PREVALENCE AND MOLECULAR IDENTIFICATION OF THIRD GENERATION CEPHALOSPORIN-RESISTANT KLEBSIELLA PNEUMONIAE ISOLATED FROM WOUNDS OF PATIENTS ATTENDING SELECTED HOSPITALS IN MINNA, NIGERIA

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ABSTRACT

In recent years, the emergence and circulation of third generation cephalosporin-resistant *Klebsiella pneumoniae* due to the production of extended spectrum beta- lactamases in both hospital and community settings is increasing worldwide resulting in high morbidity and mortality. This study determined the prevalence and molecular identity of third generation cephalosporin-resistant *K. pneumoniae* isolated from wounds of patients attending selected Hospitals in Minna, Nigeria. A total of 500 wound samples were collected in General Hospital and Ibrahim Badamasi Babangida Specialist Hospital Minna, between the period of June and December 2019. Wound samples were cultured on MacConkey Agar and Nutrient Agar. *Klebsiella. Pneumoniae* isolates were identified based on colonial morphology, motility, Gram's staining, detailed biochemical tests and molecular techniques. The prevalence of *K. pneumoniae* was 57 (11.40 %). The distribution of *K. pneumoniae* on the basis of age showed 24.5 %, 56.2 %, and 19.3 % for age groups 0-30, 31-60 and 61-90 years respectively. The prevalence of *K. pneumoniae* wound infection based on gender indicated 77.2 % and 22.8 % for male and female gender respectively. Antibiotic susceptibility profile of 57 *K. pneumoniae* isolates was evaluated by Kirby-Bauer modified disc Agar diffusion technique. The antibiogram showed that all the 57 (100 %) *K. pneumoniae* isolates demonstrated different and high levels of resistance to third generation cephalosporin (cefotaxime 66.7 %, ceftazidime 45.6 %, ceftriaxone 38.6 %, cefixime 43.9 %, cefpodoxime 47.4 % and cefdinir 98.2 %). Most 50 (87.7 %) of the isolates also showed high level of co-resistance to other antibacterial agent like penicillin (ampicillin 80.7 %), aminoglycoside (gentamycin 47.4 %) and fluoroquinolone (ciprofloxacin 28.1 %), while the least resistance was shown against carbapenem (imipenem 3.5 %). Multidrug resistance was observed in 27 (47.4 %) of the isolates while the multiple antibiotic resistance index analysis for each of the isolates ranged from 0.1-1.0. All the 3GC-R *K. pneumoniae* isolates were phenotypically screened for the presence of Extended Spectrum Beta- Lactamases (ESBLs) using Double Disc Synergy Test (DDST). The prevalence of ESBL producing 3GC-R *K. pneumoniae* isolates was 23/57 (40.4 %). Molecular analysis showed that five representatives of ESBL producing 3GC-R *K. pneumoniae* isolates were identified as *K. pneumoniae* strains MBT51, RBT40, MO1, J42 and NPK3-1-39. Five isolates carried CTX-M and TEM genes while only two of the isolates harboured SHV gene. The rate of wound colonization by ESBL producing 3GC-R *K. pneumoniae* was found to be high underscoring the need for public health measures to prevent wound infection. Hence there is need for regular screening and monitoring of 3GC-R and ESBL producing *K. pneumoniae* isolates in General Hospital and IBB Specialist Hospital Minna.

TABLE OF CONTENTS

CHAPTER FIVE

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Third generation cephalosporins are beta-lactam antibiotics with excellent activity against Gram-negative aerobic bacteria. However, some penicillin resistant strains of *K. pneumoniae* are also resistant to third-generation cephalosporins (Wohlwend *et al*., 2015).

Third generation cephalosporin–resistant (3GC-R) *Klebsiella pneumoniae* has a worldwide distribution with high degree of prevalence in both hospital and community settings posing a serious threat to the successful treatment of bacterial infections globally (Aljanaby and Alhasnawi, 2017). Generally, antibiotic-resistant organisms particularly *Enterobacteriaceae* are reported as threat to human health throughout the world (Wei *et al*., 2018; Müller-Schulte *et al*., 2020). To further aggravate the problem of antibiotic resistance, the indiscriminate use of antibacterial agents account for the emergence of antibiotic resistance genes and the evolution of antibiotic-resistant bacteria (Wei *et al*., 2018; Müller-Schulte *et al*., 2020).

Klebsiella pneumoniae is an enteric, opportunistic, non-motile, rod-shaped, lactose fermenting and Gram-negative bacterium with a prominent polysaccharide capsule that is thick, giving the colonies their glistening and mucoid appearance on agar plates (Al-Agha *et al*., 2017; Moradigaravand *et al*., 2017). The organism belongs to the family of *Enterobacteriaceae*, and it is a natural inhabitant of the skin, gastrointestinal tract and the nasopharynx of healthy humans and animals as well as different environmental abodes (Venezia *et al*., 2017; Bidewell *et al*., 2018; Da Silva *et al*., 2019; Effah *et al*., 2020).

Klebsiella pneumoniae has been identified as one of the leading causes of nosocomial and community acquired infections such as sepsis, bacterial pneumonia, wound infections, urinary tract infections (UTIs), pyrogenic liver abscesses, bacteraemia,

endocarditis, and the pathogen primarily cause infections in immunocompromised individuals, neonates and the elderly accounting for approximately one third of infections caused by Gram-negative bacteria worldwide (Kothari *et al*., 2013; Biradar and Roopa, 2015; Huynh *et al*., 2017; Venezia *et al*., 2017; Heidary *et al*., 2018).

Third generation cephalosporin-resistant (3GC-R) *K. pneumoniae* is one of the most frequently encountered bacteria from burn and surgical site wounds. Colonization of wound by this organism is endemic in hospital environment and prolong hospitalization is one of the risk factors for wound infections (Perween *et al*., 2015).

Data from the European Antimicrobial Surveillance Resistance Network (EASRN) between 2005- 2015 revealed varying rates of resistance to third generation cephalosporins by *K. pneumoniae.* The resistance rates ranged from 2.3 % for strains in Sweden to 81 % for those in Bulgaria, 13.6 % in Belgium, and 25.3 % in France (Renvoisé *et al*., 2013; WHO, 2014; Venezia *et al*., 2017). In recent years, *K. pneumoniae* has rapidly acquire plasmid encoding for Extended Spectrum Beta-lactamase (ESBL) genes namely; CTX-M (cefotaxime hydrolyzing), TEM (Temoneira), and SHV (sulfhydryl variable). Thus, remarkable evidence established that resistance to 3GC among strains of *K. pnuemoniae* is chiefly mediated by the production of Extended Spectrum Beta-Lactamase enzymes (ESBLs), these break the oxyimino-beta-lactam ring in beta-lactam antibiotics such as cephalosporins, penicillins, and monobactam. However, they are inhibited by clavulanic acid (Kothari *et al*., 2013; Long *et al*., 2017; Adabara *et al*., 2020).

1.2 Statement of the Research Problem

Klebsiella pneumoniae is one of the Multi-drug resistant organisms identified as an urgent threat to human health by the World Health Organization, the United States Centres for Disease Control and Prevention and the United Kingdom Department of Health (Kidd *et al*., 2017). Of greater consequence is the emergence and circulation of

third generation cephalosporin-resistant *K. pneumoniae* due to the production of extended spectrum beta-lactamases (ESBLs) in both hospital and community settings increasing worldwide, resulting in high morbidity and mortality (Calbo and Garau, 2015). According to World Health Organization's report in 2014, 50–60 % of multidrug resistant *K. pneumoniae* as a consequence of ESBL production are not susceptible to third generation cephalosporins (WHO, 2014; Venezia *et al*., 2017). The overall antibacterial resistance among pathogens of wound infections is alarming. Similarly, the incidence of wound infections caused by 3GC-R *K. pneumoniae* have further complicated therapeutic options, causing a great concern to the medical world (Sani *et al*., 2012).

Third generation cephalosporin resistant- *K. pneumoniae* is a serious clinical and public health problem as some of its strains are resistant to about 95 % antibiotics in use (Gashe *et al*., 2018; Da Silva *et al*., 2019). This poses a negative economic impact on low income countries like Nigeria where infectious disease control is a major challenge.

1.3 Justification for the Study

For many years, third generation cephalosporins (3GC) have been used as the major and potent drugs to treat infections due to *Enterobacteriaceae* (Renvoisé *et al*., 2013). These drugs were specifically designed to overcome the hydrolytic effect of major beta-lactamases (Qadeer *et al*., 2013; Da Silva *et al*., 2019)**.** However, growing resistance to these antibiotics makes the rapid detection of such resistance very important (Renvoisé *et al*., 2013).

Although there is increasing awareness of 3GC-R *K. pneumoniae* as well as its prevalence in different countries in the world and some parts of Nigeria, however, there is limited knowledge or research available on this subject matter in the study area.

This study is believed to have aided in understanding the molecular epidemiology of *K. pneumoniae* strains and also established a relationship between ESBL encoding genes responsible for ESBL enzymes causing increased resistance to 3GC among *K. pneumoniae* isolates of wound infection in the study area. Therefore, this study was necessary to determine the prevalence and molecular characteristics of third generation cephalosporin resistant *K. pneumoniae* isolated from wounds of patients attending selected Hospitals in Minna, Nigeria.

1.4 Aim and Objectives of the Study

The aim of this study was to determine the prevalence and molecular identity of third generation cephalosporin-resistant *Klebsiella pneumoniae* from wounds of patients attending selected Hospitals in Minna, Nigeria.

Objectives of the study

The objectives of this research were to:

i. isolate and identify *Klebsiella pneumoniae* from wound samples of patients attending selected Hospitals in Minna.

ii. determine the antibiotic susceptibility profile of *Klebsiella pneumoniae* isolates.

iii. screen 3GC-R *K. pneumoniae* for the production of Extended Spectrum Beta-Lactamases (ESBLs).

iv. detect and identify ESBL encoding genes among ESBL producing 3GC-R *K. pneumoniae* using molecular techniques.

