2012). *K. pneumoniae* O antigens prevents access of complement components to activators (porins and rough lipopolysaccharides) and thus contributes to bacterial resistance against complement-mediated killing (Bei *et al*., 2014). The small number of different *Klebsiella* O-types is a great advantage with respect to their applicability as vaccines. Thus, each *Klebsiella* vaccine composed of O antigens has to be rendered safe by sufficient detoxification of the lipopolysaccharides (Podschun & Ullman, 1998).

K .pneumoniae isolates with shortened or absent O antigens (rough lipopolysaccharides) are sensitive to serum complement, while isolates with full

length O-antigen (smooth lipopolysaccharides) are resistant to serum complement (Rebekah & Bachman, 2018).

2.3.3.2 Lipid A

Lipid A is a glucosamine based phospholipid that makes the outer membrane of the outer membrane of Gram negative bacteria (Raetz & Whitfield, 2002). *K. pneumoniae* lipid A modification contributes to resistance to host innate defenses (Bei *et al*., 2014). The modification of lipid A helps pathogens to survive in the hostile host by camouflaging the pathogen from host immune detection, promoting antimicrobial peptide resistance and by altering outer membrane properties (Llobet *et al.,* 2015)

2.3.3.3 Core

The core region of LPS, which links the O antigen onto the lipid A molecule, contains a small number of mono-, di- or oligosaccharides (including two to three 3-deoxy-D-manno-octulosonic acid) (Min & Xuefeng, 2015). Two types (type 1 and type 2) of core polysaccharide have been characterized for *K. pneumoniae*. (Bei *et al*., 2014).

2.3.4 Siderophores or iron acquisition

Iron is a limited resource that is required by *K .pneumoniae* and must be acquired from environment to thrive during infection (Paczosa & mecsas, 2016). Siderophores are organic compounds with low molecular produced by microorganisms under low iron conditions (Ahmed & Holmstrom, 2004). Iron is essential for bacterial growth both in vitro and in vivo (Bei *et al*., 2014). It plays a key role as a cofactor for the electron transport chain and for various other enzymes. However, in aerobic conditions and mammalian tissues, the majority of iron is found as Fe3+; iron entirely sequestered by transferrin and lactoferrin, to grow successfully in host tissues, bacteria must be able to obtain iron from these host transport proteins (Al-Muhanna *et al.,* 2014).

The growth of bacteria in host tissue is limited not only by the host defense mechanisms but also by its supply of available iron. Iron is an essential factor in bacterial growth, functioning mainly as a redox catalyst in proteins participating in oxygen and electron transport processes (Podschun & Ullman, 1998). The supply of free iron available to bacteria in the host milieu is extremely low, since this element is bound intracellularly to proteins such as hemoglobin, ferritin, hemosiderin, and myoglobin and extracellularly to high-affinity iron binding proteins such as lactoferrin and transferrin (Bei *et al*., 2014; Podschun & Ullman, 1998).

The siderophores secreted by *K. pneumoniae* contributes to the inflammation and bacterial dissemination during pneumonia (Holden *et al.,* 2016). The siderophores secreted by *K. pneumoniae* during lung infection induce stabilization of the transcription factor HIF-1α, increase the expression of proinflammatory cytokines in the lung, and promote dissemination of *K. pneumoniae* to the spleen (Behnsen & Raffatellu, 2016; Holden *et al.,* 2016). *K. pneumonia* makes three siderophores called enterobactin (Ent), aerobactin and yersiniabactin (Ybt) (Lawlor *et al.,* 2007). Enterobactin (also known as enterochelin) is a catecholate siderophore produced by *K. pneumonia* and has greater affinity for iron than host molecules, such as transferrin and lactoferrin (Behnsen & Raffatellu, 2016) . To combat this, the host has evolved to produce lipocalin-2 (also known as neutrophil gelatinase associated lipocalin, siderocalin, and 24p3), an antimicrobial protein that binds to iron laden enterobactin, thereby preventing its reuptake by bacteria (Behnsen & Raffatellu, 2016), (Rebekah & Bachman, 2018).

K. pneumoniae produce a prototypical siderophore called aerobactin, which has the highest iron affinity compared with any other known iron chelators. Mammals have evolved to secrete lipocalin 2 in order to sequester enterobactin, leading to the blocking of siderophore based iron acquisition as a part of their innate immune response (Bei *et al*., 2014). Lawlor *et al*., (2007) reported that *K. pneumoniae* yersiniabactin production is increased during pulmonary infection while during in vitro iron-limiting growth conditions. Enterobactin is produced at higher levels when compared to yersiniabactin (Lawlor *et al*., 2007)

2.3.5 Others: Nitrogen source utilization

2.3.5.1 Urease enzyme

Urease is a virulence factor found in various pathogen bacteria. It is essential in colonization of a host organism and in maintenance of bacterial cells in tissues. Urease is capable of urea hydrolysis (Konieczna et al., 2012). *K. pneumoniae* can produce urease as a putative virulence factor for isolates that cause urinary tract infections (Min & Xuefeng, 2015) and produce cytoplasmic urease in order to hydrolyze urea to ammonia and carbon dioxide as a source of nitrogen for growth (Bei *et al*., 2014).

2.3.5.2 Allantoin metabolism

Allantoin has been identified as a source of nitrogen in various bacterial species and as both a nitrogen source and a carbone source in *K. pneumoniae.* An allantoin utilization operon has been associated with hypervirulent *K. pneumoniae* strains that cause pyogenic liver abscess (Rebekah & Bachman, 2018). A 22-kb chromosomal all gene locus responsible for allantoin metabolism may play a role in *K. pneumoniae* liver infection and serve as a marker for rapid identification (Chou *et al.*, 2004). Pyogenic liver abscesses caused by hypervirulent *K. pneumoniae* frequently occur in diabetes mellitus patients with an increased allantoin concentration (Bei *et al*., 2014). Figure 2.1 shows the *Klebsiella* pathogenicity factors.

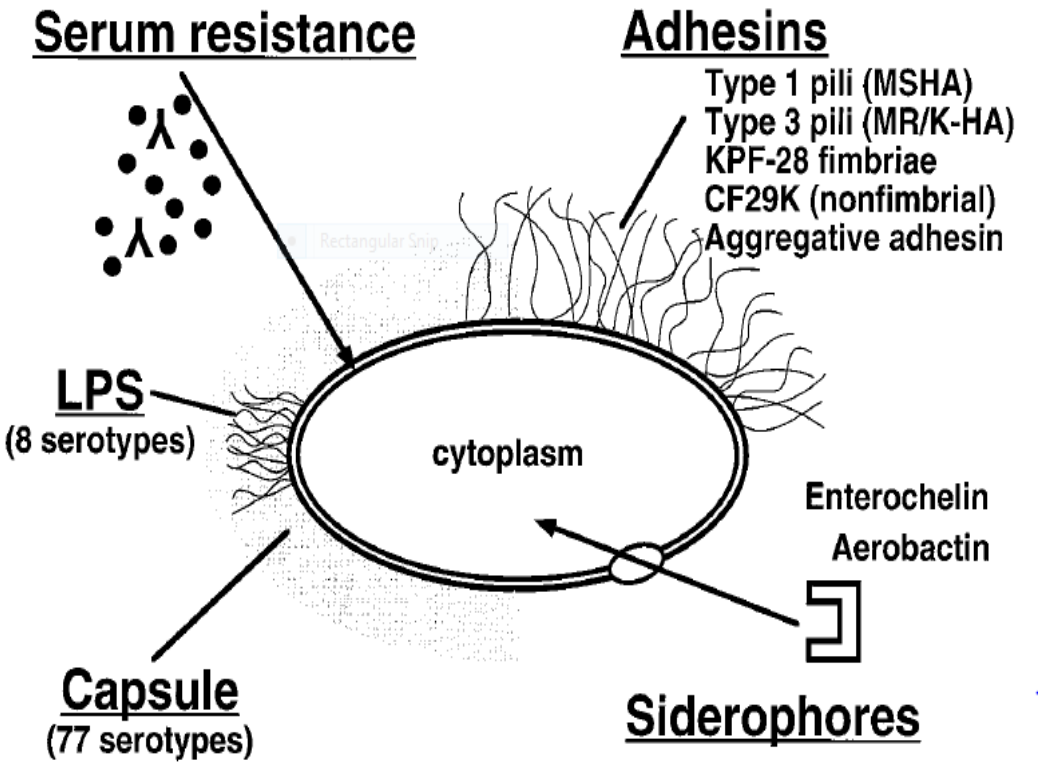

Figure 2.1: Schematic representation of *Klebsiella* pathogenicity factors

(Podschum & Ullman, 1998)

2.4. Mechanisms of antibiotic resistance

Bacteria resist to antibiotics by using the following genetic strategies: a) producing destructive enzymes to neutralize antibiotics, b) modifying antimicrobial targets by mutation that drugs cannot recognize them, c) removing antimicrobial agents by pumping them out (efflux), d) preventing antibiotics from entering by creating a biofilm or otherwise reducing permeability, e) creating by passes that allow bacteria to function without the enzymes targeted by antibiotics (Hellen *et al*., 2015 ; Munita & Arias, 2016)

2.4.1 Beta lactamase enzymes

K. pneumonia is known to secrete a variety of β lactamases, which inactivate the majority of β lactam antibiotics (Satish *et al*., 2016). The Ambler scheme classifies β-lactamases into four classes according to the protein homology of enzymes. β lactamases of classes A, C and D are serine and class B enzyme are metallo β-lactamases (Shaikh *et al.*, 2015; Singh-Moodley & Perovic, 2016); each of which is also divided into several different subgroups (Öztürk *et al*., 2015). Two classification schemes for β lactamases are currently in use.

The most used classification scheme for β lactamases is the Ambler structural which is based on the amino acid sequence and divides β lactamases into 4 class: the classes A, C, and D of serine β lactamases and the class B of metallo-β-lactamases (Silveira *et al.*, 2018). Three major groups of enzymes are defined by their substrate and inhibitors profiles: group 1 cephalosporinases are not well inhibited by clavulanic acid; group 2 penicillinases, cephalosporinases, and broad spectrum β lactamases are not inhibited by active site directed β lactamases inhibitors; group 3 metallo β lactamases that hydrolyze penicillins, cephalosporins and carbapenems and that are not inhibited by almost all β lactam containing molecule. This classification scheme is of more relevance to physicians or microbiologists in diagnostic laboratory because it considers β-lactamase inhibitor and β-lactam substrates that are clinically relevant

2.4.1.1 Extended spectrum β-lactamases

The emergence of extended spectrum β lactamases (ESBL) producing *K. pneumonia* is becoming a critical concern as it increases the bacterium's resistance to therapy. ESBLs are composed of three major genetic groups: Temoniera (TEM), Sulfhydryl variable (SHV) and Cefotaxime (CTX-M types). Nosocomial infections due to TEM and SHV-producing *K. pneumoniae* strains have been frequently documented as important ESBL producers (Zhang *et al.*, 2016). The spread of ESBL genes is associated with various mobile genetic elements, such as transposons, insertion sequences and integrons. The most common enzyme contributing to antibiotic drug

resistance in *K. pneumoniae* is an SHV-type β-lactamase (Min & Xuefeng, 2015). ESBL are still associated with major outbreaks of β lactam resistance (Bush, 2010). ESBL (class A) are so named due to their ability to hydrolyze a wide spectrum of β-lactam drugs. ESBL are β lactamases capable of conferring bacterial resistance to the penicillins, first, second and third generation cephalosporin's and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics (Gupta et *al*., 2003), and which are inhibited by β-lactamase inhibitors such as clavulanic acid, tazobactam or sulbactam (Shaikh *et al.*, 2015).

ESBL are often encoded by genes located on large plasmids and these also carry genes for resistance to other antimicrobial agents (Deepthi & Deepti , 2010).

The SHV-1 β lactamases is responsible for up to 20% of the plasmid mediated ampicillin resistance in *K. pneumoniae* (Clarivet *et al.*, 2016 ; Shaikh *et al.*, 2015). The CTX-M producing isolates were frequently resistant to cefotaxime and ceftriaxone (Bush, 2010). TEM-1 is capable of hydrolyzing penicillins and first generation cephalosporins but is unable to attack the oxyimino cephalosporins and the first TEM variant with increased activity against extended spectrum cephalosporins was TEM-3 (Shaikh *et al.*, 2015).

2.4.1.2 AmpC β- lactamases

AmpC β-lactamases are cephalosporinases that confer resistance to a wide variety of beta lactam drugs, including α methoxy-β-lactams, such as cefoxitin, broad spectrum cephalosporins and aztreonam (Coudron *et al.*, 2000). Class C cephalosporinases can abolish the antibacterial activity of cephalosporins and can also demonstrate inactivating capabilities toward beta lactams (Bush, 2010). AmpC β lactamase hydrolyze narrow broad and expand spectrum cephalosporins and cephamycins and resist inhibition by clavulanate, sulbactam and tazobactam (Thomson, 2010). Genes for the production of AmpC can exist on bacterial chromosomes or plasmids. AmpC genes on bacterial chromosome produce low levels "re-pressed" but can become "de-repressed" by induction by antibacterial such as cefoxitin (Marsik &Nambiar, 2011).