CHAPTER TWO

2.0 LITERATURE REVIEW

2 .1 *Klebesiella pneumoniae*

Klebsiella pneumoniae is an enteric, encapsulated, Gram-negative, rod shaped, non-motile, lactose fermenting, opportunistic and facultative anaerobe (Aly *et al.*, 2014; Al-Agha, *et al*., 2017; Shi *et al.*, 2018). The bacterium is surrounded by a prominent polysaccharide capsule that encases the whole cell surface providing resistance against many host defence mechanisms, the capsule also protects the cell from drying (Qureshi, 2019). It belongs to genus *Klebsiella* of the tribe *Klebsiellae* and the *Enterobacteriaceae* family (Bidewell *et al*., 2018; Qureshi, 2019).

The organism inhabits the mouth, skin, and intestinal tract of humans and animals as normal micro flora and may also be isolated from different environmental sources (Al-Agha *et al*., 2017; Bidewel *et al*., 2018). *Klebsiella pneumoniae* was first described by Carl Friedlander in 1882 as a bacterium isolated from the lungs of patients who had died from pneumonia and was later named after Edwin Klebs (1834-1913), a 19th century German microbiologist (Bengoechea and Sa Pessoa, 2019). *Klebsiella pneumoniae* can cause a wide range of infections in people of all age groups with particular reference to neonates, the elderly, immunocompromised persons, diabetic patients, alcoholics and those who have extended stay in the hospital, particularly Intensive Care Units (Bengoechea and Sa Pessoa, 2019).

However, hypervirulent *K. pneumoniae* strains with raised production of capsular polysaccharide can affect healthy individuals causing life-threatening community-acquired infections (Li *et al*., 2014). It is described an opportunistic pathogen due to its ability to colonise the mucosal surfaces without causing pathology, however, from mucosae, *K. pneumoniae* may disseminate to other tissues causing serious infections like

pneumonia, urinary tract infections (UTIs), wound infections, bloodstream infections and sepsis (Paczosa and Mecsas, 2016).

The genus *Klebsiella* is currently grouped into 7 species with demonstrated similarities in DNA homology known. These include *Klebsiella pneumoniae, Klebsiella oxytoca, Klebsiella ozaenae, Klebsiella rhinoscleromatis, Klebsiella planticola, Klebsiella ornithinolytica* and *Klebsiella terrigena*. *Klebsiella pneumoniae* is the most medically important specie of this genus. *Klebsiella oxytoca* and *Klebsiella rhinoscleromatis* have also been encountered from human clinical specimens (Biradar and Roopa, 2015; Al-Agha *et al*., 2017).

These bacteria may spread horizontally among patients in the hospital via hospital personnel and equipment (Kothari *et al*., 2013). Presently, *K. pneumoniae* has been declared by several international healthcare organizations as an "urgent threat to human health," and represents the "K" of the "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species). The six most significant and dangerous causes of hospital-acquired multidrug resistant infections (CDCP, 2013).

Although, traditionally, *K. pneumoniae* has been regarded as an opportunistic pathogen however, it is also a causative agent of hospital-acquired infections accounting for high morbidity and mortality. Furthermore, an increasing number of community-acquired invasive *K. pneumoniae* infections are being reported globally (Marcoleta *et al*., 2018).

2.2 Clinical Importance of *Klebsiella pneumoniae*

Klebsiella pneumoniae is a major human pathogen that has been implicated in infections in healthcare settings over the past few decades. Antimicrobial treatment of *K. pneumoniae* infections has become increasingly difficult as a consequence of the

emergence and spread of strains that are resistant to multiple antimicrobials (Moradigaravand *et al*., 2017; Kidd *et al*., 2017; Ferreira *et al*. 2019). Members of the *Klebsiella* genus typically express 2 types of antigens on their cell surface. The first is a lipopolysaccharide (O antigen); the other is a capsular polysaccharide (K antigen). Both antigens contribute to pathogenicity. About 77 K antigens and 9 O antigen have been identified in *K. pneumoniae*. The structural variability of these antigens forms the basis for classification into various serotypes.

The virulence of all serotypes appears to be similar, and three species are associated with disease in humans (Qureshi, 2019). As an opportunistic pathogen, *K. pneumoniae* has been incriminated as the causative agent of both nosocomial and community acquired infections (Shi *et al.*, 2018; Bengoechea and Sa Pessoa, 2019). In humans, the common infections caused by *K. pneumoniae* include surgical site or wound infections, urinary tract infection (UTIs), pneumonia, bacteraemia osteomyelitis, meningitis, necrosis and endophthalmitis in patients with liver abscesses (Siu *et al*., 2012; Struve *et al*., 2015; Biradar and Roopa, 2015; Proskesch *et al*., 2016; Gorrie *et al*., 2018). Sepsis and septic shock ensue following entry of the bacteria into the blood. Though *K. pneumoniae* accounts for a small percentage of pneumonia cases, the case fatality rates are high as 90 % in untreated cases. *Klebsiella oxytoca* has also been incriminated in neonatal bacteraemia, especially among premature infants and neonates in intensive care units (Qureshi, 2019).

2.3 Epidemiology of *Klesiella pnuemoniae*

Klebsiella pneumoniae is ubiquitous in nature, in humans, *K. pneumoniae* exist as a saprophyte and may colonize the skin, gastrointestinal tract or pharynx. It also colonises sterile wounds, urinary tract and may be regarded as normal flora in many parts of the colon, intestinal and biliary tract. Carriage rates vary with different studies (Al-Agha *et*

al., 2017; Qureshi, 2019). *Klebsiella pneumoniae* and *K. oxytoca* are the 2 members of this genus responsible for most human infections. They are opportunistic pathogens found in the environment and in mammalian mucosal surfaces. The principal pathogenic reservoirs of infection are the gastrointestinal tract of patients and the hands of hospital personnel. These Organisms can spread rapidly, often leading to nosocomial outbreaks (Biradar and Roopa, 2015; Al-Agha *et al*., 2017; Qureshi, 2019).

In some parts of the world, *K pneumoniae* is an important cause of community-acquired pneumonia in elderly persons (Moradigaravand *et al*., 2017). Studies conducted in Malaysia and Japan estimated the incidence rate in elderly persons to be 15-40 %, which is equal to, if not greater than, that of *Haemophilus influenzae*. However, in the United States, these figures are different. Persons with alcoholism are the main population at risk, and they constitute 66 % of people affected by this disease. Mortality rates are as high as 50 % and approach 100 % in persons with alcoholism and bacteraemia. *Klebsiella pneumoniae* is also important in nosocomial infections among adult and paediatric populations. *Klebsiella* spp account for approximately 8 % of all hospital-acquired infections (Qureshi, 2019).

In Nigeria, various studies have reported different epidemiological prevalence of *K. pneumoniae.* For instance, Iregbu *et al.* (2013) in Abuja, North central reported the prevalence of *K. pneumoniae* to be 13 %, a study conducted in southwest by Oli *et al*. (2017) reported the incidence of 14.28 % for *K. pneumoniae,* 20 % was reported in Northwest by Giwa *et al*. (2019). Another study conducted in Southwest published the prevalence of *K. pneumoniae* infection as 26.8 % (Fadeyi *et al*., 2016). Idakwo *et al*. (2015) and Adabara *et al*. (2012) reported the prevalence of 26.95 and 39.1 % in Minna, North Central Nigeria respectively. Also, Nas *et al*. (2019) documented the incidence of *K. pneumonaie* to be 52.1 % in Kano, Nigeria.

2.4 Third Generation Cephalosporin

Cephalosporins are beta-lactam antibiotics derived from the mould *Acremonium* previously called *Cephalosporium*. They are bactericidal and have the same mode of action as other beta-lactam antibiotics; such as penicillins. cephalosporins disrupt the synthesis of the peptidoglycan layer of bacterial cell walls by binding to enzymes called penicillin binding proteins (PBPs). The peptidoglycan is an important substance for cell wall structural integrity, making it essential for the synthesis of the bacterial cell wall (Shabbaz, 2017).

Cephalosporins have similar structure and share mechanisms of action with penicillins in that they both have the same four-member “core” beta-lactam ring, however, cephalosporins have an additional atom in the side ring. Penicillin-susceptible pathogens are usually cephalosporin-susceptible, with exception of *Listeria* and *Pasteurella* sp likewise some penicillin resistant strains of *K. pneumoniae* are also resistant to third-generation cephalosporin (Harrison and Bratcher, 2008).

Cephalosporins are also called broad-spectrum antibiotics or extended spectrum cephalosporin due to their effectiveness against a wide range of Gram negative and Gram positive bacteria (Moore, 2017). Since the first cephalosporin was discovered in 1945, scientists have been improving the structure of cephalosporins to make them more effective against a wider range of bacteria. Each time the structure is modified, a new "generation" of cephalosporins are made. Till date, there are five generations of cephalosporins: first generation, second generation, third generation, fourth generation and fifth generation cephalosporins (Dowling *et al*., 2017). Third generation cephalosporin were the third generation of cephalosporin to be developed hence the name (Fookes, 2018)**.**

Third generation cephalosporin were introduced in the early 80s in clinical practice in response to increasing prevalence and spread of beta-lactamases in certain organisms, for example, ampicillin hydrolyzing TEM-1 and SHV-1 beta-lactamases in *Klebsiella pneumoniae* and *Escherichia coli* and the spread of these beta-lactamases into new hosts such as *Haemophilus influenza* and *Neisseria gonorrhoeae* (Paterson and Bonomo, 2005).

Beta lactamases are a group of enzymes that confer resistance to almost all the beta-lactam antibiotic by disrupting the peptide bond of the beta- lactam ring, they are grouped into four based on substrate inhibition and phenotypic characteristics such as molecular weight and isoelectric point (Paterson and Bonomo, 2005; Abdolhamid and Akbar, 2016; Da silva *et al*., 2019). However, the extended spectrum beta-lactamases are the most important group of these beta-lactamases (Abdolhamid and Akbar, 2016). Since these ESBLs were first reported in the mid-80s in Europe, ESBL producing organisms have evolved worldwide and are now one of the leading causes of hospital-acquired infections with increased morbidity and mortality rate (Harris *et al*., 2007; Kothari *et al*., 2013; Adabara *et al*., 2020).

Third generation cephalosporin have excellent efficacy against Gram-negative aerobic bacteria and the usual pathogens responsible for bacterial meningitis among children (Dowling *et al*., 2017).

In addition, third generation cephalosporins have greater *in vitro* activity against Gram-negative organisms, especially those with beta-lactamases when compared with earlier generations. The typical microbial targets of cefpodoxime include *Klebsiella pneumoniae, E. coli, Serratia, Acineobacter, Enterobacter, Proteus, Providencia, Morganella* and *Neisseria*. Only ceftazidime and cefoperazone that are much more efficient against *Pseudomonas aeruginosa* with ceftazidime having the greatest activity (Dowling *et al*., 2017).

Third generation cephalosporin represents important antibiotics for the treatment of serious infections caused by *K. pneumoniae* before the use of last-resort carbapenems (Wohlwend *et al*., 2015). The rationale for the use of extended-spectrum cephalosporin is based on their better ability to penetrate the central nervous system, eye, and prostate, and the possibility that they may prevent metastatic infections (Wohlwend *et al*., 2015).

Third generation cephalosporin are used for the treatment of the following types of infections when caused by susceptible strains of bacteria; bacteraemia, septicaemia, Skin and wound infections, bone and joint infections, central nervous system infections, gynaecological infections, Intra-abdominal infections, urinary tract infections and lower respiratory tract infections. Cephalosporins are not usually used as a first-line antibiotics, they tend to be reserved for use when other antibiotics like aminoglycoside, penicllins and fluroquinolones fail (Fookes, 2018).

Cephalosporins have been classified into five generations based on structural modification and wide range of activities against both Gram positive and Gram negative bacteria (Given in Table 2.1).

Table 2.1: Classification of cephalosporins

First generation cephalosporin	Second generation cephalosporin	Third generation cephalosporin	Fourth generation cephalosporin	Fifth generation cephalosporin
Cefazolin	Ceprozil	Ceftazidime	Cefepime	Ceftaroline
Cefadroxil	Cefaclor	Cefotaxime	Cefpirome	
Cephalexin	Cefuroxime	Ceftriaxone		
Cephradine	Cefoxitin	Cefixime		
	Cephamycins	Cefpodoxime		
	Laracarbef	Cefdinir		
	Cefotetan	Ceftibutin		
		Ceftizoxime		
		Maxalactam		
		Cefoperazone,		
		Cefditoren		

Source: Harrison and Bratcher (2008); Bartlett *et al*. (2010).

2.5 Wound and Wound Infection

Wound is defined as any physical injury involving a break on the skin or in the bone (Aftab *et al*., 2014; Negut *et al*., 2018). A wound can also be a simple or a severe disruption of the normal continuity of bodily structures such as the skin or a tissue due to trauma which can spread to other tissues and anatomical structures such as subcutaneous tissue, muscles, tendons, nerves, vessels, and even to the bone (Aftab *et al*., 2014; Negut *et al*., 2018).

The basic function of an intact skin is to prevent the invasion of microorganisms and colonization of the underlying tissues of the skin (Bowler *et al*., 2001). Exposure of subcutaneous tissue following a loss of skin integrity (wound) creates a moist, warm and nutritious environment that is conducive for microbial colonization and proliferation. Thus, any wound is at risk of becoming infected most frequently by more than one microbe which maybe potentially pathogenic (Bowler *et al*., 2001; Haesler and Ousey, 2018).

Wound infection is the presence of pus or purulence in a lesion as well as the general or local features of sepsis such as pyrexia, pain and induration. Infection is believed to occur when virulence and pathogenic factors expressed by one or more microorganisms in a wound out-compete the host natural immune system (Pondei *et al*., 2012; Haesler and Ousey, 2018). Infected wounds are characterized by bacterial burden, chronic inflammation, and unbalanced cellular defence mechanism (Mohammed *et al*., 2017**)**.

The microorganisms implicated in wound infection include; Gram positive bacteria which range from *Staphylococcus aureus, Streptococcus pyogenes* to *Enterococcus faecalis*. Members of the Gram negative pathogens include *Pseudomonas aeruginosa, Klebsiella* species, *Escherichia coli*, *Proteus* species, *Enterobacter* species, *Bacteriodes*, and *Clostridium*, Fungi associated with wound infection are *Candida* species and *Aspergillus*

species (Aftab *et al*., 2014; Pujji *et al*., 2019). Some wounds harbour more than one microorganisms, such wounds are said to be polymicrobial especially in the margin of wounds and in chronic wounds (Jerry *et al*., 2018).

Wound infections is reported as one of the most common hospital acquired infections and are an important cause of morbidity that has accounted for 70-80 % mortality in patients irrespective of the cause of the wound (Jerry *et al*., 2018). Wound infection remains a great challenge to the clinical management of wound regardless of the improvement made in surgical interventions and chemotherapy (Pondei *et al*., 2012).

2.5.1 Classification of wounds

Wounds are largely classified as having either an acute or a chronic aetiology. Acute wounds may occur suddenly rather than over time and are caused by external damage to intact skin and may include surgical wounds, bites, burns, minor cuts and abrasions, and more severe traumatic wounds such as lacerations and those caused by crush or gunshot injuries (Bowler *et al*., 2001).

Irrespective of the nature of the cutaneous injury, acute wounds are expected to heal within a predictable time frame, although the treatment required to facilitate healing will vary according to the type, site, and depth of a wound. While chronic wounds develop when acute wounds fail to heal within a stipulated time frame. When a chronic wound displays signs of delayed healing it might take a longer time to heal (Haesler and Ousey, 2018). Chronic wounds are most frequently caused by endogenous mechanisms associated with a predisposing condition that ultimately compromises the integrity of dermal and epidermal tissue. Fewer examples of chronic wounds include ulcer, decubitus and burn wounds (Bowler *et al.*, 2001). When wound healing does not progress normally, it may result in a chronic wound and this cumulates significant burden to both the patient and the clinicians (Han and Ceilley, 2017).

2.5.2 Risk factors associated with wound infection

The progression of a wound to an infected state involves a multitude of factors including breakage on the skin, old age, repeated trauma, blood perfusion or homeostasis, immune suppression and coexisting morbidity, all of which impair wound healing, increasing the risk of infection. Other risk factors reported include surgical technique, diabetes, obesity, prolonged hospitalisation which increases risk of infection by multidrug resistant organisms, and length of operation. The type, site, size, and depth of the wound and the combined level of virulence expressed by the types of microorganisms involved have also been reported to facilitate wound infection. Thus, knowledge of risk factors associated with infections could help strengthen efforts to reduce their occurrence, thus beating down morbidity and mortality for these infections (Sani *et al*., 2012; Kihla *et al*., 2014; Hussein *et al*., 2016).

2.6 Antimicrobial Resistance

Antibacterial resistance is the reduction in effectiveness of a drug such as antibiotics in curing infection or a condition (Shaikh *et al*., 2015). Antimicrobial resistance has been identified by the World Health Organization (WHO) in 2019, as one of the ten threats since it affects healthcare system and the successful prevention and treatment of infectious diseases (Barchitta *et al*., 2019). Internationally, there is a growing concern over antimicrobial resistance (AMR) which is currently estimated to be responsible for more than 700,000 deaths per annum worldwide. If drastic and proactive measures are not taken to stop its progress, antimicrobial resistance will cost approximately 10 million lives and about 100 trillion dollars per year by 2050 (Tadesse *et al*., 2017). Furthermore, with the increased use and misuse of antibiotics, bacterial resistance has emerged among common pathogens and are threatening the effectiveness of even the most potent and reliable antibiotics (Adekunle, 2012).

The emergence and spread of multidrug resistant (MDR) bacteria, especially members of the *Enterobacteriaeceae* such as *Klebsiella pneumoniae* and *Esherichia coli* have further complicated therapeutic options, leading to serious clinical and public health crises worldwide (Mulani *et al*., 2019). Multidrug resistant is defined as bacteria exhibiting resistance to at least one agent in three or more antibiotics classes (Basak *et al*., 2016; Venezia *et al*., 2017; Da Silva *et al*., 2019).

Infections with multidrug-resistant *K. pneumoniae* strains and *E. coli* can result in extended hospital stay, treatment failure and higher mortality rates and incurred financial burden (Kim *et al*., 2016; Kidd *et al*., 2017; Venezia *et al*., 2017). Of recent, World Health Organization (WHO) has also listed ESKAPE pathogens in the list of 12 bacteria against which new antibiotics are urgently needed (Tacconelli *et al*., 2018). They describe three categories of pathogens which include critical, high and medium priority, according to the urgency of need for new antibiotics. Carbapenem resistant *A. baumannii* and *P. aeruginosa* along with extended spectrum beta-lactamase (ESBL) or carbapenem resistant *K. pneumoniae* and *Enterobacte*r spp. are placed in the critical priority list of pathogens; whereas, vancomycin resistant *E. faecium* (VRE) and methicillin and vancomycin resistant *S. aureus* (MRSA and VRSA) are in the list of high priority group (Mulani *et al*., 2019).