2.4.1.3 Carbapenemases enzymes

The most representative carbapenemase is classified in three classes: i) class A (Klebsiella pneumoniae carbapenemase (KPC);ii) class B: Imipenemase (IMP), Verona Integron encoded Metallo β lactamase (VIM) and New Delhi Metallo β Lactamase (NDM) ;iii) class D oxacillinase (OXA-48) (Bakthavatchalam *et al.*, 2016 ; Stoesser *et al.*, 2017). Class A, B and D are serine based enzymes; however class B is metallo-β-lactamase that relies on a water molecule coordinated to a divalent cation (Zn^{2+}) to activate and break the β-lactam ring (Lee *et al.*, 2015).

In 2001, the first KPC-producing *K pneumonia* isolate was reported in North Carolina (Arnold *et al.*, 2011). The production of carbapenemases is the most important mechanism of enzymatic resistance in *K. pneumoniae* (Bina *et al.*, 2015).

K. pneumoniae carbapenemases (KPC) inactivate all β lactam antibiotics whereas MBL are able to hydrolyze all β lactam except tazobactam and are not inhibited by aforementioned inhibitors (Meletis , 2016). Oxacillinase (class D) has the ability to hydrolyze oxacillin much faster than benzylpenicillin (Antunes & Fisher, 2014). Ambler structural based on the amino acid was indicated in Figure 2.1. The functional classification scheme updated is based on the proposal by Bush *et al.* (1995) was indicated in Table 2.2

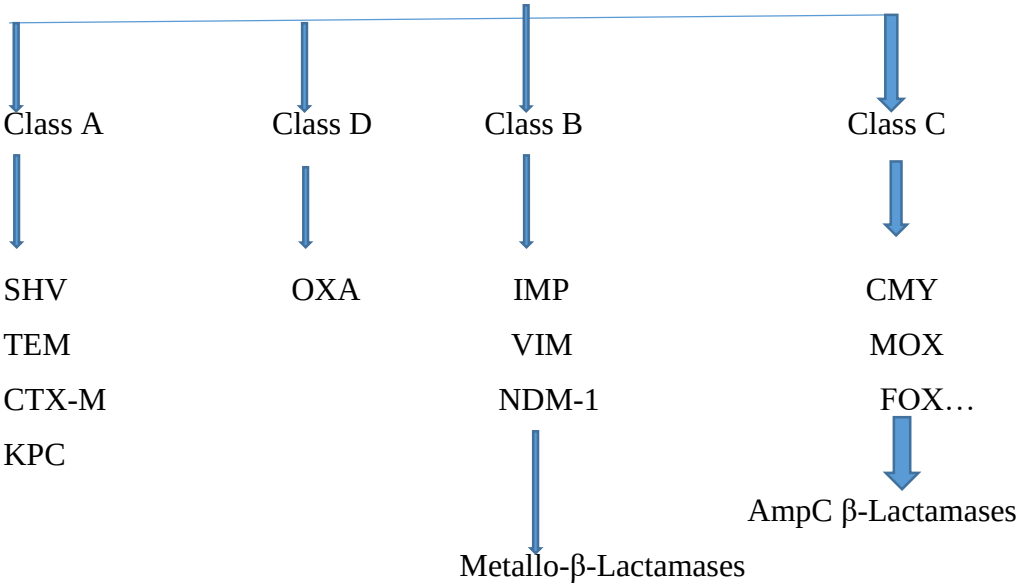

Figure 2.2: Molecular classification of β- lactamases

Table 2.2: Classification schemes of β- lactamases (Bush *et al*., 1995)

Bush-Jacoby group(2009)	Molecular class (Subclass)	Distinctive Substrate(s)	Defining Characteristics	Representative enzyme(s)
1	C	Cephalosporins	greater hydolysis of cephalosporins than benzylpenicillin;hydrolyzes cephamycins	E.coli AmpC,P99 ACT-1,CMY-2, FOX-1,MIR-1
1e	C	Cephalosporins	Increased hydrolysis of Ceftazidime and often other oxyimino-β-lactams	GC1,CMY-37
2a	A	Penicillins	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	A	Penicillins,early cephalosporins	similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1,TEM-2 SHV-1
2be	A	Extend spectrum Cephalosporins, monobactams	Increased hydrolysis of Oxyimino-β-lactams(cefotaxime,ceftazidime ceftriaxone,cefepime,aztreonam)	TEM-3,SHV-2 CTX-M-15,PER-1 VEB-1
2br	A	Penicillins	Resistance to clavulanic acid Sulbactam and tazobactam	TEM-30,SHV-10
2ber	A	Extend spectrum Cephalosporins, monobactams	Increased hydrolysis of Oxyimino-β-lactams combined with resistance to clavulanic acid,sulbactam and Tazobactam	TEM-50
2c	A	carbenicillin	Increased hydrolysis of carbenicillin	PSE-1,CARB-3
2ce	A	Carbenicillin cefepime	Increased hydrolysis of carbenicillin cefepime and cefpirome	RTG-4
2d	D	Cloxacillin	Increased hydrolysis of cloxacillin and oxacillin	OXA-1,OXA-10
2de	D	Extended Spectrum cephalosporins	Increased hydrolysis of cloxacillin and oxacillin	OXA-11,OXA-15
2df	D	Carbapenems	Increased hydrolysis of cloxacillin oxacillin and carbapenems	OXA-23,OXA-48
2e	A	Extended spectrum cephalosporins	Hydrolyzes cephalosporins.Inhibited by calvulanic acid but not aztreonam	CepA
2f	A	Carbapenems	Increased hydrolysis of carbapenems Oxymino-β-lactams,cephamycins	KPC-2,IMI-1, SME-1
3a	B(B1) B(B3)	Carbapenems	Broad spectrum hydrolysis including carbapenems but not monobactams	IMP-1,VIM-1 CcrA,IND-1 L1,CAU-1,GOB-1 FEZ-1
3b	B(B2)	Carbapenems	preferential hydrolysis of carbapems	CphA,Sfh-1

2.4.2 Efflux pumps

Bacterial efflux pumps are an important source of multidrug and export antibiotics from the cell, increasing their resistance (Wen *et al.*, 2018). Efflux pump system in *K. pneumoniae* include AcrB and mdtK systems and play a major role as a mechanism of antimicrobial resistance (Wasfi *et al.*, 2016).

K. pneumoniae expresses the efflux pump AcrAB, which contributes to the export of not only antibiotics (e.g., quinolones and β-lactams), but also host derived antimicrobial agents (e.g., the antimicrobial agents present in human (Webber & Piddock, 2003). The inactivation of AcrAB not only leads to a multidrug resistance phenotype, but also to a reduced capacity to cause pneumonia in a murine model; the expression of another *K. pneumoniae* efflux pump, namely EefABC, is not related to any antibiotic resistance phenotype, but it confers acid tolerance in vitro and high competition potential in the host gastro intestinal tract (Bei *et al*., 2014)

2.4.3 Biofilms

Biofilms are complex communities of microorganisms attached to a surface or interface enclosed in an exopolysaccharide matrix of microbial and host origin to produce a spatially organized three dimensional structures (Maldonado *et al*., 2007). Biofilm formation is a complex process that has been divided into a number of stages involving attachment, micro colony production, mature biofilm and release of free planktonic bacteria from the biofilm (Clegg & Murphy, 2016). *K. pneumoniae* remains one of the major biofilm forming nosocomial pathogens and grow attached on the surface of such devices and infected tissues persisting for long periods of time in spite of antimicrobial therapy (Alcantar-Curiel *et al.*, 2018). Patel *et al*. (2016) reported the biofilm producing Gram negative bacilli in vascular catheters of cancer patients. Bacterial biofilms are frequently observed on tissue surfaces at the site of persistent infections, indicating that biofilms are associated with virulence and chronic infection; the presence of indwelling urinary devices leads to an accumulation in situ of host derived material on the catheter surfaces (Clegg & Murphy, 2016).

Once in the biofilm, extracellular polymeric substances protect bacteria from opsonization and phagocytosis, making eradication of bacteria extremely difficult (Min & Xuefeng, 2015). Biofilms are the crucial factors responsible for the evolution of multidrug resistant strains and pose a risk for dissemination between patients and throughout the hospital (Alcantar-Curiel *et al.,* 2018).

2. 4.4 Outer membrane proteins

The outer membrane protein (OMP) is essential for cell viability and serves as permeability barrier preventing the entry of harmful toxic (Ebbensgaard *et al.,* 2018; Kapoor *et al.,*2017). However the permeability of this barrier have a major impact on the susceptibility of the microorganism to antibiotic (Ghai & Ghai, 2018).
Multiple antibiotic-resistant mutants of *K. pneumonia* may show altered levels of outer-membrane proteins. These mutants may arise spontaneously and show resistance to a range of antimicrobials, including nalidixic acid, trimethoprim, chloramphenicol and β-lactams (Delcour, 2009). The antibiotic resistant properties of OMP can be grouped into the two fundamental functions: one is reducing OM permeability to prevent the uptake of antibiotic (Yao *et al.*, 2018)

2.4.5 The resistance associated mobile genetic elements

K. pneumoniae acquires resistance to multiple antibacterial drugs mainly through horizontal transfer of mobile genetic elements (WHO, 2014).The acquisition of spread of resistance associated genes, as well as the wider evolution of bacteria, is strongly associated with certains types of mobile genetic elements including plasmid, transposons, integrons and associated genes cassettes, prophages, integrating conjugative elements and genomic islands (Bi *et al.*, 2015)

2.4. 5.1 Plasmid mediated antibiotic resistance in *K. pneumoniae*

Numerous virulence factors as well as antibiotic resistance genes are usually part of plasmids or genetic elements located in plasmids that have the capability to

disseminate at the molecular level such as integrons or transposons (Ramirez *et al*., 2014).

Plasmids play a central role in the dissemination and acquisition of the resistant determinants in these bacteria (WHO, 2014). Plasmids harbor genes coding for specific functions including virulence factors and antibiotic resistance that permit bacteria to survive the hostile environment found in the host and resist treatment (Jiang *et al.*, 2010). *K .pneumoniae* strains harbor more than one plasmid, including small high copy and low copy number plasmids that are usually large that carry resistance and virulence genes that keep increasing its ability to resist the main antibiotics such as cephalosporins, carbapenems, penicillins, aminoglycosides and fluoroquinolones (Ramirez *et al*., 2014)

2.4.5.2 Integrons associated cassettes genes

Integrons are genetic elements that capture and integrate gene cassettes by site specific recombination and convert them to functional genes (Odumosu *et al.*, 2013). Two major groups of integrons have been described: chromosomal integrons and mobile integrons (Stalder *et al.*, 2012).

The integron is genetic elements that incorporate mobile genes termed gene cassettes into reserved genetic site specific recombination capable of integrating and expressing open reading frames (ORFs) contained in modular structures called gene cassettes (Rapa & Labbate, 2013).

The integron is defined by three components: an integrase gene (int I) that encodes a site specific recombinase, an attachment site (att I) and a promoter (Pc), which promotes the expression of any suitably integrated gene (Odumosu *et al.*, 2013 ; Partridge *et al.*, 2009). The integration of genes cassettes is facilitated by an integrase mediated recombination between att I and att C and less commonly att C and att C. The accumulation of multiple resistance gene cassettes has associated these elements with multidrug resistant (Rapa & Labbate, 2013).

2.4.5.3 Integrative and conjugative elements

Integrating conjugative elements (ICE) are mobile genetic elements that can be excised from their host's chromosome and self-transferred by conjugation to a new host to be reintegrated into its chromosome and play an important role in the incorporation and dissemination of genes encoding a variety of properties including antibiotic resistance(Carraro & Burrus,2015; Johnson & Grossman, 2015).
ICE SXT of the MPF_F type, ICE Eclc (MPF_G), $MISym^{R7A}$ (MPF_T), Tn916 and ICEBs1 (MPF_{FA}), CTn DOT (MPF_B) are characterized and these elements encode traits associated with pathogenicity, mutualism, or the spread of antibiotic resistance (Cury *et al.,* 2017). SXT element was the first discovered and encodes for resistance to a number of antibiotics, including trimethoprim-sulfamethoxazole (Zakharova & Viktorov, 2015).

2.4.5.4 Transposons

Transposons are a group of mobile genetic elements that are defined as a DNA sequence and can jump into different places of the genome; for this reason, they are called jumping genes (Babakhani & Oloomi, 2018). Two classes of transposons have been identified: class I (Retro-transposons) and class II (DNA transposons) and can be described as either "copy-paste" for class I or "cut and paste" for class II (Iyer *et al.,* 2013). When transferred between bacteria, transposons can carry antibiotic resistance genes within them and are major drivers of resistance spreading (Rubio-Cosials *et al.,* 2018).

2.4.5.5 Genomic islands

Genomic islands are large regions on the chromosome that have a base composition which is different from the whole genome, encode an integrase and are inserted at tRNA loci (Doaa , 2014)**.** Virulence and antimicrobial resistance genes are encoded on genomic islands (GIs) where they can excise from a host chromosome, transfer and integrate into a new chromosome (Juhas *et al.,* 2009).

Genomes of bacterial species can evolve through a variety of process including mutations, rearrangements or horizontal transfer (Schimidt & Hensel, 2004).