2.6.1 General mechanism of antibacterial resistance

Antimicrobial compounds were chemically and biologically synthesized to kill (bactericidal) or inhibit (bacteriostatic) bacterial growth (Knopp, 2018). However, over the years, bacteria have developed several resistance mechanisms by which they can thwart antimicrobial agents, rendering them ineffective (Da Silva *et al*., 2019). Generally, the mechanisms of antimicrobial resistance in bacteria are broadly categorised into four groups; enzymatic inactivation or alteration of the antimicrobial agent commonly by an

irreversible cleavage, increased efflux pumps of the antibiotics, reduced drug accumulation due to decreased permeability of the cell wall and modification of the target site where the antibiotics usually bind (Santajit and Indrawattana, 2016). Among these mechanisms, the enzymatic degradation and efflux pump systems play an important role in the development of multidrug resistance in *K. pneumoniae. Klebsiella pneumoniae* strains produce enzymes, including extended-spectrum beta-lactamases, metallo-b-lactamases, oxacillinases, and carbapenemases, that can degrade beta-lactam antibiotics (Kim *et al*., 2016).

2.6.1.1 Intrinsic (Natural) resistance

In intrinsic resistance, microorganisms naturally do not possess target sites for the antimicrobial agents or they naturally have low permeability to those agents because of the differences in the chemical nature of the drug and the bacterial membrane structures especially for those that require entry into the bacterial cell in order to exert their action. Thus, with intrinsic resistance the organism possesses properties that makes it naturally resistant to certain assaults thereby incapacitating the antibacterial agents. Since bacteria tend to have a natural process that encourages resistance, this resistance process can occur through gene level mutations whereby antibiotics induce selective pressure and the genes act in association with selective pressure (Zaman *et al*., 2017).

2.6.1.2 Acquired resistance

Acquired resistance is whereby an organism that is naturally susceptible to antimicrobials gains resistance mechanism and becomes non-susceptible to the antimicrobial agents (Dowling *et al*., 2017). Hence bacteria possess the ability to directly transfer genetic material between each other via plasmids. This implies that natural selection is not the only mechanism by which resistance evolves (Zaman *et al*., 2017).

2.6.1.3 Enzymatic inactivation or alteration of the antimicrobial agents

Most bacteria acquire high level of resistance by functional inactivation of the antibiotic, this can be done via chemical modification of the antibiotic that is catalysed by enzymes expressed in the resistant cell (Knopp, 2018). In *K. pneumoniae*, the most potent and significant mechanism of antibacterial resistance is enzymatic hydrolysis of wide range of antibiotics such as bet-lactams, quinolones, aminoglycosides and macrolides (Qamar *et al*., 2019).

2.6.1.4 Modification of target sites

Antibiotics exert their antibacterial effect by specifically binding to their target sites. To prevent this binding, bacteria can undergo mutations in the target sites to decrease its affinity to the antibiotic without disrupting its function. This resistance mechanism typically arises by chromosomal mutations and is therefore not laterally transferred. For example, mutations in the target-encoding genes *rpsL*, *fusA*, *rpoB* or *gyrA* can increase resistance towards streptomycin, fusidic acid, rifampicin or ciprofloxacin, respectively. Another resistance mechanism to reduce antibiotic binding is target modification, where the gene encoding the cellular target is unaltered, but the protein is post-transnationally modified to cause a reduction in binding affinity. Besides a direct alteration of the antibiotic target, resistance can also be caused by laterally transferred homologous genes with a low affinity for the antibiotic that can functionally replace the inhibited cellular target. This type of resistance is frequent among methicillin resistant *Staphylococcus aureus*. In a susceptible strain, the penicillin binding protein (PBP) 2a is inhibited by methicillin, causing cell death. Resistant strains often encode an additional PBP2a. This variant has a lower binding affinity towards beta-lactam antibiotics including methicillin, causing loss of susceptibility. For certain antibiotics, resistance can be achieved by overexpression of the target (Knopp, 2018).

2.6.1.5 Increased efflux

Efflux pumps are transport proteins that are involved in expulsion of toxic substrates from within the cells into the environment. These substances include all classes of clinically important antibiotics. The pumps are present in both Gram-positive and Gram – negative bacteria including eukaryotic cells. Pumps that transport multiple antibiotics are usually associated with Multi-drug resistance (MDR). There are five principal families of efflux transporters, Resistance–Nodulation Division (RNA), Major Facilitator (MF), Small Multi-drug Resistance (SMR), Multi-drug and Toxic Efflux (MATE), and ATP binding cassette (ABC).The AcrA and B-TolC efflux pump, belonging to the RND family, is the most important and relevant efflux system in *Klebsiella pneumoniae* and *E. coli* (Da Silva *et al*., 2019). These efflux pumps are made up of efflux protein such as *Acr B* encoded by *Acr B* gene located in cytoplasmic membrane of the bacterial cell. Also, there is an accessory protein also called membrane fusion protein, example, *Acr A* encoded by *Acr A* gene which is located in the periplasmic space and an outer membrane protein also known as outer membrane protein channel, such as TolC encoded by TolC genes, also located in the outer membrane of bacteria (Krishnamoorthy *et al*., 2013).

All *K. pneumoniae* strains harbour numerous MDR efflux pumps such as AcrAB, KexD, KdeA, KmrA, kpnEF and kpnGH genes including CmeA, CmeB, MATE, MFS, MacA, MarcB, MarA, OML, and RND conferring resistance to wide spectrum of antimicrobial agents (Dsouza *et al*., 2017; Founou *et al*., 2019). The efflux pumps, belonging to the resistance-nodulation-division (RND) family, can extrude amphiphilic and charged antibiotics such as beta-lactams, aminoglycosides and fluoroquinolones (Kim *et al*., 2016).

Figure 2.1 gives an illustration of the resistance mechanisms in both Gram-negative and Gram-positive bacteria.

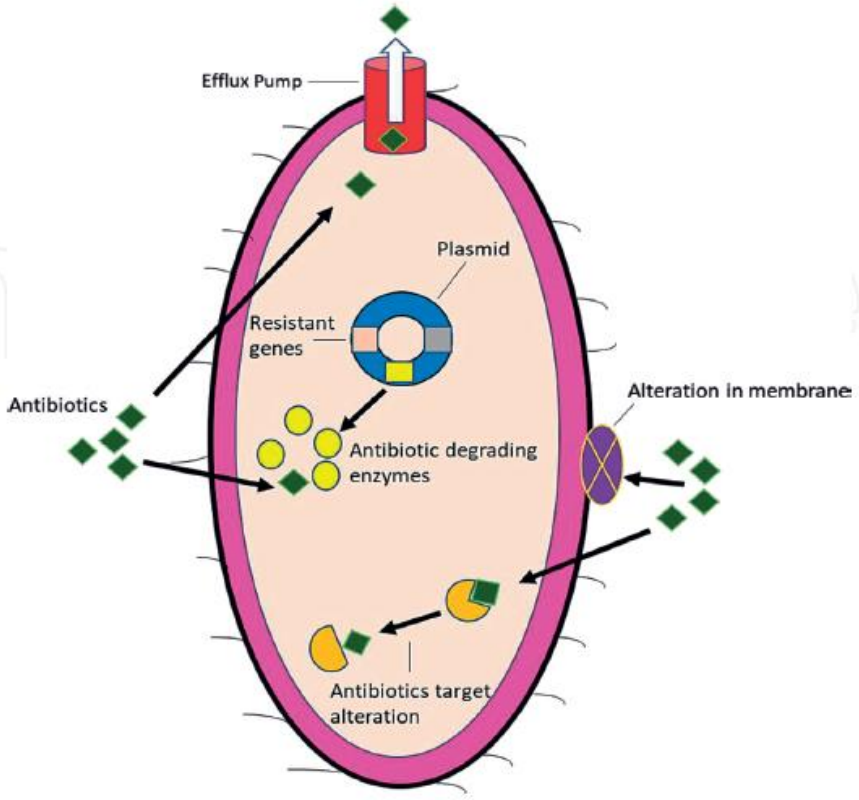

Figure 2.1: Antimicrobial resistance mechanisms.

Source: Qamar *et al*. (2019)

2.6.2 Resistance to third generation cephalosporin

The emergence of resistance to third generation in *K. pneumoniae* and other *Enterobacteriaceae* is a serious public health challenge (Venezia *et al*., 2017). One of the most important mechanisms of resistance to 3GC in *K. pneumoniae* and *E. coli* is the synthesis of Extended Spectrum Beta-lactamases (ESBLs), a family of beta-lactamase enzymes conferring resistance to nearly all beta-lactam antibiotics by hydrolysing beta-lactam ring except carbapenems and cephamycins however, they are inhibited by clavulanic acid (Da Silva *et al*., 2019; Ibrahim *et al*., 2019). Different researchers have reported the prevalence of ESBL positive strains in several continents of the world and this varies from one hospital in a particular region to another. This may be as a consequence of wide spread use of third generation cephalosporin leading to mutation in genes producing ESBL enzymes (Kothari *et al*., 2013).

Genes encoding ESBLs are typically found on plasmids that also carry other antibiotic resistance genes, often rendering ESBL-producing strains multidrug-resistant. In addition, not all resistance to third generation cephalosporin among *K. pneumoniae* is mediated by ESBLs. Some *K. pneumoniae* strains may have acquired plasmids harbouring AmpC beta-lactamases (Livermore and Brown, 2005). Thus, resistance to 3GC in *Enterobacteriacea*e could also be caused by other plasmid and chromosomally encoded enzymes, such as carbapenemases (Logan *et al*., 2014; Wei *et al*., 2018; Ibrahim *et al*., 2019).