The schematic life-style of mobile genomic islands would thus consist of the following steps: i) acquisition of the genomic islands by a host through horizontal gene transfer**,** ii**)** integration of the genomic islands into the host chromosome by site specific recombination**,** iii) development of the genomic rearrangements, gene loss or acquisition of other mobile genetic elements, iv) excision of the genomic islands from the chromosome, v) transfer of the genomic islands to another recipient (Juhas *et al.,* 2009). The spread of successful multidrug resistance moble genetic elements between bacteria is the main driving force in the dissemination of acquired antibiotic resistance genes (Cloeckaert *et al.*, 2017).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was carried out at The Nairobi Hospital Laboratory (Microbiology and parasitology unit), Nairobi county, Kenya. The Nairobi Hospital is located on Argwings Kodhek Road. The Nairobi hospital has bed capacity of more than 750 and offering quality general medical services, specialist clinics and high-tech diagnostic services. This Hospital is a tertiary health care and receives referrals for specialized medical care and diagnostic from various hospitals and clinics in the region. The Nairobi Hospital is a private hospital opened on 9 April 1954, as an exclusively European Hospital, which was the colony of the United Kingdom. On October 1961, it began serving non-Europeans and the name was changed to The Nairobi Hospital (The Nairobi Hospital, 2017), (Nyanchama, 2018).

3.2 Study design

This was a cross sectional study conducted from May 2017 to April 2018.

3.3 Target population

The study included the clinical samples previously tested positive for *K .pneumoniae* with standards methods. Demographic characteristics were recorded from laboratory request forms.

3.4 Sample size determination

Sample size was determined according to Fisher *et al* (1998) formula.

$$\boldsymbol{n = \frac{Z^2(1-\alpha)pq}{e^2}}$$

Where:
n is the sample size,

Z is the abscissa of the normal curve that cuts off an area α at the tails,

(1 – α) equals the desired confidence level (e.g., 95%);

e is the desired level of precision, 0.05 in this study;

p is prevalence of a previous study 23%

= 0.23 Probability of pathogen isolation.

q is 1-p.

The value for Z is found in statistical tables which contain the area under the normal curve. e. g. Z = 1.96 for 95 % level of confidence.

The prevalence used was from a study conducted at Moi Teaching and Referral Hospital, Eldoret, Kenya which was 23 % (Apondi *et al.*,2016)

$n = (1.96)^2 0.23(1-0.23)/ (0.05)^2 = 272$

The minimum samples size of positive clinical samples for *K. pneumoniae* required was 272.

3.5. Sampling Technique

Samples were allocated numbers in addition to laboratory identification number**.** A consecutive method was used for sampling technique. Positive clinical samples for *K. pneumoniae* were recruited until the sample size was achieved.

3.6 Inclusion criteria

Non-duplicate positive clinical samples for *K. pneumoniae* from outpatients /inpatients, all ages and both sexes were analyzed.

3.7 Exclusion criteria

Stool samples tested positive for *K. pneumoniae*.

3.8 Methodology of data collection

3.8.1 Demographic characteristics collection

Demographic characteristics were obtained from laboratory request forms. Only details of age, sex, inpatients/outpatients were recorded.

3.8.2 Laboratory procedures

3.8.2.1 Isolation of *K. pneumoniae* from clinical samples

Out of 272 non duplicate *K. pneumoniae* isolates collected from various clinical samples; 171 (62.86 %) were isolated from urine, 41(15.07 %) from swab, 17 (6.25 %) from sputum, 17 (6.25 %) from blood, 11(4.04 %) from tracheal aspirate, 9(3.30 %) from Catheter tip, 3(1.10 %) from body fluid and 3(1.10 %) from bile. All positive clinical samples for *K. pneumoniae* were cultured as follows: Urine samples were cultured on Cystine Lactose Electrolyte Deficient (CLED) agar (Oxoid, UK) while other clinical samples (pus swab, sputum, blood, tracheal aspirate, catheter tip, body fluid and bile) were cultured on MacConkey agar (Oxoid, UK). The inoculum was streaked aseptically using loop over the agar surface using parallel streaking method on MacConkey agar and nutrient agar (quadrant) and Zigzag streaking method on CLED agar(quadrant) and incubated at 37 ºC for 18 hours (Batra, 2018 ;Sanders, 2012). The media preparation protocol was indicate Appendix I. Colony characteristics were noted. *K. pneumoniae* colonies are lactose fermenter, mucoid/viscosity, circular and smooth. During study period, non duplicate samples were analyzed.

3.8.2.2 Identification and antimicrobial susceptibility test of *K. pneumoniae*

Pink and yellow mucoid colonies suspect of *K. pneumoniae* were identified based on their Gram stain reaction (Sagar , 2018) and biochemical reaction characteristics using VITEK 2 system (BioMerieux, USA; version VT-R01.02). Gram stain protocol was indicated in appendix II.

Non duplicate tests were performed on *K. pneumoniae* isolates. Sterile test tubes (ID and AST test tubes) used to prepare inoculums were filled with 3ml of 0.45% saline water and placed in a cassette. The identification (ID) test tube was used to prepare inoculum from the pure colonies and mixed thoroughly using a vortex until a suspension of 0.5 – 0.63 McFarland was formed. The McFarland was determined using Densichek (BioMerieux, USA). A volume of 145µl of the inoculum from the ID test tube was pipetted into the antibiotic susceptibility testing (AST) test tube and mixed thoroughly.

The Gram negative (GN) ID test cards and AST test cards were inserted in the respective test tubes and loaded into the VITEK 2 instrument (version VT-R01.02). While in the VITEK 2 instrument, the cards were filled, sealed and incubated in the VITEK 2 system incubator until results were generated by the expert advanced system of the VITEK 2 system (BioMerieux, USA, version VT-R01.02).

The culture suspension was inoculated into GN card with the help of a vacuum device inside the filling chamber .The cards were later transferred into loading chamber, where the cards were sealed and were incubated in a rotating carousel at 37º C. Each loaded card was removed from the carousel for every 15 minutes, transported to the optical system for reaction readings and returned to the carousel incubator until the next read time (Hackman *et al.*, 2013; Simgamsetty *et al.*, 2016).

The VITEK 2 system monitors the kinetics of bacterial growth and calculate Minimum Inhibitory Concentration(MIC)Using a unique algorithm. In the current study, the card "VITEK 2 GN" was used for identification of *K .pneumoniae* based on established biochemical tests and developed substrates measuring carbon source utilization and enzymatic activities.

Antimicrobial susceptibility testing with the VITEK 2 system was performed using an AST-GN 83 Card. Antibiotics tested in AST-GN 83 card included ampicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, piperacillin/tazobactam, cefazolin, cefuroxime, cefoxitin, cefotaxime, ceftazidime, ceftriaxone, cefepime, Aztreonam, meropenem, amikacin, gentamycin, ciprofloxacin, nitrofurantoin and trimethoprim/sulfamethazole.

The VITEK 2 system automatically processes the antimicrobial susceptibility cards until MIC are obtained in accordance with the internal database of possible phenotypes for *K. pneumoniae* antimicrobial agents combinations. *E.coli* ATCC 35218 was used as quality control. The results were interpreted according to the breakpoints provided by the CLSI, 2017 (Table 3.1). The *K. pneumoniae* isolates were treated the same ways as E.coli ATCC, the antibiograms generated were used to cluster the isolates into various susceptibility profiles ranging from fully sensitive to resistant. Identification, and antibiotic susceptibility test protocol was indicated in appendix IV.

Table 3.1: MIC interpretive by Clinical Laboratory Standards Institute

Antibiotics	S	I	R
Ampicillin(AMP 10µg)	≤8	16	≥32
Amoxicillin/Clavulanic acid(A/C 20/10µg)	≤8/4	16/8	≥32/16
Ampicillin/Sulbactam(A/S 10/10 µg)	≤8/4	16/8	≥32/16
Piperacillin/Tazobactam (PT100/10µg)	≤16/4	32/4-64/4	≥128/4
Cefazolin(CFZ 30µg)	≤2	4	≥8
Cefuroxime(CFX 30µg)	≤8	16	≥32
Cefoxitin(CX 30µg)	≤8	16	≥32
Cefotaxime(CTX 30µg)	≤1	2	≤1
Ceftazidime(CAZ 30µg)	≤4	8	≥16
Ceftriaxone(CTR 30µg)	≤1	2	≥4
Cefepime(CPE 30µg)	≤2	4-8	≥16
Aztreonam(ATM 30µg)	≤4	8	≥16
Meropenem(MP 10µg)	≤1	2	≥4
Amikacin(AMK 30µg)	≤16	32	≥64
Gentamycin(CN 10µg)	≤4	8	≥16
Ciprofloxacin(CIP 5µg)	≤0.06	0.12-0.5	≥1
Nitrofurantoin(NIT 300µg)	≤32	64	≥128
Trimethoprim/Sulfamethoxazole(SXT 1.25/23.75µg)	≤2/38		≥4/76

Key: ≤: less than or equal to, ≥: greater than or equal, µg: microgram, S: Sensitive, I: intermediate, R: Resistant, MIC: minimum inhibitory concentration

3.8.2.3 ESBL, Amp C and carbapenemase screening

The detection of ESBLs, AmpC and carbapenemase production was performed on non duplicate *K. pneumoniae* isolates by VITEK 2 system (version VT-R01.02) with VITEK AST- GN83 card (BioMerieux, U.S.A). The VITEK 2 (version VT-R01.02) automated susceptibility system has introduced an ESBL test on their system whereby ceftazidime and cefotaxime are tested alone and in combination with clavulanic acid/ or on simultaneous assessment of the inhibitory effects of cefepime, cefotaxime and ceftazidime alone and in presence of clavulanic acid. Logarithmic reduction in growth within the well containing clavulanic acid compared to the well not containing clavulanic acid indicates expression of an ESBL (Spanu *et al.,* 2006).
VITEK 2 System (version VT-R01.02) analyzed the carbapenem susceptibility results to identify isolates suspected of carbapenemase production base on reduction of susceptibility to meropenem (Pasteran *et al.* , 2011). Reduced susceptibility to cefoxitin is taken as indicator of AmpC production (Li *et al.,* 2015; Polsfuss *et al.,* 2011).
The VITEK 2 system AES compares each MIC result for the tested isolate with the modal MIC distribution of *K. pneumoniae* with known resistance mechanisms included in the database .Based on this comparison, the system indicates the potential presence of β lactamases. Phenotypic ESBL, AmpC and carbapenemases producers was indicated in appendix IV. *K. pneumoniae* isolates, once identified and beta lactamases screened, were stocked in 1.5 ml capacity vials containing stocking media at -20 ^{0}C. The stocking media was prepared in a mixture of colonies grown on BHI broth (Hi Media, India) and glycerol 30 %. The preparation of glycerol 30 % and storage of *K. pneumoniae* was indicated in appendix III.

3.8.2.4 DNA extraction

The stored *K. pneumoniae* isolates were picked from the stock culture and sub cultured onto MacConkey agar plates at 37^{o} C for 24 hours for revival.

Colonies of *K. pneumoniae* from MacConkey agar plates were sub cultured onto Nutrient agar (Oxoid, UK) plates at 37^0C for 24 hours for DNA extraction. *K. pneumoniae* pellets ((1-5 *10^6 colonies) was suspend in 200 µl of DNA Elution Buffer in combination with 200 µl of Biofluid and cell buffer and 20µl of Proteinase K; and boiled for 10 minutes at 55°C using a heating block. Following lysis, equal volume of genomic binding buffer was added to the digested sample, centrifugation was then done at 15,000 rpm for 1 minute. Contaminants were removed using wash steps and DNA was eluted with an elution buffer (ZymoResearch, USA). DNA products were stocked at -20 ^{0}C for further application. Schematic representation of DNA extraction was indicated in Figure 3.1 and DNA extraction protocol in appendix V.

Thermo Scientific NanoDrop was used to quantify and assess purity of DNA. DNA concentration was performed with 2µl of DNA products. DNA concentration was estimated by measurement the absorbance at 260 nm (A260) and the number generated allowed to estimate the concentration of the solution. A sample was diluted 50 X. For a 1 cm pathlength, 50 µg /mL solution of ds DNA was the optical density at 260 nm (OD_{260}). Concentration (µg/ml) = (A260 reading x dilution factor x 50µg/ml. The DNA purity was performed by measure absorbance at 260 nm divided by reading at 280 nm (A260 nm/A280nm). The calculated ration of 1.7- 2.0 was an indicator as good quality of DNA (Barbas *et al,* 2007; Desjardins & Conklin, 2010). Concentration DNA was confirmed by gel electrophoresis. 2 µl sample of undiluted DNA loaded on the gel to visualize the DNA bands by comparing their intensity and the intensity of 100 ng standard. If the gel has the same approximate intensity as the 100 ng standard, then the solution concentration was 50ng/µl (100ng divided by 2 µl) (Desjardins & Conklin, 2010).

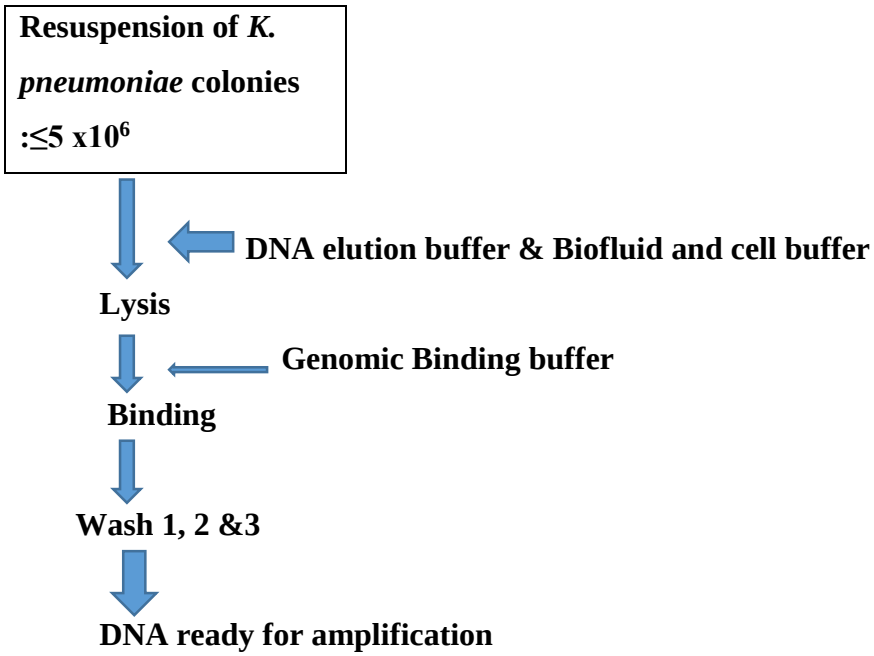

Figure 3.1: Schematic representation of DNA extraction

3.8.2.5 Polymerase chain reaction (PCR) amplification of resistance genes

PCR amplification was used to detect ESBL and carbapenemase genes using PloFlex System. Primer sequences, annealing temperature, their size for detection of genes in this study were acquired from a previous studies (Table3.2). The sequences were compared to reference sequence in the National Center for Biotechnology Information GenBank database (http:// www.ncbi.nlm.nih.gov//).

Stock solution of each primer (100 µM) was prepared according the manufacturer's instructions (BioLabs, UK). Working solution was prepared from the stock solution with sterile nuclease free water as instructed by manufacturer. Then for 10µM, I dissolved 10µl of each primer in 90µl of nuclease free water. PCR amplification was carried out in 25 µL reaction volumes containing Taq DNA polymerase Master Mix with standard buffer 12.5µl, template DNA 2.5 µl, 10 µM forward primer 0.5 µl, 10 µM reverse primer 0.5 µl and nuclease-free water to 25µl.

PCR conditions were Initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing temperatures (Table 3.2) for 50

seconds, extension at 72°C for 1 minute 30 seconds and final extension at 72 °C for 7 minutes. The PCR products were stored at 4°C.

Table 3.2: Oligonucleotides primers used for genes detection

Gene	Nucleotide sequence(5'-3')	AT	F.L	References
Bla $_{TEM}$	F TCGGGGAAATGTGCGCG R TGCTTAATCAGTGAGGCACC	54	972	Sima *et al*., 2016
Bla SHV	F GGGTTATTCTTATTTGTCGC R TTAGCGTTGCCAGTGCTC	56	928	Sima *et al.*, 2016
Bla CTX-M	F CGCTTTGCGATGTGCAG R ACCGCGATATCGTTGGT	54	557	Mousse *et al.*,2016
Bla KPC	F CATTCAAGGGCTTTCTTGCTGC R ACGACGGCATAGTCATTTGC	52	538	Mushi *et al*., 2014
Bla VIM	F GATGGTGTTTGGTCGCATA R CGAATGCGCAGCACCAG	55	390	Zowawi, *et al*., 2014
Bla IMP	F GGAATAGAGTGGCTTAATTCTC R-CCAAACCACTACGTTATC	55	189	Li *et al*, 2012
Bla OXA -48	F TTGGTGGCATCGATTATCGG R GAGCACTTCTTTTGTGATGGC	55	741	Li *et al*, 2012

Key: *Bla*: β-lactamase coding gene, AT: annealing temperature, F: forward primer, R: reverse primer, FL: fragment length.

3.8.2.6 Gel electrophoresis

Gel electrophoresis is a simple, rapid and highly sensitive method that is widely used to separate DNA/RNA molecules by size. DNA and RNA nucleic acids are negatively charged so they migrate through the gel in the direction toward the positive electrode of the electric field.

Therefore, smaller molecules move faster along the gel matrix than larger molecules and so molecules of different length are separated by size (Lee *et al.*,2012). Electrophoresis was carried out in 1.2% agarose in TAE buffer.

A volume of 5µl EZ vision in gel was added to the agarose. 8µl of the PCR product was mixed with 2µl of loading buffer and added to the wells for electrophoresis.
A volume of 5µl of molecular weight marker was loaded in the first lane of the gel to aid in the estimation of the PCR product sizes. Electrophoresis was performed at 110 V for 35 minutes. Gels were visualized under UV light and image captured using a gel imaging system (UVITEC, Cambridge). Gel electrophoresis protocol was indicated in appendix VI.

3.9 Data Management and analysis

Data was entered into computer using MS Excel. The data was stored into following formats: laboratory note book and Flash disc. The MS excel data was coded and analyzed with Statistical Package for Social Sciences (SPSS, version 21). A Chi-Square (Fichers'Exact or Pearson's Exact test where applicable) was used to assess the difference between beta lactamase producers and beta lactamase non producers with regard to antimicrobial resistance in *K. pneumoniae* isolates. In all cases, $p<0.05$ was regarded as statistically significant. Data was presented using tables and graphs.

3.10 Ethics approval

Laboratory procedures were performed in accordance to the standard operating procedures. The bacterial strains used in study were isolated from the routine clinical specimens. This study was approved by the Bioethics and Research committee of The Nairobi Hospital (Ref: TNH/ADMIN/CEO/08/12/17) as it was indicated in Appendix VII

CHAPTER FOUR

RESULTS

4.1 Proportion of *K. pneumoniae* isolates by gender, age groups and in /outpatients

Out of 272 *K. pneumoniae* isolates from various clinical samples, 54 % (147/272) were collected from females while 46 % (125/272) were collected from males. *K. pneumoniae* isolates was more collected in females as compared to males in almost age sets except at age group of 0-10 years. The proportion of *K. pneumoniae* isolates collected from outpatients (50.7 %) was slightly more than that from collected in inpatients (49.3 %) (Table 4.1).

Table 4.1: Proportion of *K. pneumoniae* isolates by their characteristics

Patients	Frequency	%	
Outpatients	138	50.7%	
Inpatients	134	49.3%	
Age groups	Frequency (%)	Gender (n, %)	
		M (125, 46.0%)	F (147, 54.0%)
0-10	32(11.8)	18(56.3)	14(43.7)
11-20	8(2.9)	2(25.0)	6(75.0)
21-30	15(5.5)	5(33.3)	10(66.7)
31-40	40(14.7)	17(42.5)	23(57.5)
41-50	55(20.2)	26(47.3)	29(52.7)
50+	122(44.9)	57(46.5)	65(53.5)

Key: M: Male, F: Female, %: percentage

4.2 Antimicrobial susceptibility patterns of *K. pneumoniae* isolates

Highest resistance rates were recorded to Trimethoprim/Sulfamethoxazole (56.6%), ceftazidime (57.7 %), cefotaxime (58.5 %), cefazolin (62.9 %) and ampicillin (100 %).

The less resistance rates were observed to amikacin (18.0%), gentamicin (21.0%), nitrofurantoin (21%), meropenem (21.7%) and ciprofloxacin (25.4%) (Table 4.2).

Table 4.2: Antimicrobial susceptibility patterns for *K. pneumoniae* isolates

Antibiotics	S (%)	I (%)	R (%)
Ampicillin(10µg)	0	0	272(100)
Amoxicillin/Clavulanic acid(20/10µg)	128(47.1)	25(9.2)	119(43.8)
Ampicillin/Sulbactam(10/10 µg)	116(42.6)	25(9.2)	131(48.2)
Piperacillin/Tazobactam(100/10µg)	110(40.4)	51(18.8)	111(40.8)
Cefazolin(30µg)	92(33.8)	9(3.3)	171(62.9)
Cefuroxime(30µg)	104(38.2)	7(2.6)	101(37.1)
Cefoxitin(30µg)	164(60.3)	7(2.6)	94(34.6)
Cefotaxime(30µg)	108(39.7)	5(1.8)	159(58.5)
Ceftazidime(30µg)	107(39.3)	8(7.0)	157(57.7)
Ceftriaxone(30µg)	107(39.3)	19(7.0)	146(53.7)
Cefepime(30µg)	112(41.2)	10(3.7)	150(55.1)
Aztreonam(30µg)	139(51.1)	18(6.6)	115(42.3)
Meropenem(10µg)	210(77.2)	3(1.1)	59(21.7)
Amikacin(30µg)	208(76.5)	15(5.5)	49(18.0)
Gentamycin(10µg)	205(75.3)	10(3.7)	57(21.0)
Ciprofloxacin(5µg)	192(70.6)	11(4.0)	69(25.4)
Nitrofurantoin(300µg)	124(45.5)	91(33.5)	57(21.0)
Trimethoprim/Sulfamethoxazole(1.25/23.75µg)	108(39.7)	10(3.7)	154(56.6)

Key: %: percentage, S: Sensitive, I: intermediate, R: Resistant, µg: microgram

4.3 Proportion of phenotypic ESBL, AmpC and carbapenemase producing *K. pneumoniae*.

The proportion of ESBL producing *K. pneumoniae* isolates was predominant (29.8%) compared to other β-lactamases. The presence of β-lactamases singly or in combination were recorded (Table 4. 3).

Table 4.3: Distribution of β- lactamases

Beta-Lactamases	Frequency	%
ESBL	81	29.8
Carbapenemases	19	7.0
ESBL+ carbapenemases	14	5.1
AmpC+ carbapenemases	5	1.8
ESBL+ carbapenemases+ AmpC	7	2.6
ESBL+Amp C	2	0.7

4.4. Comparison of resistance pattern for β-lactamase and non β-lactamase producing *K. pneumoniae*.

The antimicrobial susceptibility pattern of *K. pneumoniae* isolates revealed that the ESBL producing isolates were more resistant than the non ESBL producing K. *pneumoniae* isolates ($p \leq 0.05$). High resistance was detected with 3rd generation cephalosporins: cefotaxime (97.5%), ceftazidime (97.5%) and ceftriaxone (96.3%). Carbapenemase producing with *K. pneumoniae* showed the significant resistance to beta lactam antibiotics and to gentamicin ($p \leq 0.05$). The difference in antimicrobial resistance between coproduction and non coproduction producing isolates was significant for gentamicin ($p = 0.001$), amikacin ($p = 0.001$) and ciprofloxacin ($p = 0.001$) in *K. pneumoniae* isolates producing all the three beta lactamase enzymes (Table 4.4).

Table 4. 4: Resistance Pattern of *K. pneumoniae* producing single & mixed enzymes

AB	ESBLs Producers (n=102)	Non ESBLs Producers (n=170)	p	Carbapenemases Producers (n=41)	Non carbapenemases Producers (n=231)	p	Co-production Enzymes (n=28)	Non coproduction Enzymes (n=244)	p
A/C	40 (49.4%)	79 (41.4%)	0.001	13 (68.4%)	106 (41.9%)	0.018			
A/S	48 (59.3%)	83 (43.5)	0.003	13 (68.4%)	118 (46.6%)	0.046			
PT	48 (59.3%)	63 (33.0%)	0.001	14 (73.7%)	97 (46.6%)	0.010			
Cfz	77 (95.1%)	94 (49.2%)	0.001	17 (89.5%)	154 (60.9%)	0.024			
Cfx	75 (92.6%)	86 (45.0%)	0.001	17 (89.5%)	144 (56.9%)	0.020			
Cx	33 (40.7%)	68 (35.6%)	0.027						
Ctx	79 (97.5%)	80 (41.9%)	0.001	18 (94.7%)	141 (55.7%)	0.004			
Caz	79 (97.5%)	78 (40.8%)	0.001	18 (94.7%)	139 (54.9%)	0.003			
Ctr	78 (96.3%)	68 (35.6%)	0.001	17 (89.5%)	129 (51.0%)	0.004			
Cpe	76 (93.8%)	74 (38.7%)	0.001	17 (89.5%)	133 (52.6%)	0.004			
Azm	42 (51.9%)	73 (38.2%)	0.044	13 (68.4%)	102 (40.3%)	0.025			
Mp	7 (8.6%)	26 (13.6%)		17 (89.5%)	42 (16.6%)	0.001			
Sxt	49 (60.5%)	105(55.0%)	0.042						
CN				7 (36.8%)	50(19.8%)	0.039	24(85.7%)	33(13.5%)	0.001
Amk							22(78.6%)	27(11.1%)	0.001
Cip							24(85.7%)	45(18.4%)	0.001

Key: AB: antibiotic, A/C: ampicillin /clavulanic acid, A/S: ampicillin/sulbactam, PT: piperacillin/tazobactam, Cfz: cefazolin, Cfx: cefuroxime, Cx: cefoxitin, Ctx: cefotaxime, Caz: ceftazidime, Ctr: ceftriaxone, Cpe: cefepime, Azm: aztreonam, Mp: meropenem, Sxt: trimethoprim/sulfamethoxazole, CN: gentamicin, Amk: amikacin, Cip: ciprofloxacin, ESBL: Extended Spectrum β-Lactamase, p: p value, %: percentage

4.5 Distribution of resistance genes in ESBL producers

The ESBL genes including *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{OXA-48}$ were detected (Fig 4.2, Fig 4.3, Fig 4.4, Fig 4.5). There was a high proportion of *bla*$_{TEM}$/*bla*$_{SHV}$/*bla*$_{CTX-M}$ among the ESBL producers *K. pneumoniae* isolates (52.88 %) (Figures 4.5).