Third generation cephalosporins were specifically designed to resist the hydrolytic action of major β-lactamases (Qadeer *et al*., 2013; Da Silva *et al*., 2019)**.** However, the mutations of the classical CTX-M types, TEM-1 and SHV-1 beta-lactamases have rendered these Extended Spectrum Cephalosporin ineffective, resulting in increased morbidity and mortality. Furthermore, the high level of resistance to third generation cephalosporins reported in *K. pneumoniae* and *E. coli* strains is an indication that treatment of severe infections caused by these members of the *Enterobacteriaceae* may rely on carbapenems, the last resort for the treatment of severe community and hospital acquired infections. However, the prevalence of carbapenem-resistant *K. pneumoniae* (CR-KP) has also emerged in recent years. Several countries have reported more than 10 % *K. pneumoniae* resistance to carbapenem resulting to relevant public health concern worldwide (Barchitta *et al*., 2019; Meng *et al*., 2019). *Escherichia coli* and *K. pneumoniae* have the ability to produce ESBLs in very large quantities. The genes encoding the ESBLs are found on plasmids and have a great propensity to spread between bacteria (Giske *et al*., 2008). These plasmids also harbour resistance genes for other antibiotics groups. The most frequent co-resistance found in ESBL producing organisms are to aminoglycosides, fluoroquinolones, tetracyclines and chloramphenicol (Chatterjee *et al*., 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 The Study Area

The research was conducted in Minna, Minna is a city in North Central region of Nigeria with an estimated population of 304,113 in 2006 (NPC, 2006). It is located at 9.62 latitude and 6.55 longitude and the city is raised 243 metres above sea level. It is the biggest city in Niger State. The State has two seasons the wet (rainy) and the dry season. The State has an area of 76,363 km^2. The major indigenous tribes in the State are Nupe, Gbagyi, Kambari, Dakawa, Hausa, and Koro (Adabara *et al*., 2012).

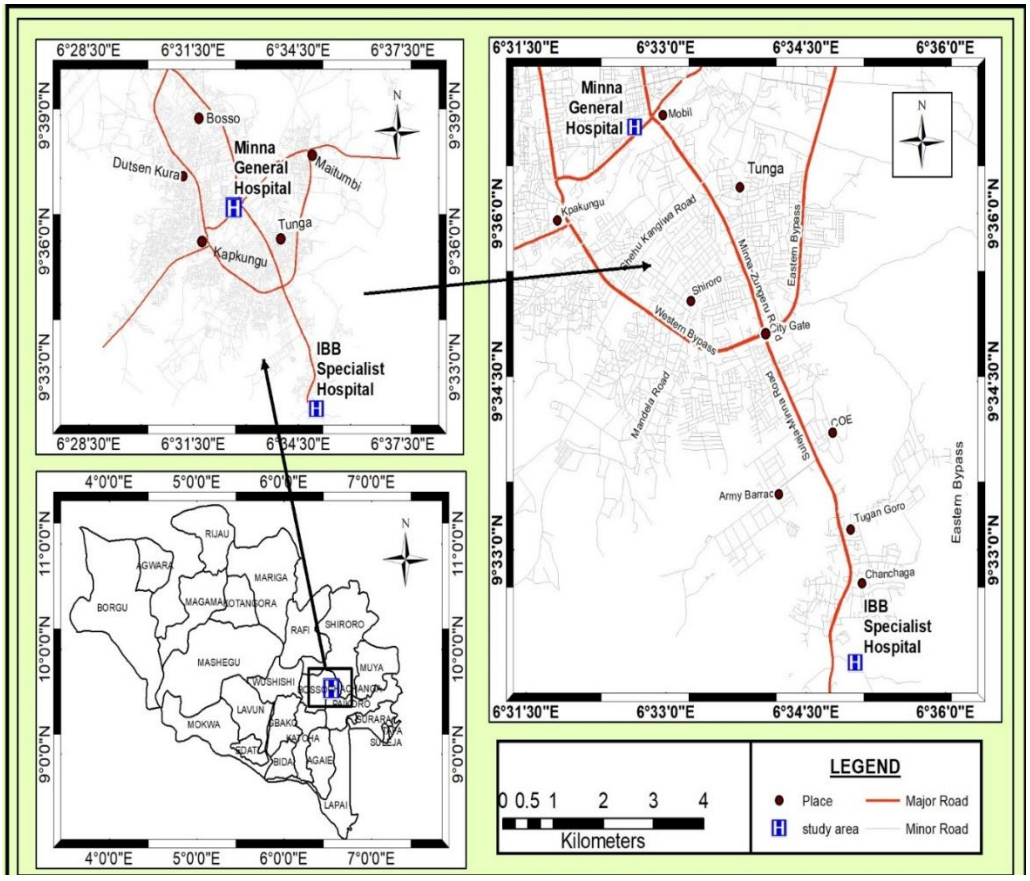

Figure 3.1: Map of the study area

Source: GIS (2021)

3.2 Ethical Clearance

The study procedure was carefully reviewed and approved by the ethical committees of General Hospital and IBB Specialist Hospital Minna.

3.3 The Study Population

The studied population consisted of male and female patients between the ages of 0 (less than one year of age) and 90 years old with suspected wound infections in General Hospital and IBB Specialist Hospital Minna, Nigeria. This study was conducted between June and December 2019. Informed consent of the patients was obtained prior to specimen collection.

3.4 Sample Size

The sample size for this study was decided by using a standard epidemiology formula for minimum sample size calculation (Nas *et al*., 2019).

The sample size was determined in equation 3.1

$$N = \frac{(Z\text{-}a)^2 \text{ X } P\ (1\text{-}P)}{D^2} \quad \ldots\ldots\ldots\ldots \text{ equation 3.1}$$

Where

N = required minimum sample size

Z_1-a = Confidence level at 95 % was found to be 1.96

P = the estimated prevalence of *K. pneumoniae* infection in Minna obtained from previous studies by Idakwo *et al*. (2015) was 26.95 % (0.2695)

D= Precision error at 5 % was 0.05

$$N = \frac{(1.96)^2 * 0.2695(1\text{-}0.2695)}{0.05^2} = \frac{3.8416 * 0.1971}{0.0025} = \frac{0.757}{0.0025} = 302.8 \text{ samples}$$

The minimum required number of samples for this study was 302.8. However, to create padding effect for data attrition, the number of samples were increased to 500 in this study.

3.5 Inclusion Criteria

Wounds of patients from age 0 to 90 years attending General Hospital and IBB Specialist Hospital Minna were included in this study.

3.6 Exclusion Criteria

Wounds of patients who were not attendees of General Hospital and IBB Specialist Hospital Minna were not included in this study.

3.7 Sample Collection

A total of five hundred (500) wound samples were aseptically collected from indoor and outdoor patients attending General Hospital and Ibrahim Badamasi Specialist Hospital Minna, Nigeria. The skin surrounding the wounds was cleaned with normal saline before swabbing with sterile swab sticks to avoid contamination from skin normal flora. The samples were transported within few hours of collection to "Step B" Laboratory complex, Federal University of Technology Minna, Niger State Nigeria, in an ice pack under sterile condition.

3.8 Inoculation of Wound Swabs for *K. pneumoniae* Isolation

Wound samples were inoculated on MacConkey agar plates by making a pool and carefully streaking out the surface of the Agar plates with a standard flamed wire loop, the plates were incubated aerobically at 37 $^{\circ}$C for 24 hours. After 24 hours, discrete, large, raised, viscous, mucoid, lactose-fermenting and pink coloured colonies suggestive of *K. pneumoniae* were carefully picked and subcultured on Nutrient Agar plates and were

further incubated for another 24 hours at 37 °C in order to obtain pure isolates. Pure isolates were subcultured on Nutrient Agar slant and stored for further analysis.

The identity of *K. pneumoniae* isolates were ascertained by Gram staining, motility and detailed biochemical tests such as; catalase, coagulase, urea hydrolysis, oxidase, Simmon's citrate utilization, sugar fermentation, indole production test, methyl red, triple sugar iron (TSI) agar and Voges-Proskauer (Cheesbrough, 2010). The identity of *K. pneumoniae* were confirmed by molecular analyses.

3.8.1 Gram staining

Gram staining of each isolate was carried out as described by (Cheesbrough, 2010). A thin smear of each of the test organism from an overnight culture was prepared using a sterile wire loop to pick a colony onto a clean slide with a loopful of normal saline, thereafter, the smear was allowed to air-dry and heat fixed. This was stained with 0.5 % of crystal violet for 60 seconds, then the water was drained off while lugol's iodine was added to the smear for another 60 seconds and was rinse with clean water. After which, acetone was used to decolourise the smear rapidly until the original visible colour of the primary dye disappeared and it was rinsed immediately with clean water. The smear was counterstained with safranin solution for 30 seconds and it was rinsed with distilled water and air-dried. The stained slide was examined under oil immersion microscopically using (x 100) objective lens (Chessbrough, 2010).

3.8.2 Motility test

The test isolates were inoculated into tube containing semi-solid agar by stabbing the agar butt in the centre and down the tube with a flamed-sterilized straight needle, the inoculating needle was vertically withdrawn to avoid spreading the inoculum beyond the original stab line. The tubes were incubated at 37 °C for 24-48 hours, after 24-48 hours of incubation, the pattern of turbidity was examined. Growth occurring only along the

line of inoculation was considered as positive result for non-motile bacterial isolates while growth occurring throughout the agar was regarded as motile isolates. Triphenyl Tetrazolium Chloride (TTC) was added to the motility butt to show where growth had occurred. The bacteria reduced the TTC which is red in colour (Cheesbrough, 2010).

3.8.3 Biochemical tests

Catalase test: A microscope slide was placed inside a sterile Petri dish, exactly one drop of 3 % H_2O_2 was placed on the clean slide with the aid of Pasteur pipette. Using a sterile and standardized inoculating loop, a loopful of the test organism was emulsified on the hydrogen peroxide. The Petri dish was covered immediately with a lid to prevent aerosols. Rapid and immediate bubble formation was observed for positive test (Cheesbrough, 2010).

Coagulase test: A drop of distilled water was placed on each end of a clean grease-free slide, followed by emulsification of colonies of the test organism to make two thick suspensions. A loopful of plasma were added to one of the suspensions and mixed gently, clumping of the test organism was observed within 10 seconds, indicating positive results. However, no plasma was added to the second suspension, it was used as control to distinguish granular appearance of the test organism from the actual coagulase clumping (Cheesbrough, 2010).