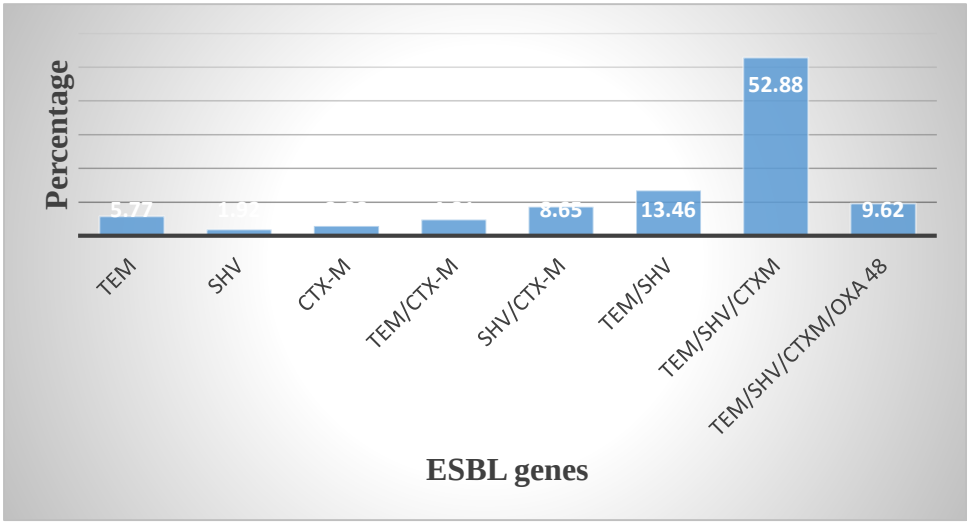

Figure 4.1: Resistance genes *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{OXA-48}$

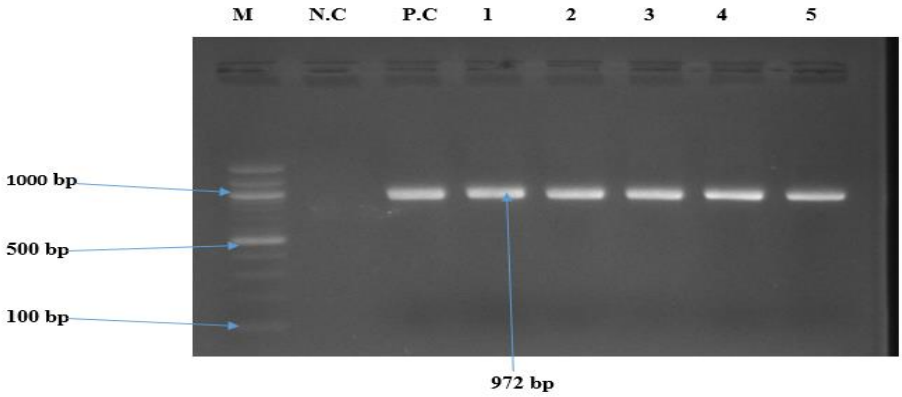

Figure 4.2: Agarose gel electrophoresis of *bla$_{TEM}$* genes

Lane M: Ladder (100-1500bp), N.C: Negative control (*E.coli* ATCC 35218), P.C: Positive control (*K. pneumoniae* ATCC 700603), Lanes 1-5: The 972 bp by PCR products of *bla$_{TEM,}$* bp: base pairs.

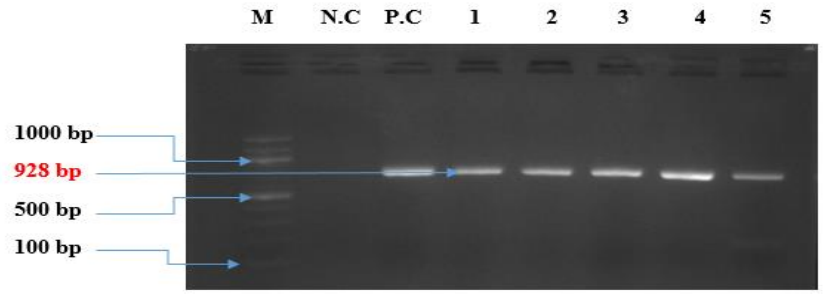

Figure 4.3: Agarose gel electrophoresis *of bla*$_{SHV}$ genes

Lane M: Ladder (100-1500bp), N.C: Negative control (*E.coli* ATCC 35218), P.C: Positive control (*K. pneumoniae* ATCC 700603), Lanes 1-5: The 928 bp by PCR products of *bla$_{SHV,}$ bp:* base pairs.

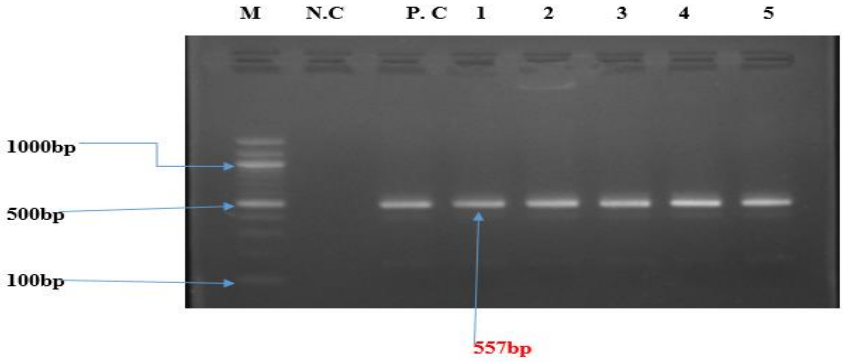

Figure 4.4: **Agarose gel electrophoresis of *bla*$_{CTX-M}$ genes**

Lane M: Ladder (100-1500bp), N.C: Negative control (*E.coli* ATCC 35218), P.C: Positive control (*K. pneumoniae* ATCC 700603), Lanes 1-5: The 557 bp by PCR product of *bla*$_{CTX-M}$, *bp:* base pairs.

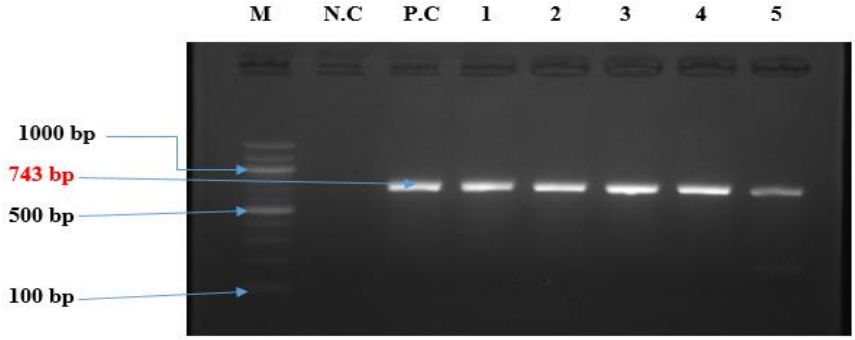

Figure 4.5: Agarose gel electrophoresis of *bla*$_{OXA-48}$ genes

Lane M: Ladder (100-1500bp), N.C: Negative control (*E.coli* 35218), P.C: Positive control (*K. pneumoniae* ATCC 13883), Lanes 1-5. The 743 bp by PCR product of *bla*$_{OXA-48}$, bp: base pairs.

4.6 Distribution of resistance genes in carbapenemase producers

Bla$_{OXA-48}$ and bla$_{KPC}$ (Fig 4.5) and (Fig 4.7) genes were detected with a high proportion of *bla$_{OXA-48}$* (62.2 %) among carbapenemase producers *K. pneumoniae* isolates. *Bla$_{VIM}$* and *bla$_{IMP}$* genes were not detected (Fig 4.6).

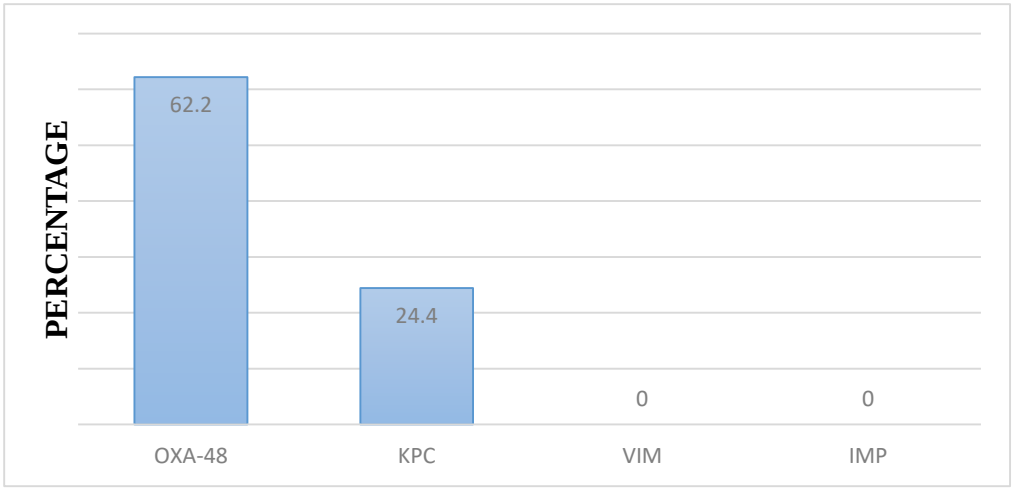

Figure 4.6: Resistance genes *bla$_{KPC}$*, *bla$_{VIM}$*, *bla$_{IMP}$* and *bla $_{OXA-48}$*

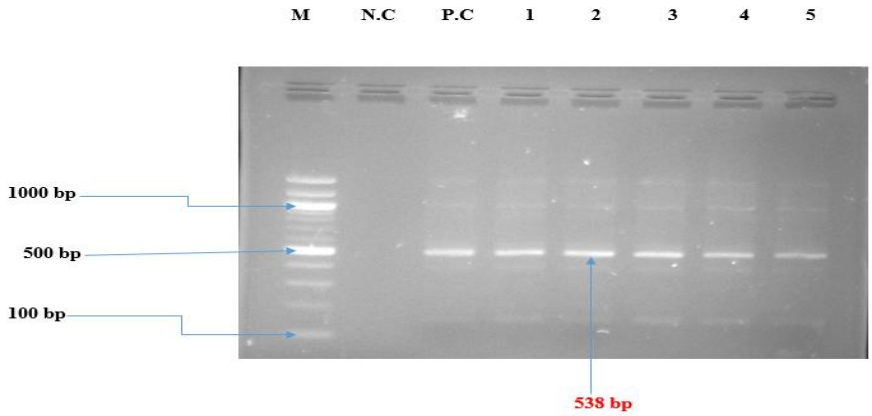

Figure 4.7: Agarose gel electrophoresis of *bla*$_{KPC}$ genes

Lane M: Ladder (100-1500 bp), N.C: Negative control (*E.coli* ATCC 35218), P.C: Positive control (*K. pneumoniae* ATCC 13883), Lanes 1-5: The 538 bp by PCR product of *bla*$_{KPC,}$ bp: base pairs.

CHAPTER FIVE

DISCUSSION

5.1 Demographic Characteristics of patients with *K. pneumoniae* infections

In this study, 54 % (147/272) of *K. pneumoniae* isolates were collected from females while 46 % (125/272) were collected from males. In Kenya, Apondi *et al.* (2016) reported similar findings from blood samples with a high rate of females with *K. pneumoniae* (64.8 %) from the study conducted at Tertiary Teaching Hospital. In Ghana, Afriyie *et al.,* (2015) reported a higher proportion of *K. pneumoniae* from urine samples in females (75 %) than in males (25 %). In Nigeria, females were observed to have higher cases of *K. pneumoniae* infections (62.5 %) than males (37.5 %) from the study done by Sule & Kumurya (2016) from urines samples at Murtala Muhammad Specialist hospital. In South East Region of Bangladesh, a study conducted by Akter *et al.* (2014) reported that males (57 %) had a higher proportion of *K. pneumoniae* infections compared to females (43 %). In India, Deshmukh *et al.,* (2016) reported similar findings with a proportion of females (74.20 %) and males (25.80 %) from urine samples collected from different pathological laboratories of Amravati and Maharashtra.

In this study, females were more affected as compared to males in almost age sets except at age group of 0-10 years in this study. In Nigeria, the age group with highest prevalence of *K. pneumoniae* was 21-30 with 16.50 % in the study conducted by Sule & Kumurya (2016) from urine samples at Murtala Muhammad Specialist hospital. In Pakistan, for the age group 20-30, the females (13.69 %) showed more than the double the proportion of urinary tract infections cases in males (5.33 %) (Bashir *et al.,* (2008). The same study reported that the cases of UTIs were more in boys than girls of less than ten years of age. The recognized risk factors such as sex (female) for *K. pneumoniae* community acquired pneumonia were reported by other researchers (Inghammar *et al.,* 2018; Rammaert *et al.,* 2012).