Urea hydrolysis test: Colonies of the test organism were inoculated on urea based agar medium in glass test tubes, streaked and incubated at 37 $^{\circ}$C for 24 hours. A change in colour from yellow/orange- pink indicated urease production by the isolates. Hence urease positive result was confirmed. Urease positive organisms were able to hydrolyse urea with the help of the urease enzyme. Whereas no pink colour change was concluded as negative urease test (Cheesbrough, 2010).

Oxidase test: A piece of filter paper was soaked with a few drops of oxidase reagent. A colony of the test organism from a fresh (24 hours culture) was then smeared on the filter paper. An oxidase reagent strip was also used. Uniform colonies of the test organism was smeared on the oxidase reagent strip. The oxidase-producing organisms oxidized phenylenediamine in the reagent in to a deep purple colour (Cheesbrough, 2010).

Citrate utilisation test: Exactly 24.8 g of Simmon's Citrate Agar was dissolved in 1000 mL of water. 10 mL each was dispensed aseptically into test tubes and were sterilized for 15 minutes at 121 °C, the tubes were allowed to set as slants. A sterile wire loop was used to pick isolated colonies of the test organism from lawn culture and was inoculated on Simmon's citrate Agar slant by stabbing the butt and streaking the slant lightly in a zigzag manner. The slants were incubated at 37 °C for 2-4 days. A change in colour from green to intense blue with visible growth along the slant indicated citrate utilization by the isolates due to the reaction of the bromothymol blue indicator with the streak of growth colonies. Hence the test was positive. However, lack of colour change in the test medium was regarded as a negative result.

Sugar fermentation test: The bacterial isolates were tested for sugar fermentation using six (6) sugars individually; Glucose, lactose, fructose, sucrose, galactose and mannitol. Nutrient broth containing 0.5 % of each of the sugars, phenol red indicator and inverted Durham tubes were prepared and sterilized by boiling at 121 °C for 15 minutes. The test isolates were inoculated in each mixture of the sugar solution and incubated for 24 hours at 37 °C. Colour change from red to yellow as a result of acid production indicated sugar fermentation while gas production was demonstrated by the presence of bubbles trapped in the inverted Durham tubes. However, if there was no colour change of the medium it was regarded as a negative result (Cheesbrough, 2010).

Indole production test: Exactly 0.5 mL (five drops) of Kovac's indole reagent was added to a 24 hours old nutrient broth culture of the test organism in test tubes. The tubes were shaken gently and allowed to stand. Formation of red colour in the reagent layer at the tip of the medium within some seconds in the presence of indole indicated positive result while appearance of orange or yellow colour depicted negative result which confirmed the presence *K. pneumoniae* (Cheesbrough, 2010).

Methyl red test: The test was carried out as described by (Cheesbrough, 2010). A loopful of the test isolates were inoculated in 5 mL of glucose phosphate peptone water in test tubes and were incubated for 24-48 hours at 37 °C. Three drops of methyl red indicator were added to the medium and mixed gently. Reactions were observed, appearance of a bright red colour upon addition of methyl red solution was regarded as positive methyl red test while yellow colour indicated negative result.

Triple sugar iron test (TSI): The test organism was inoculated on TSI slants by stabbing through the centre of the agar to the butt and then streaking on the surface of the agar slopes using a straight inoculating loop. The slants were incubated for 24 hours at 37 °C. The change in colour of the medium from red to yellow both at the butt and slant indicated acid production from glucose. Gas production was observed by bubbles and cracks in the medium. Blackening of the medium was confirmed as H_2S production (Cheesbrough, 2010).

Voges-Proskauer test: A loopful of the test organisms were inoculated into 5 mL of peptone water in test tubes and were incubated at 37 °C for 24 hours. After incubation, 1 mL of 40 % KOH and 3 mL of alpha-naphtol were added to the suspensions and allowed to stand for at least 15 minutes. The formation of an eosin pink colour depicted a positive V-P test result while negative result showed no colour change in the medium (Cheesbrough, 2010).

3.9 Antibiotic Susceptibility Test

Antibiotic Susceptibility profile of fifty seven (57) *K. pneumoniae* isolates from wounds was determined using ten (10) commercially prepared antibiotics used for the treatment of bacterial infections. The antibiotics tested were cefotaxime, ceftazidime, ceftriaxone, Cefixime, Cefpodoxime, Cefdinir, Imipenem, Gentamycin, Ciprofloxacin, and Ampicillin (Table 3.1). The antibiotic susceptibility profile of (57) *K. pneumoniae* was evaluated by Kirby-Bauer modified disc agar diffusion (DAD) technique as recommended by Clinical and Laboratory Standard Institute guidelines (CLSI, 2018). Discrete colonies of *K. pneumoniae* isolates from 24 hours culture of Nutrient Agar plates were suspended in 3 mL of sterile physiological saline and the turbidity was adjusted to 0.5 Mcfarland standard (10^{-8} cfu/mL). Sterile swab sticks were used to inoculate the bacterial suspensions evenly on the surface of the Mueller Hinton agar (MHA) plates in three directions, rotating the plates at 60 °C. The inoculated plates were allowed to dry for 15 minutes. Sterile forceps were used to place the antibiotic disc on the surface of the agar. The plates were inverted after 30 minutes of disc application and were incubated aerobically for 18-24 hours at 37 °C.

In accordance with the diameter of the zones of growth inhibition around the antibiotics discs, the *K. pneumoniae* strains were interpreted as sensitive, intermediate and resistant to particular antibiotics (Table 3.2) (CLSI, 2018). Multidrug resistance (MDR) of *K. pneumoniae* isolates was defined as resistance to at least 1 agent in 3 or more antibacterial classes (CLSI, 2018). Multiple Antibiotic Resistance Index (MARI) of each of the *K. pneumoniae* isolates was defined as a/b, where "a" was the number of antibiotics to which the test isolate was resistant to and "b" represented the total number of antibiotics used against each isolate (Liberto *et al*., 2009). All the antibiotic discs were ordered from Oxoid Ltd, UK.

Table 3.1: Classes of antibiotics used and their examples

Antibiotic classes	Examples of antibiotics
Third generation Cephalosporin	Cefotaxime (30 μg), Ceftazidime (30 μg), Ceftriaxone (30 μg), Cefixime(5 μg), Cefpodoxime (10 μg) and Cefdinir (5 μg)
Carbapenem	Imipenem (10 μg)
Penicillin	Ampicllin (10 μg)
Aminoglycoside	Gentamycin (10 μg)
Fluoroquinolone	Ciprofloxacin (5 μg)

Table 3.2: Clinical and Laboratory Standard Institute (CLSI) 2018 interpretative zone chart

Antibacterial agent	Susceptibility	Intermediate	Resistance
Cefotaxime (CTX)	≥ 26	23-25	≤ 22
Ceftazidime (CAZ)	≥ 21	18-20	≤ 17
Ceftriaxone (CRO)	≥ 23	20-22	≤ 19
Cefixime (CFM)	≥ 19	16-18	≤ 15
Cefpodoxime (CPD)	≥ 21	18-20	≤ 17
Cefdinir (CDR)	≥ 20	17-19	≤ 16
Imipenem (IMP)	≥ 23	20-22	≤ 19
Gentamycin (CN)	≥ 15	13-14	≤ 12
Ciprofloxacin (CPX)	≥ 21	16-20	≤ 15
Ampicillin (PN)	≥ 17	14-16	≤ 13

Source: CLSI (2018)

3.10 Phenotypic screening for the Production of Extended-Spectrum Beta-Lactamase Enzymes (ESBL) using Double Disc Synergy (DDST) test Method

The 57 *Klebsiella pneumoniae* isolates demonstrated resistance to at least one of the six (6) third generation cephalosporins tested, hence, they were all screened and confirmed for the production of extended spectrum beta-lactamase enzymes (ESBLs) using double disc synergy test method (DDST) as described by (CLSI, 2018).

A 24 hours culture of the test organism was inoculated in physiological saline and incubated at 37 °C for 2-6 hours to obtain a cell concentration equal to that of 0.5 McFarland standard. A sterile cotton swab was used to seed the inoculum concentration on the surface of Mueller-Hinton Agar plates. Discs of (20 μg) of amoxicillin + (10 μg) of clavulanic acid were centrally placed and surrounded by discs of 3rd-generation cephalosporin: cefotaxime (30 μg), ceftazidime (30 μg) and cefpodoxime (10 μg) at a distance of at least 20 mm apart from the centrally placed disc of amoxicillin (20 μg) /clavulanic (10 μg). Plates were incubated at 37 °C for 18-24 hours and zones of inhibition were observed. The production of ESBL was interpreted as the 3rd-generation cephalosporin disc inhibition zone shape enhanced or increased towards the Amoxicillin + Clavulanic acid disc, confirming that some of the 3GC-R *K. pneumoniae* isolates were ESBL producers. Also, the presence of ESBLs among 3GC-R *K. pneumoniae* isolates were further confirmed by measuring the diameter of the zones of growth inhibition of third generation cephalosporin discs alone and comparing with the size of the zones of 3rd-generation cephalosporin disc combination with amoxicillin (20 μg) /clavulanic (10 μg) disc. Bacterial isolates which exhibited the increase of ≥5 mm in zone of inhibition for ceftazidime, cefotaxime and cefpodoxime tested in combination with clavulanic acid compared to zone of third generation cephalosporin when tested alone were confirmed as ESBL producing 3GC-R *K. pneumoniae*. Whereas, negative ESBL reaction was

evaluated by lack of enhanced or increased zone of inhibition towards the amoxicillin /clavulanic acid. (CLSI, 2018). The enhancement in zone of inhibition after the introduction of amoxicillin (20 μg) /clavulanic acid (10 μg) is due to the presence of clavulate in the amoxicillin disc that inactivated the ESBL enzymes produced by the test isolates.