The reasons of high proportion of *K. pneumoniae* in females were the shorter urethras close to anus which allows *K. pneumoniae* quick access to the bladder, sexual activity in young women, pregnancy, aspects of personal hygiene or birth control bill, low concentration of lactobacilli in elderly women (Behzadi *et al.,* 2010 ;Tajbakhsh *et al.,* 2015).

In the current study, the highest proportion of *K. pneumoniae* isolates was more collected in age groups of over 50 years (44.9 %) as compared to other age groups. In Pakistan, Bashir *et al*. (2008 reported a proportion of 50 % in age group over 50 years from urine samples. This could be due to the weakened immune system and /or underlying conditions such as cancer, diabetes in elderly people (Meatherall *et al.,* 2009; Wang *et al.,* 2019). In this study, the proportion of *K. pneumoniae* isolates collected from outpatients (50.7 %) was slightly more than that collected from inpatients (49.3 %). In Rwanda, Muvunyi *et al.,* (2013) reported the relatively higher proportion of *Klebsiella spp*. in inpatients than in outpatients (Muvunyi *et al.,* 2013). In Nigeria, Nwosu *et al. (*2014) reported a higher proportion of *K. pneumoniae* in outpatients (77.6 %) than in outpatients (22.4 %). *K. pneumoniae* is a common hospital-acquired infection pathogen and also a potential community acquired pathogen.

5.2 Antimicrobial susceptibility patterns of *K. pneumoniae* isolates

K. pneumoniae is one of the most common bacteria that causes infections in patients. Choosing the best antibiotic is very important for treatment of nosocomial infections caused by *K. pneumoniae* (Babakhani *et al.,* 2015). In the current study, all *K. pneumoniae* isolates were resistant to ampicillin (100 %). Higher resistance levels to ampicillin have been reported in previous studies in clinical samples (Ali *et al.*2014 ; Deshmukh *et al.*2016; Ghanem *et al.*2017 ;Moini *et al*.2015,Ntirenganya *et al.*2015). *K. pneumoniae* is naturally resistant to ampicillin due to a constitutively expressed chromosomal class β lactamases (Varghese *et al.,* 2016) . The frequent use of trimethoprim/sulfamethoxazole for the treatment or as prophylactic antimicrobial has led to higher resistance levels (Varghese *et al.,* 2016).

In this study the resistance rate to trimethoprim/sulfamethoxazole was 56.6). In Tanzania ,Mshana *et al.,* (2009) reported 48.3 % as resistance to Trimethoprim/Sulfamethoxazole from clinical specimens. In Uganda, the resistance rate to trimethoprim/sulfamethoxazole was 65 % from pastoralist communities (Stanley *et al*., 2016). Lower resistance to amikacin (18 %) was observed in this study. Kumar, (2013) reported the lowest antibiotic resistance to amikacin (11.9 %) of *K . pneumoniae* isolated from pus. Less resistance rates to amikacin were reported in previous studies in India from urine samples and in Iran from clinical isolates of *K. pneumoniae* (Manikanda & Amsath (2013) ;Mansury *et al.* (2016). This may be explained by the absence of routine use of amikacin in infections as empirical therapy (Abyneh *et al.,* 2018). In Kenya, least resistance to amikacin (21.0 %) and meropenem(7 %) and high resistance to ceftriaxone (87.2 %) ,gentamicin(82.8%) ,cefipime(85.4 %) ,ceftazidime(69.7%) were observed in *K.pneumoniae* from blood samples (Apondi *et al*.(2016). The findings in the study conducted on *K. pneumoniae* from stool and urine samples in Kampala district revealed high rates resistance to commonly used antibiotics such as ampicillin, and lower resistance rates to amoxicillin/ clavulanate, ciprofloxacin and gentamicin (Najjuka *et al.,* 2016).

Barakzahi *et al.(*2014) reported the most of antibiotic resistance of *K. pneumoniae* was detected to cefotaxime(81%). In Teheran, Feizabadi *et al*.(2007) reported the antibiotic resistance in two hospitals and the highest antibiotic resistance was reported for amoxicillin-clavulanic acid (81.81 %) and aztreonam(78.78%); the lower resistance was for piperacillin/tazobactam(15.15 %). In the present study, to meropenem was 21.7 %. In Yasuji city, Dehshiri *et al.(*2018) reported that the resistance rate to imipenem was 1 %. In a study by Bina *et al*.,(2015), in order to determine *K. pneumoniae* carbapenemase in clinical samples,the resistance rate to meropenem was 13.9 %. In Rwanda, resistance rates were reported in a study conducted on susceptible patterns of pathogens responsible for both community and hospital acquired UTIs to antibacterial agents currently used to treat UTIs and were varying between 50-70.2 % for amoxicillin clavulanic acid ,6.9-38.3 % for ceftriaxone, 4.2-31.9 % to ceftazidime, 31.9-57.4 % to ciprofloxacin,

26.4-29.8 % to nitrofurantoin, 36.1-46.8 % to gentamicin, 45.8-46.8 % to amikacin, 80.6 - 95.7 % to trimethoprim/sulfamethoxazole and none of the isolates were resistant to imipenem in the outpatients and inpatients respectively (Muvunyi *et al.,* 2011). In Uganda, resistance rates of *K. pneumoniae* from blood specimens were to ceftriaxone (75 %), ciprofloxacin (77 %) ,piperacillin / tazobactam (36 %) and Imipenem (20 %) (Kajumbula *et al.,* 2018). In Iran, Yekani *et al.*,(2018) reported high resistance rates on *K. pneumoniae* isolates from urinary specimens to cefotaxime (57.2 %), ceftazidime (57.2 %), ceftriaxone (53.6 %), cefoxitin (25 %), meropenem (21.4 %), imipenem (21.4 %), cefepime (64.3 %) ,ampicillin (96.4 %), cefazolin (75 %), cefuroxime (60.7 %), aztreonam (60.7 %) and nitrofurantoin (64.3 %). In Turkey, Kirac *et al. (*2014) reported the resistance rate of *Klebsiella* species from urines samples to cefepime (7 %), aztreonam (7 %), cefuroxime (20 %), trimethoprim/sulfamethoxazole (20 %), cefazolin (13 %), ampicillin (73 %).

Kalaskar & Venkataramana, (2012) reported *K. pneumoniae* resistance to cephalosporins 1-4 generations close to 100 % in 2012. Over the past two decades, there has been a wide use of extended broad spectrum antimicrobial agents to meet the emerging challenge of treating infections due to Gram negative bacilli (Varghese *et al.,* 2016). In Africa, in both outpatients and inpatients ,the antimicrobial susceptibilty of *Klebsiella* species varied at the following rate:amoxicillin/clavulanic acid (29-82%); cefotaxime (74-84%), %); ciprofloxacin (54-94%); gentamicin (73-100%); amikacin (73-100%), trimethoprim/sulfamethoxazole (1-88%); nitrofurantoin (6-79%) from the data of 14 countries on susceptibility of *Enterobacteriaceae* causing UTI (Tansarli *et al*. (2013) Antibiotic resistance patterns varied in different hospitals, cities and nations. Based on the results of this study in comparison with other studies regarding antibiotics resistance,the difference of the resistance rates could be due to the sample size,sampling method, the types of antimicrobial agents commonly used in certain areas and the rate at which antibiotics are prescribed for treatment of various infectious diseases causing gene mutation leading to β lactamases production. Therefore, antimicrobial resistance could lead to limited therapeutic options, costs to healthcare, push more people into extreme poverty, economic loss and high rate morbidity and mortality.

5.3 Proportion of phenotypic ESBL, AmpC and carbapenemase producing *K. pneumoniae*

The antibiotic resistance mechanisms include extended-spectrum β-lactamases (ESBLs), AmpC β-lactamase and carbapenemases. ESBLs production was detected in 29.8 % (81 /272) in this study. Previous studies conducted in India and Iran reported different rates of ESBL production in *K. pneumoniae* clinical isolates (34 % ; 64.7-74.5 % ; 43.1 % ; 50 % ; 35.16 % ; 9 %; 38.5 % and 3 % respectively) (Anusuya &Rajesh, 2016; Chatterjee *et al.,* 2010;Chavan *et al* .,2016, Doddaiah & Dhanalakshmi, 2014; Oberoi *et al.,* 2013; Rawat *et al.*,2013 ; Shahandeh *et al.*, 2016; Vijaya & Achut ,2017). In the current study, the level of carbapenemase production was 7.0 % (19/272). In India, Anusuya & Rajesh (2016) reported 8.0 % of the isolates. This study reported a lower proportion of carbapenemase among *K. pneumoniae* isolates in clinical samples when compared to report from Iran, India and Pakistan (16.3 %, 10.98 %,17.4 %, 33.3 % respectively) (Doddaiah &Dhanalakshmi, 2014 ; Oberoi *et al.* 2013; Satish *et al*.,2013; Wadekar *et al*.,2013). However, carbapenemase enzymes were not detected from clinical isolates in the study by Rawat *et al.*, (2013).

The proportion of ESBL and Amp C co-production was 0.7 % (2/272) in this study. The rate of ESBLs and Amp C was 71.3% in the study conducted by Doddaiah & Dhanalakshmi (2014) while ESBLs and Amp C were coproduced in 27.7 % of isolates in the study done by Rawat *et al*.(2013). In Nigeria,Yusuf *et al*. (2014) reported 17.6 % of ESBL and Amp C co-production in clinical samples. In India, Vijaya & Achut (2017) reported 9.8 % of ESBL and Amp C co-production while Chivan *et al.* (2016) reported 9.7 % of the total of *Klebsiella* species.

The proportion of ESBL and carbapenemase co-production was 5.1% (14/272) in this study. In India, a different co-production rate of the two enzymes (16 %) was reported by Anusuya & Rajesh (2016). However, the proportion of ESBLs and carbapenemase producers in this study is close to the findings from a study done by

Doddaiah & Dhanalakshmi (2014) where the proportion was 6.52 %. ESBL and carbapenemases were not detected in the study done by Rawat *et al* (2013).
The rate of Amp C and carbapenemase was 1.8 % (5/272) in the present study which is somewhat comparable to a study done from Nigeria by Yusuf *et al.* (2014) who reported the proportion of Amp C and carbapenemases for 1.5% in *K. pneumoniae* isolates. In India, Amp C and carbapenemase co-production were not detected from clinical isolates in the study done by Archana *et al.* (2016).

ESBL, AmpC and carbapenemases co-occurred in 2.6 % (7/272) of *K. pneumoniae* isolates in the present study which contrasts to the report from a study done by Chatterjee *et al.* (2010) where the co-occurrence of the three enzymes was 29.4 % from *K. pneumoniae* isolates bacteria in India. Infections caused by organisms producing such enzymes(ESBL, AmpC and carbapenemases) have resulted in poor outcomes, reduced rate of clinical and microbiological responses, longer hospital stays , greater hospital expenses and are a major threat worldwide (Anusuya &Rajesh, 2016).

5.4 Antimicrobial resistance between β-lactamase producers and non β-lactamase producers producing *K. pneumoniae*

K. pneumoniae have been developed multiple antimicrobial resisstance mechanisms including alteration of the drug of plasmided-mediated β-lactamases (Tenover,2006). In the current study, the difference in antibiotic resistance between ESBL producers and non ESBL producers was statistically significant for all beta lactam (except meropenem) and trimethoprim/ sulfamethoxazole. It was due to the fact that ESBL producers commonly harbor resistance determinants to other classes of antibiotics including cefepime aminoglycosides and fluoroquinolones (Turner, 2005).
The highest resistance rates among ESBL producers were observed to ceftazidime and cefotaxime (97.5 %), ceftriaxone (96.3 %), ceftazidime (95.1 %), cefepime (93.8 %). In Ethiopia, Resistance rates to ceftazidime, ceftriaxone and cefotaxime were 100 %, 100 % and 70.6 % respectively from *K. pneumoniae* isolates (Abyneh *et al*., 2018).

In South Africa, Buys *et al.* (2016) reported a significant resistant to 2nd, 3rd and 4th generation cephalosporins and Cotrimoxazole in *K. pneumoniae* bloodstream infections. ESBL are modified β- lactamases that impart resistance to third generation cephalosporins and make all β lactam antibiotics (except meropenem) and cephalosporins useless in the therapy (Procop *et al.,* 2003). ESBL producing *K. pneumoniae* isolates was well recognized problem and explained the high resistance rates to commonly used antimicrobials agents including aminoglycosides and fluoroquinolones as it was reported in previous studies (Henson *et al.,* 2017; Varghese *et al.,* 2016). Carbapenem antibiotics (meropenem) are considered the treatment of choice for serious infections caused by ESBL producing and /or AmpC β-lactamase-producing *Enterobacteriaceae* because of their high stability to β-lactamase hydrolysis and the relatively high susceptibility of ESBL producers to carbapenems (Colodner *et al.,* 2004; Varghese *et al.,* 2016).

In the current study, the difference in antibiotic resistance between carbapenemase producers and non carbapenemase producers was statistically significant for all β lactam and gentamicin. The vast majority of carbapenem resistant *K. pneumoniae* had additional resistance to fluoroquinolones and aminoglycosides (D'Aleo *et al.,* 2018). The highest resistance rates among carbapenemases producers were observed to cefotaxime (94.7 %), ceftazidime (94.7 %), cefuroxime (89.5 %), ceftriaxone (89.5 %), cefepime (89.5 %) and meropenem (89.5 %). The study conducted at Italian general hospital from blood samples showed rates resistance to ertapenem (96.6 %), imipenem (75.9 %), meropenem (75.9 %), ceftazidime (100 %), cefepime (96.6 %), gentamicin (58.6 %), amikacin (51.7 %) (Ferranti *et al.,* 2018). Some carbapenemase such as the *K. pneumoniae* carbapenemase degrade virtually all classes of β-lactams (Kiiru *et al.,* 2012). Carbapenems drug are the last line but its efficacy is threatened by bacteria that produce carbapenemase enzymes (Rendani *et al.,* 2015). At this time, there are limited selection of treatment options carbapenem resistance *K. pneumoniae* infections. Clinicians have been forced to re-evaluate the use of agents, which have been rarely used due to efficacy and /or toxicity concerns such as polymixins, fosfomycin (Morrill *et al.,* 2015).

In the present study, the resistance to amikacin, gentamicin and ciprofloxacin was significant in co-occurrence of β- lactamase compared to when there is no co-production. Similar findings were observed in the study done by Chatterjee *et al*. (2010) where the resistance was significant to the same antibiotics in *K. pneumoniae* isolates producing all three β lactamases. Fluoroquinolones and aminoglycosides resistance has been attributed to plasmid encoded *qnr* genes, as well as to mutant aminoglycoside-modifying gene *aac-6'-Ib-cr* and their mobilization onto plasmids involve integrons (Rice *et al.,* 2008). The resistance of these antibiotics could be to these antibiotics are important alternative antibiotics for treating β-lactamase producers and /or used in combination therapy with β-lactam antibiotics and the mutants resistant may be selected as it was demonstrated by Ansari *et al.,*(2016). Knowledge on local antimicrobial resistance trends is important not only in guiding clinicians to prescribe appropriate antibiotics but also for evidence based recommendations in empirical antibiotic treatment.

5.5 Proportion of resistance genes in ESBL producers

In current study, ESBL genes including bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ and $bla_{OXA\text{-}48}$ were detected singly or in combination among *K. pneumoniae* isolates.

In Kenya, The prevalence of bla_{TEM} and bla_{SHV} was 4% and 2% respectively and all *K. pneumoniae* isolates did not carry any the two genes in the study done by Juma *et al.,* (2016) whereas Kiiru *et al., (*2012) reported the association of $bla_{TEM\text{-}1}$ + $bla_{OXA\text{-}1}$ from *E. coli* isolates. In South Africa, Vasaikar *et al*. (2017) reported the combination of bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ 56.11 % (78 /139) of *K. pneumoniae* isolates.

In India, Bora *et al.* (2014) reported a combination of bla_{TEM} + bla_{SHV} + $bla_{CTX\text{-}M}$ (20.11 %), bla_{TEM} + bla_{SHV} (13.79 %), bla_{TEM} + $bla_{CTX\text{-}M}$ (27.58 %), bla_{SHV}+ $bla_{CTX\text{-}M}$ (8.62 %) while the proportion of bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ was 16.09 %, 8.04 % and 5.04% respectively in *K. pneumoniae* isolates . Diagbouga *et al.* (2016) reported 14.28 % of bla_{TEM}, 10.71 % of bla_{SHV}, and 25 % of $bla_{CTX\text{-}M}$, 10.71 % of bla_{TEM} and bla_{SHV}, and 3.57 % of bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ of K. *pneumoniae* isolates.

The presence of *bla$_{TEM}$*, *bla$_{SHV}$* and *bla$_{TEM}$* genes was 3.39 %, 4.23 % and 11 % respectively in the study done by Alibi *et al.,* (2015) in ESBL producing *K. pneumoniae* strains . In Brazil, Jaskulski *et al.* (2013) reported that 16.67 % (2/12) of ESBLs producing *K. pneumoniae* from clinical samples carried *bla$_{TEM}$*, *bla$_{CTX-M}$*, *bla$_{TEM}$* / *bla$_{SHV}$* genes; 8.33 % (8/12) carried *bla$_{SHV}$* while combinations of *bla$_{TEM}$* /*bla$_{CTX-M}$* and *bla$_{TEM}$/bla$_{SHV}$/bla$_{CTX-M}$* were detected in 25 % (3/12) and in 16.67 % (2/12) respectively. In Burkina Faso, among 28 ESBL producing *K. pneumoniae* collected from clinical isolates, 14.28 % expressed *bla$_{TEM}$*, 10.71 % expressed *bla$_{SHV}$*, 25 % expressed *bla$_{CTX-M}$*, 10.71 % expressed *bla$_{TEM}$* and *bla$_{SHV}$*, 3.57 % showed a combination of *bla$_{TEM}$*, *bla$_{SHV}$* and *bla$_{CTX}$*-M (Zongo *et al.*, 2015). In Saudi Arabia, the proportion of *bla$_{CTX-M}$*, *bla$_{SHV}$* was (7.4 %) and (14.8 %) respectively while the proportion of combination *bla$_{TEM}$* + *bla$_{CTX-M}$* (3.7 %); *bla$_{SHV}$*+ *bla$_{CTX-M}$ (*44.4 %); *bla$_{TEM}$* +*bla$_{SHV}$*+ *bla$_{CTX-M}$* (25.9 %) from *K. pneumoniae* isolates (Somily *et al.*, 2015).

In this study, the co-occurrence of *bla$_{TEM}$ /bla$_{SHV}$ /bla$_{CTX-M}$ / bla$_{OXA-48}$* was reported in 9.62 % of ESBL producing *K. pneumoniae.* As previous studies from Tanzania, North African and European countries, *bla$_{OXA-48}$* gene for carbapenem resistance has been found in ESBL producers (Mshana *et al.*, 2011; Mshana *et al.*, 2013; Potron *et al.*, 2013). In Turkey, a combination rate of *bla$_{OXA-48}$*, *bla$_{SHV}$*, *bla$_{TEM}$*, and *bla$_{CTX-M}$* (76 %) was reported from *K. pneumoniae* clinical samples (Iraz *et al.*, 2015). In Saudi Arabia, the proportion of *bla$_{CTX-M}$* +*bla$_{OXA-48}$* was 3.7 % in *K. pneumoniae* isolates (Somily *et al.*, 2015) .

Carriage of multiple β-lactamases genes in the same strain contribute to the selective success of this pathogen. This may be due to either: i) carriage of an antibiotic an array antibiotic resistance gene due the acquisition of transposons containing different *bla* genes on the same plasmid; ii) co-carriage of more than one antibiotic resistance plasmid (Navon-Venezia *et al.*, 2017). The association between *bla$_{OXA-48}$* and others ESBL genes could potentially lead to pan-β-lactam resistance (Potron *et al.*, 2013).

5.6 Proportion of resistance genes in carbapenemase producers

Bla$_{OXA-48}$ was the most dominant gene (62.2 %) in carbapenemase producing *K. pneumoniae* in this study. In Sudan, Abdelhakam & Al-Fadhil (2017) reported 34/96 (35.4 %) the carriage of *bla$_{OXA-48}$* of carbapenemase producing *K. pneumoniae* from clinical samples. In Tanzania, the proportion of *bla$_{OXA-48}$* was 5.88 % in study done by Mushi *et al.* (2014) from clinical isolates. In Brazil, Flores *et al.* (2016) reported a prevalence of 16 % from patients at an intensive care unit. The prevalence of *bla$_{KPC}$* genes was 24.4% from the current study. In Tanzania, Mushi *et al.,* (2014) reported 4.41 % from clinical samples. The studies conducted in others countries including, Sudan, Egypt, and Italy reported different proportion of *bla$_{KPC}$* genes (59.4 %, 47.8 %, 77 %, 91 %) (Abdelhakam & Al-Fadhil, 2017; Dalia & Doaa, 2017; Sakariouki *et al.*, 2017; Wang *et al*., 2012).

All *K .pneumoniae* isolates were negative for *bla$_{VIM}$* and *bla$_{IMP}$* genes in the present study. These findings concur the findings from previous studies done in Brazil, Iran and Turkey in clinical samples (Flores *et al.*,2016; Moghadampour *et al*.,2018; Sahin *et al.*, 2015). Infections caused by carbapenemase genes are associated with high morbidity and mortality (Rendani *et al.*, 2015). The frequencies of the genes responsible for production of ESBL and carbapenemase vary among themselves and among bacterial species. These variations make each region have its own characteristics (Flores *et al.*,2016).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

From the findings of the study the following conclusions were drawn.

1. *K. pneumoniae* is a common hospital-acquired infection pathogen and also a community acquired pathogen. It was more collected in females as compared to males in almost age sets except at age group of 0-10 years. The highest isolates proportion was found in the age group more than 50 years.
2. The proportion of *K. pneumoniae* resistant to observed to amikacin (18 %), gentamicin (21 %), nitrofurantoin (21 %), meropenem (21.7 %) and ciprofloxacin (25.4 %) was lower compared to other antibiotics. All *K. pneumoniae* isolates were resistant to ampicillin (100 %) indicating that this antibiotic should not be prescribed.
3. ESBL, AmpC and Carbapenemase phenotypes and ESBL and carbapenemase resistance genes are the major cause of antibiotic resistance in the hospital. The Co-production of two or more of the enzymes occurred among *K. pneumoniae* not only limits the therapeutic options but also poses diagnostic challenges. Carriage of multiple ESBL genes in the same strain contribute to the selective success of *K. pneumoniae.*

6.2 RECOMMENDATIONS

1. This study highlights the need to establish an antimicrobial resistance surveillance and β-lactamases producing *K. pneumoniae* to monitor the trends and new types of resistance mechanisms emerging in hospital.
2. Health education on personal practices by regularly washing hands and by keeping clean instruments and environment.
3. Healthcare professionals must talk to the patients how to take antibiotic, the dangers of antibiotic resistance and preventing infections.

4. The study recommends an integrated system of action of clinicians and microbiologist in deciding the antibiotic treatment; maintaining proper sanitation; antimicrobial policy and surveillance of drug resistance, hand hygiene and food hygiene. This will help in controlling and preventing the spread of these resistant bugs in the hospital environment.
5. Prudent use of antibiotics in developing countries should advocated and in such countries, the existing empiric treatment regimes should be revised occasionally in order to reflect prevailing resistance phenotypes.
6. It very clear that antimicrobial resistance cannot be controlled by the health sector alone. Drug resistance recognizes no borders, as it is transmitted to human through the food chain and through environmental degradation .All governments sectors, from health to trade to agriculture, must be engaged urgently.
7. Though, the molecular methods are not possible to carry out routinely in the laboratories of developing countries, some extra-efforts such as PCR should be carried out for the correct identification of the genes involved in antibiotic resistance. WHO recommended five ways to overcome multi drug resistant problem (Archana & Harsh, 2011):
    - ✓ Committing to a comprehensive, financed national plan with lines of accountability and community engagement
    - ✓ Strengthening surveillance and laboratory capacity
    - ✓ Ensuring a regular supply of good quality medicines
    - ✓ Enhancing infection prevention and control in healthcare settings
    - ✓ Fostering innovation, research and development

This study forms a strong basis of future larger studies that should include whole genomic epidemiology that can unravel transmission routes.

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APPENDICES

Appendix I: Bacteria media preparation

A. MACCONKEY AGAR

1. Formula	Gram/Litre
Peptic digest of animal tissue	20.0
Lactose	10.0
Bile salts	5.0
Sodium Chloride	5.0
Neutral red	0.070
Agar	15.0
Final pH	7.5±0.2

2. Preparation

Suspend 55.07 grams in 1000ml distilled water .Heat to boiling with gentle swirling to dissolve the medium completely. Sterilized by autoclaving at 15Ibs pressure (121^{o}C) for 15 minutes. Cool to 45-50^{o}C and pour into sterile petri dishes.

3. Use: For selection of enteric bacteria.

B CLED AGAR

1. Formula	Grams/Litre
Peptic digest of animal tissue	4.0
Casein enzymic hydrolysate	4.0
Beef extract	3.0
Lactose	10.0
L-cystine	0.128
Agar	15.0
Final pH	7.3±0.2

2. Preparation

Suspend 36.1grams in 998 ml of distilled water. Add rehydrated contents of 1 vial of bromothymol blue supplement (FD091). Heat, to boiling, to dissolve the medium

completely .Sterilize by autoclaving at 15Ibs (121^{o}C) for 15 minutes. Mix well and pour into sterile petri dishes.

1. Use: Isolation of bacterial flora in urinary tract

C. Nutrient Agar

1. Formula	**Grams/Litre**
Lab-Lemco powder	1.0
Yeast Extract	2.0
Peptone	5.0
Sodium Chloride	5.0
Agar	15.0
pH	7.0±0.2

2. Preparation

Suspend 28 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121oC for 15 minutes.