3.11 Selection Criteria of the Isolates for Molecular Analysis

The selection of ESBL producing 3GC-R *K. pneumoniae* for molecular analysis was based on multiple antibiotic resistance index analysis of the 23 ESBL producers. The ESBL producers with higher MARI between (0.6-1.0) were categorised in to five groups.

Extended spectrum beta-Lactamase producer (GH205) with MARI of 0.6 was characterised by resistance to (Third generation cephalosporin and aminoglycoside) based on this resistance index, GH205 was selected from group one (1). Similarly, ESBL producer (IBB164) with MARI of 0.7 expressed resistance to (3GC, Fluoroquinolone and Penicillin) hence, IBB164 was extracted from group two (2). Also, ESBL producer (GH22) having multiple antibiotic resistance index of 0.8 demonstrated resistance to (3GC, Aminoglycoside and Penicillin), based on this resistance index, GH22 was extracted from group three (3). Also, IBB16 with MARI of 0.9 exhibited resistance to (3GC, Aminoglycoside, Fluoroquinolone and Penicillin). Based on this MARI, ESBL producer (IBB16) was selected from group four (4). Finally, ESBL producer (GH107) with multiple antibiotic resistance index of 1.0 exhibited resistance to all the antibiotic categories (3GC, Carbapenem, Aminoglycoside, Fluoroquinolone and Penicillin) tested in this study, based on this MARI, GH107 was selected from group five (5). Table 3.3 shows the selection criteria of the ESBL producing 3GC- *K. pneumoniae* for molecular analysis.

Table 3.3: Selection criteria of the isolates for molecular analysis

Group	ESBL producers	No. Resistance	No. Antibiotic class	MARI
1	GH205	6	2	0.6
2	IBB164	7	3	0.7
3	GH22	8	3	0.8
4	IBB16	9	4	0.9
5	GH107	10	5	1.0

Key**:** No = Number

3.12 Molecular Identification of ESBL-Producing 3GC-R *K. pneumoniae* by Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a technique used for amplification and analysis of short sequence of Deoxyribonucleic acid (DNA) or Ribonucleic acid (RNA) even in specimens containing minute quantities of DNA or RNA. Usually, three main steps are involved in PCR. The steps are repeated for 30-40 cycles. The cycles are performed on an automated cycler, a device which rapidly heats and cools the test tubes containing the reaction mixture. Each step denaturation (alteration or modification of DNA structure), annealing (Joining of primers to the DNA sequence to be copied) and extension (attachment and copying of templates by polymerase enzymes) takes place at different temperatures (Cox, 2015).

3.12.1 DNA extraction

DNA (Deoxyribonuleic acid) was extracted using the protocol stated by (Trindade *et al*., 2007). Briefly, single colonies grown on medium were transferred to 1.5 mL of liquid medium and cultures were grown on a shaker for 48 hours at 28 ºC. After this period, cultures were centrifuged at 4600 g for 5 (five) minutes. The resulting pellets were re-suspended in 520 µL of TAE (Tris Ethylene Diamine Tetra acetic acid) buffer (10 mMTris-HCl, 1 mM EDTA and pH 8.0). Fifteen microliters of 20 % SDS (Sodium Dodecyl sulfate) and 3 µL of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 (one) hour at 37 ºC, then 100 µL of 5 M NaCl and 80 µL of a 10 % CTAB (Cetyltrimethylammonium bromide) solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 minutes at 65 ºC and kept on ice for 15 minutes. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 minutes and centrifugation at 7200 g for 20 minutes. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated

at –20 ºC for 16 hours. DNA was collected by centrifugation at 13000 g for 10 minutes, washed with 500 µL of 70 % ethanol, air-dried at room temperature (25°C) for approximately three hours and finally dissolved in 50 µL of TAE (Tris Ethylene Diamine Tetra acetic acid) buffer.

3.12.2 Detection by PCR

Polymerase Chain Reaction sequencing preparation cocktail consisted of 10 µL of 5x GoTaq colourless reaction, 3 µL of 25 mM MgCl2, 1 µL of 10 mM of dNTPs mix, 1 µL of 10 mol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µL with sterile distilled water 8 µL DNA template. The Polymerase chain reaction was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94 °C for 5 minutes, followed by a 30 cycles consisting of 94 °C for 30 seconds, 50 °C for 60 seconds and 72 °C for 1 minute 30 seconds; and a final termination at 72 °C for 10 minutes. And chill at 4 °C (Wawrik *et al*., 2005; Frank *et al*., 2008).

3.12.3 Integrity of the extracted DNA

The integrity of the amplified DNA of about 1.5Mb gene fragment was checked on a 1.5 % Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5 % Agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60 °C and stained with 3 µL of 0.5 g/mL ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliters (2 µL) of 10X blue gel loading dye (which gives colour

and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4 µL of each PCR product and loaded into the wells after the 100 bp DNA ladder was loaded into well 1 (one). The gel was electrophoresed at 120 V for 45 minutes, visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100 bp molecular weight ladder that was ran alongside experimental samples in the gel (Frank *et al*., 2008).

3.12.4 Purification of amplified (PCR) product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µL of Na acetate 3 M and 240 µL of 95 % ethanol were added to each about 40 µL. The PCR amplified product in a new sterile 1.5 µL tube Eppendorf was mixed thoroughly by vortexing and was kept at -20° C for at least 30 minutes. Centrifuged for 10 minutes at 13000 g and 4 °C followed by removal of supernatant (inverted tube on trash once) after which the pellet were washed by adding 150 µL of 70 % ethanol and were mixed, then centrifuged for 15 minutes at 7500 g and 4° C. Again supernatant was removed and tubes were inverted on paper tissue and allowed to dry in the fume hood at room temperature (25 °C) for 10-15 minutes. Then supernatant was re-suspended with 20 µL of sterile distilled water and kept in -20 ˚C prior to sequencing. The purified fragment was checked on a 1.5 % Agarose gel ran on a voltage of 110 V for about 1 hour as previous, to confirm the presence of the purified product and was quantified using a nanodrop of model 2000 from thermo scientific (Trindade *et al*., 2007).

3.12.5 Sequencing and blasting

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis.

Identities and accession numbers of *K. pneumoniae* strains were determined by uploading the edited sequences to National Centre for Biotechnology Information (NCBI) data base using Basic Local Alignment Search tool (BLAST) from the Gene Bank and accession numbers were generated by providing the necessary information of the sequence properties to the data base (Frank *et al*., 2008).

3.12.6 Molecular detection of CTX-M, TEM, and SHV coding genes of ESBL producing 3GC-R *K. pneumoniae* isolates

The five (5) extracted Deoxyribonucleic acid templates identified as *K. pneumoniae* isolates were characterised for ESBL coding genes. The samples were first of all identified through sequencing and blasting 16S ribosomal RNA.

The Molecular characterization of CTX-M, TEM, and SHV coding genes in the 5 selected *K. pneumoniae* isolates was by simple PCR on the extracted DNA using CTX-M, TEM, and SHV individual coding regions specific primers. The primer sequences are documented in (Table 3.5). Reaction cocktail used for all PCR per primer set included (Reagent Volume µL) - 5X PCR SYBR green buffer (2.5), MgCl2 (0.75), 10pM DNTP (0.25), 10pM of each forward and backwards primer (0.25), 8000U of taq DNA polymerase (0.06) and made up to 10.5 with sterile distilled water to which 2 µL template was added. Buffer control was added to abrogate the probability of false application. The PCR was done in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) using the appropriate profile as designed for each primer pair (Frank *et al*., 2008).

Table 3.4: Primer sequences used for detection and molecular characterization of ESBL genes of 3GC-R *K. pneumoniae*.

ESBL genes	Primer	Target Sequence	Band size	PCR Profile	Reference
CTX-M	CTX-M-F	CGCTTTGCG ATGTGCAG	550bp	An initial denaturing 5 minutes at 94 ºC, then 35 cycles of 94 ºC for 30 seconds 60 ºC for 30 seconds and 72 ºC for 60 seconds and terminate at 72 ºC for 10 minutes	Bonnet *et al*., 2003
	CTX-M-R	ACCGCGAT ATCGTTGGT			
TEM	TEM-F	TTTCGTGTC GCCCTTATT C	700bp	An initial denaturing 5 minutes at 94 ºC, then 35 cycles of 94 ºC for 30 seconds 60 ºC for 30 seconds and 72 ºC for 60 seconds and terminate at 72 ºC for 10 minutes	Xin *et al*., 2019
	TEM-R	CCGGCTCCA GATTTATCA GC			
SHV	SHV-F	TCAGCGAA AAACACCTT G	475bp	An initial denaturing 5 minutes at 94 ºC, then 35 cycles of 94 ºC for 30 seconds, 60 ºC for 30 seconds, 72 ºC for 60 seconds and terminate at 72 ºC for 10 minutes.	Yuan *et al*., 1998
	SHV-R	TCCCGCAGA TAAATCACC A			

Source: Ajanaby and Alhasnawi, (2017)

3.13 Data Analysis

The data collected was analysed using Microsoft excel 2013 and was transferred to Statistical Package for Social Sciences (SPSS) version 21 for further analysis. Chi- Square was used to analyse the data and results were presented in percentage. P–value of less than or equal to 0.05 were considered as statistically significant.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Prevalence of *Klebsiella pneumoniae* isolates based on two (2) Hospitals in Minna

Out of the 500 wound samples investigated, 57 (11.40 %) were confirmed to be positive for *K. pneumoniae*. General Hospital Minna had higher number of positive samples for *K. pneumoniae* accounting for 36 (63.20 %) while IBB Specialist Hospital Minna had the prevalence of 21 (36.80 %) for *K. pneumoniae*. Table 4.1 shows the prevalence and distribution of *K. pneumoniae* isolates based on two hospitals in Minna.