3. Use: purity of bacteria

D. Brain Heart Infusion Broth (BHI)

1. Formula	**Grams/Litre**
HM infusion powder	12.5
BHI powder	5.0
Proteose peptone	10.0
Dextrose (glucose)	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
pH	7.4±0.2

2. Preparation

Suspend 37.0g in 1000 ml distilled water. Bring to the boil to dissolve completely. Dispense into bottles and sterilize by autoclaving at 121oC for 15 minutes. Cool before use

4. **Use:** enrichment of bacteria

Appendix II: Gram Stain technique

1. Principle

The Structure of the organism's cell wall determines whether the organism is gram positive or negative. When stained with a primary stain and fixed by a mordant, some bacteria are able to retain the primary stain by resisting decolorization while others get decolorized by a decolorizer. Those bacteria which retain the primary stain are called Gram positive and those bacteria get counterstained are called Gram negative**.**

2. Reagents

a. Primary stain: Crystal violet

b. Mordant: Gram's Iodine

c.Decolorizer:95% Ethanol

d. Counterstain: Safranin

2. Procedure

a. Place slide with heat fixed smear on staining tray

b. Flood the slide with crystal violet solution for up to 1 minute. Rinse briefly with tap water. Drain

c. Flood slide with gram's Iodine solution, and allow to act (as a mordant) for 1 minute. Rinse with tap water. Drain

d. Remove excess water from slide. Flood slide with 95% ethyl alcohol drop by drop until the alcohol runs almost clear and rinse with tap water.

e. Flood slide with Safranin solution and allow to counterstain for 30 seconds. Rinse with tap water. Drain and dry the slide

f. Examine under the oil immersion lens

3. Expected results

1. Gram negative bacteria: Stain red or pink due to retaining the counter staining dye called safranin

2. Gram positive bacteria: Stain dark purple due to retaining the primary dye called crystal violet in the cell wall.

Appendix III: Preparation of glycerol and storage of *K. pneumoniae* isolates

1 Principle

Freezing is a good way to store bacteria. Generally the colder the storage temperature, the longer the culture will retain viable cells. The problem faced by bacteria stored in freezers is ice crystals. Ice can damage cells by dehydratation caused by localized increases in salt concentration. As water is converted to ice, solutes accumulate in the residual free water and this high concentration of solutes can denature biomolecules. Ice can also rupture membrane .To lessen the negative effects of freezing, glycerol is often used as a cryoprotectant.

2 Material

The identified and isolated bacterial colonies *K. pneumoniae*, 30% Glycerol, sterile water, incubator, Vortex mixer, freezer, Broth Heart Infusion (BHI)

3. Preparation 30 % glycerol

Prepare a solution of 30% by mixing 30ml of glycerol with 70 ml of distilled water. Transfer the solution to a screw cap gloss bottle and sterilize by autoclaving at 121ºC for 15 min.

4 Procedure

1. Grow a pure culture on an appropriate solid medium.
2. When the culture is fully developed, scrape it off with a loop.
3. Inoculate *K. pneumoniae* into BHI broth and incubate for 16 to 24 hours at 37ºC.
4. Add 0.6 ml of the overnight culture into a 1.5 ml cryogenic vial with 0.4 ml of 30% glycerol.
5. Mix the solution well by vortexing on a medium setting or by repeated inversions.
6. Store at -20^0C freezer.

Appendix IV: Identification, antibiotic susceptibility test

Step 1: Suspension preparations for ID and AST card: Suspension Preparation for ID card first. Transfer 3ml of saline water into tube. Select an isolate colony and dissolve it Mix well and check the density with densichek. Inoculum density for GNB should be 0.5- 0.63MCF. Then Place the ID card and tube into Cassette.

Suspension Preparation for AST Card: transfer 145ml of the ID suspension into the AST tube. Then Place the AST card and tube into the cassette.

Step 2: Filling and Loading the card into VITEK 2 System: Set all the Card and Tube with suspension in a Cassette. From PC work station print Cassette work sheet and record job ID and bar code for each card. When instrument status is OK, then press start fill button. Remove the Cassette from loading station at any time when instruments indicate. The VITEK 2GN card barcode labels were scanned and the cards were placed in the card positions next to the inoculum tubes. The AST-GN83 card barcode labels were scanned and the cards were placed in the card positions next to the empty tubes.

Step 3: Entering Specimen information Log in to window and then into VITEK 2 software with username and password. In the main view, click on the cassette icon. Find the Cassette that have been loaded in the navigation tree on the left side and enter job ID from work sheet and organism name for isolates on with AST cards only. Use define isolates button to link ID and AST cards of the same specimen. Then click the save button.

Step 4: Entering patient information in patient icon, click new patient icon. Enter patient and Specimen information and then save. Automatic identification and susceptibility testing was performed by kinetic fluorescence measurement every 15 min. The software then analyzed the data and reported the results.

Interpretation

The VITEK 2 system AES compares each MIC result for the tested isolate with the modal MIC distribution of *K. pneumoniae* with known resistance mechanisms included in the database. The VITEK 2 automated susceptibility system has introduced an ESBL test on their system whereby ceftazidime and cefotaxime are

tested alone and in combination with clavulanic acid/ or on simultaneous assessment of the inhibitory effects of cefepime, cefotaxime and ceftazidime alone and in presence of clavulanic acid.

Logarithmic reduction in growth within the well containing clavulanic acid compared to the well not containing clavulanic acid indicates expression of an ESBL.

VITEK 2 System analyzed the carbapenem susceptibility results to identify isolates suspected of carbapenemase production base on reduction of susceptibility to meropenem. Reduced susceptibility to cefoxitin is taken as indicator of AmpC production

Appendix V: Protocol of DNA extraction

Reagent preparation:

Add 1,040 µl Proteinase K storage buffer to each Proteinase K (20 mg) tube prior to use. Store at – 20^0 C.

Procedure

Resuspend *K. pneumoniae* pellets using DNA elution buffer (1-5*10^6 colonies)

1. Add up 200µl sample to a micro centrifuge tube and add:

-200 µl Biofluid and cell buffer

-20µl Proteinase K

2. Mix thoroughly or vortex 10-15 seconds and then incubate the tube at 55^0 C for 10 minutes.

3. Add 1 volume Genomic Binding buffer to the digested sample. Mix thoroughly or vortex 10-15 seconds.

E.g Add 420 µl Genomic binding buffer to the 420µl digested sample.

4. Transfer the mixture to a Zymo-Spin ™ IIC-XL column in a collection Tube. Centrifuge at ≥12,000x g for 1 minute. Discard the collection tube with the flow through.

5. Add 400 µl DNA Pre-wash buffer to the spin column in a new collection tube. Centrifuge at ≥12,000xg for 1 minute. Discard the collection tube with the flow through.

6. Add 700 µl g-DNA wash buffer to the spin column. Centrifuge at ≥12,000xg for 1 minute. Discard the collection tube with the flow through.

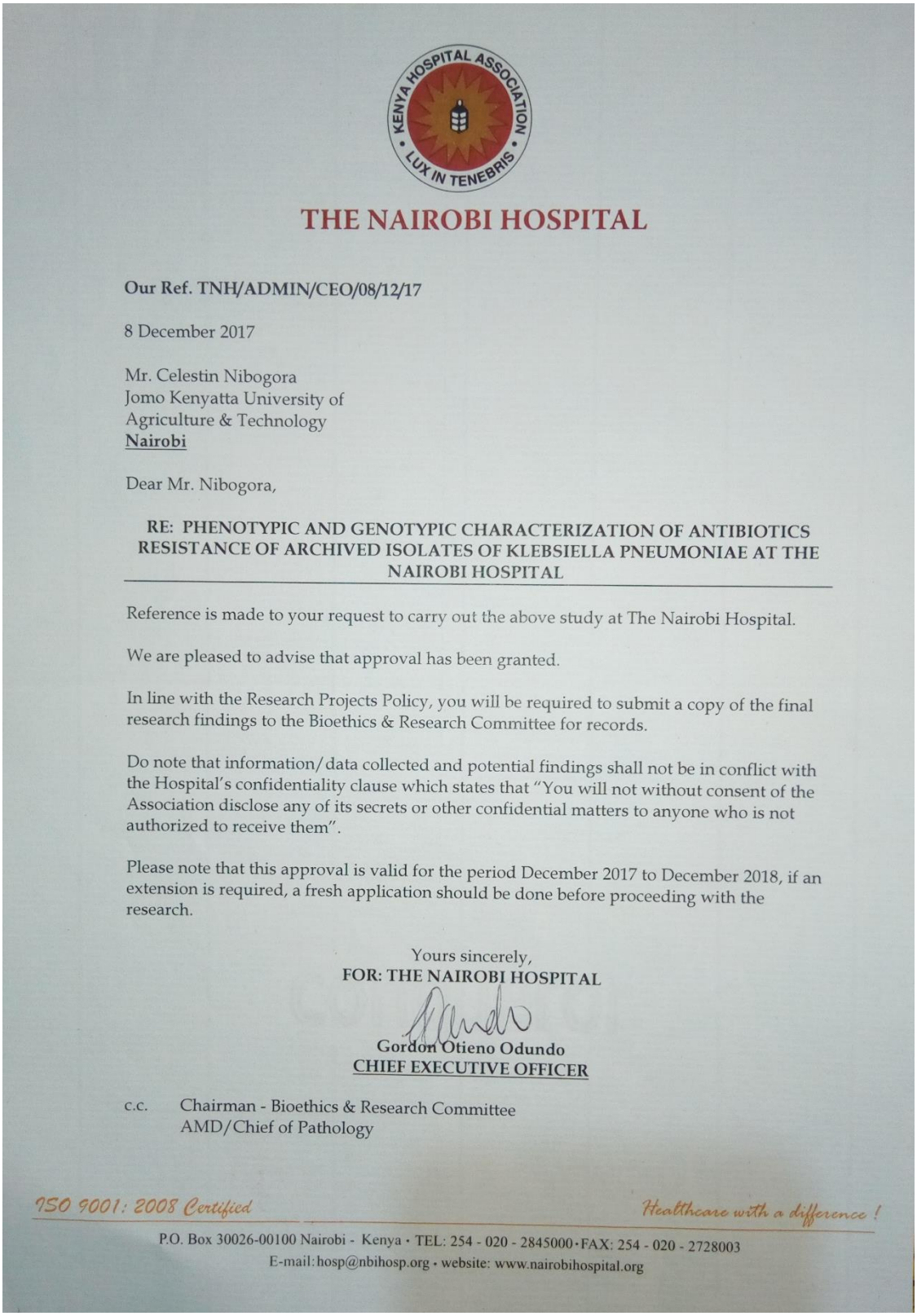
7. Add 200 µl g-DNA wash buffer to the spin column. Centrifuge at ≥12,000xg for 1 minute. Discard the collection tube with the flow through.

8. Transfer the spin column to a clean microcentrifuge tube. Add ≥50µl DNA elution buffer or water directly on the matrix. Incubate for 5 minutes at room temperature, then centrifuge at maximum speed for 1 minute to elute the DNA. The eluted DNA can be used immediately for molecular based applications or stored ≤ - 20^0 C for future use.

Appendix VI: Procedure of gel electrophoresis

1. Measure 1.2 g of agarose
2. Prepare 1xTAE buffer as work solution. (20ml 50xTAE buffer +980ml of Distilled water)
3. Prepare DNA ladder (40ml of distilled water +10 ml of Ladder +10ml of loading dye).
4. Dissolve 1.2 g agarose in 60 ml 1x TAE buffer. The agarose was dissolved by boiling the solution in microwave oven.
5. 5 µl EZ vision (Sigma-Aldrich LP, USA) was added for staining the DNA molecules.
6. The agarose-EZ vision solution was poured into the gel tray of the electrophoresis apparatus containing the combs and allowed to set for about 30 minutes.
7. 5µl of DNA Ladder was loaded into the first lane of the gel as the guide (manufacturer's instruction for the size of each band)
8. 8 µl of each PCR product was loaded into the gel wells.
9. 2µl of loading buffer was loaded into the flanking wells.
10. The electrophoresis was run at 110V for 35 minutes.
11. The gel was visualized under UV light and image (bands) captured using a digital camera.

Appendix 7: The Nairobi Hospital ethical approval



THE NAIROBI HOSPITAL

Our Ref. TNH/ADMIN/CEO/08/12/17

8 December 2017

Mr. Celestin Nibogora
Jomo Kenyatta University of
Agriculture & Technology
Nairobi

Dear Mr. Nibogora,

RE: PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF ANTIBIOTICS RESISTANCE OF ARCHIVED ISOLATES OF KLEBSIELLA PNEUMONIAE AT THE NAIROBI HOSPITAL

Reference is made to your request to carry out the above study at The Nairobi Hospital.

We are pleased to advise that approval has been granted.

In line with the Research Projects Policy, you will be required to submit a copy of the final research findings to the Bioethics & Research Committee for records.

Do note that information/data collected and potential findings shall not be in conflict with the Hospital's confidentiality clause which states that "You will not without consent of the Association disclose any of its secrets or other confidential matters to anyone who is not authorized to receive them".

Please note that this approval is valid for the period December 2017 to December 2018, if an extension is required, a fresh application should be done before proceeding with the research.

Yours sincerely,
FOR: THE NAIROBI HOSPITAL

Gordon Otieno Odundo
CHIEF EXECUTIVE OFFICER

c.c. Chairman - Bioethics & Research Committee
AMD/Chief of Pathology

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