Table 4.1: Prevalence of *K. pneumoniae* isolates based on Two (2) Hospitals in Minna

Hospitals	NSS	NPS	PP (%)
General Hospital	287	36	(63.20)
IBB Specialist Hospital	213	21	(36.80)
Total	500	57	(100)

Key:
NSS= Number of Samples Screened
NPS= Number of Positive Samples
PP= Percentage Positive (%)

4.1.2: Distribution of *K. pneumoniae* wound infection on the basis of age group in years

The distribution of *K. pneumoniae* wound infection according to age group showed that patients within the age bracket of 31-60 (56.2 %) had the highest prevalence of *K. pneumoniae* wound infection followed by patients within the age group of 0-31 (24.5 %), while the lowest prevalence was observed in age group 61-90 (19.3 %). The distribution of *K. pneumoniae* wound infection according to age is presented in Table 4.2.

Table 4.2: Prevalence of *K. pneumoniae* wound infection on the basis of age group from Two (2) Hospitals.

Age group (Years)	NSS	NPS	PP (%)	P value
0-30	163	14	(24.50)	0.018*
31-60	290	32	(56.20)	
61-90	47	11	(19.30)	
Total	500	57	(100)	

Key:
NSS = Number Samples Screened
NPS= Number of Positive Samples
PP= Percentage Positive (%)
*= Significant difference

4.1.3 Distribution of *K. pneumoniae* isolates based on gender

Out of the 500 wound samples that were analysed, 317 (Three hundred and seventeen) were obtained from male subjects while the remaining 183 (One hundred and eighty three) samples were collected from female patients. Of the 57 positive samples, the male subjects had 44 (77.2 %) while the female patients had 13 (22.8 %) positive samples. Table 4.3 shows the distribution of *K. pneumoniae* isolates according to the gender of subjects

Table 4.3: Distribution of *K. pneumoniae* isolates based on gender

Gender	NSS	NPS	PP (%)	P value
Males	317	44	(77.2)	0.022*
Females	183	13	(22.8)	
Total	500	57	(100)	

Key:
NSS = Number Samples Screened
NPS= Number of Positive Samples
PP= Percentage Positive (%)
*= Significant difference

4.1.4 Antibiotic susceptibility profile of *Klebsiella pneumoniae*

The antibiotic susceptibility profile of 57 *K. pneumoniae* isolates showed that all the isolates demonstrated varying selective and high levels of resistance to all the six third generation cephalosporins with the resistance range of (38.6-98.2 %). The highest resistance was observed against Cefdinir 56 (98.2 %) followed by ampicillin (80.7 %). On the other hand, the highest sensitivity of the isolates was expressed to imipenem with (87.7 %) while cefdinir had no activity on the isolates. Table 4.4 shows the antibiotic susceptibility profile of *K. pneumoniae* isolates.

Table 4.4: Antibiotic susceptibility profile of *Klebsiella pneumoniae*

	Number and Percentage of Isolates			
Antibiotics	**Resistant**	**Intermediate**	**Sensitive**	**P-value**
Cefotaxime	38 (66.7)	13 (22.8)	6 (10.5)	0.430
Ceftazidime	26 (45.6)	13 (22.8)	18(31.6)	0.430
Ceftriaxone	22 (38.6)	7 (12.3)	28 (49.1)	0.430
Cefixime	25 (43.9)	9 (15.8)	23 (40.4)	0.430
Cefpodoxime	27 (47.4)	17 (29.8)	13 (22.8)	0.430
Cefdinir	56 (98.2)	1 (1.8)	0 (0.0)	0.438
Imipenem	2 (3.5)	5 (8.8)	50 (87.7)	0.430
Gentamycin	27 (47.4)	14 (24.6)	16 (28.1)	0.430
Ciprofloxacin	16 (28.1)	20 (35.1)	21 (36.8)	0.430
Ampicillin	46(80.7)	8(14.0)	3 (5.3)	0.431

CLSI (2018) Interpretation zone Chart: Cefotaxime = S: ≥26, I: 23-25, R: ≤22, Ceftazidime = S: ≥21, I: 18-20, R: ≤ 17, Ceftriaxone = S: ≥23, I: 20-22, R: ≤ 19, Cefixime = S: ≥ 19, I: 16-18, R: ≤ 15, Cefpodoxime = S: ≥ 21, I: 18-20, R: ≤ 17, Cefdinir = S: ≥ 20, I: 17-19, R: ≤ 16, Imipenem= S: ≥ 23, I: 20-22, R: ≤ 19, Gentamycin = S: ≥ 15, I: 13-14, R: ≤ 12, Ciprofloxacin = S: ≥ 21, I: 16-20, R: ≤ 15, Ampicillin = S: ≥ 17, I: 14-16, R: ≤ 13.

4.1.5 Screening for ESBL production of 57 3GC-R *K. pneumoniae* isolates using double disc synergy test

A total of 57 *K. pneumoniae* isolates exhibited varying resistance to at least one or more of the 3GC. Hence, they were all screened for the production of extended spectrum beta-lactamase enzymes. However, of the 57 3GC-R *K. pneumoniae* isolates, 23 were confirmed to be positive for the production of ESBLs by virtue of increased or enhanced zones of inhibition by the third generation cephalosporin disc (Cefotaxime and ceftazidime or cefpodoxime) towards the Amox/clav acid centrally placed. The ESBL positive isolates were further confirmed by measuring the diameter of the zones of inhibition of the third generation cephalosporin discs alone and comparing with the size of zones of inhibition of 3rd-generation cephalosporin disc combination with amoxicillin (20 μg) /clavulanic acid (10 μg) disc. Isolates which exhibited increase of ≥5 mm in zones of inhibition for cefotaxime, ceftazidime and cefpodoxime tested in combination with clavulanic acid compared to zones of inhibition by third generation cephalosporin when tested alone were confirmed as ESBL producing *K. pneumoniae* isolates. Whereas, negative ESBL reaction was determined by lack of enhanced or increased zone of inhibition towards Amox/clav acid. This result is presented in Table 4.5. The total prevalence of ESBL producing 3GC-R *K. pneumoniae* was 23/57 (40.4 %). While the non ESBL producers were 34 in number representing 34/ 57 (59.6 %) in this study.

Table 4.5: Production of ESBL by 57 3GC-R *K. pneumoniae* isolates using double disc synergy test from two Hospitals

No. 3GC-R *K. pneumoniae*	PP(%) of ESBLs in 3GC-R *K. pneumoniae*	P- value
ESBL positive	23 (40.4)	0.049*
ESBL negative	34 (59.6)	
Total	57 (100)	

Key:
No= Number
PP=Percentage Prevalence (%)
*= Significant difference

4.1.6 Comparison of antibiotic resistance patterns of 23 ESBL and 34 non ESBL producing 3GC- R *K. pneumoniae*

It was observed that the ESBL producing 3GC-R *K. pneumoniae* isolates demonstrated more resistance to all the 10 antibacterial agents tested compared to their non ESBL producing counterpart in this study. The resistance rates of ESBL producers to 3GC was higher, ranging from (8.6- 100 %), while those of the non ESBLs were between (0.0- 97 %).

In this study, it was also observed that the level of resistance among ESBL producing 3GC- R *K. pneumoniae* to other groups of antibiotics such as carbapenem, aminoglycoside, fluoroquinolone, and penicillin was greater than those of the non ESBL producers. Also, statistically, there was a significant difference as p value was less than 0.05. Table 4.6 shows the comparison of antibiotics resistance patterns of 23 ESBL and 34 non ESBL producing 3GC- R *K. pneumoniae*.

Table 4.6: Comparison of antibiotics resistance patterns of 23 ESBL and 34 non ESBL producing 3GC- R *K. pneumoniae*

Antibiotics	ESBLs n=23 (%)	Non-ESBLs n=34 (%)	P-value
Cefotaxime	23(100)	15(44.1)	0.001*
Ceftazidime	23(100)	3(8.8)	0.001*
Ceftriaxone	22(95.6)	0(0.0)	0.001*
Cefixime	22(95.6)	3(8.8)	0.001*
Cefpodoxime	23(100)	4(11.7)	0.001*
Cefdinir	23(100)	33(97.0)	0.407
Imipenem	2(8.6)	0(0.0)	0.029*
Gentamycin	15(65.2)	12(35.2)	0.073
Ciprofloxacin	11(47.8)	5(14.7)	0.002*
Ampicillin	22(95.6)	24(70.5)	0.038*

4.1.7 Distribution of resistance among 57 3GC- R *K. pneumoniae* isolates according to the number of antibiotic classes used

It was observed that the distribution of antibiotic resistance based on the number of antibiotic classes for each of the 57 *K. pneumoniae* (Table 4.7) Showed that of the 5 antibiotic classes used (Third generation Cephalosporin, Carbapenem, Fluoroquinolone, Aminoglycoside and Penicillin), 7 (12.3 %) isolates were resistant to one antibiotic class, while 23 (40.40 %) isolates showed resistance to two antibiotic classes. Multidrug resistance defined as resistance to one agent in at least 3 antibiotic classes was observed in 27 (47.40 %) of the isolates where the isolates were resistant to 3, 4 and 5 antibiotic classes respectively.

In addition, 22 (95.6 %) out of the 23 (100 %) ESBL producing 3GC- R *K. pneumoniae,* were multidrug resistant.

Table 4.7: Distribution of resistance among 3GC- R 57 *K. pneumoniae* isolates according to the number of antibiotic categories used.

No. of antibiotic classes	No. of resistant isolates	(%)Resistance
1	7	12.28
2	23	40.40
3	15	26.31
4	10	17.56
5	2	3.50

Key: No= Number

4.1.8 Multiple antibiotic resistance index (MARI) analysis for each of the 57 3GC-R *K. pneumoniae* isolates

The multiple antibiotic resistance index analysis carried out for each of the 57 3GC- R *K. pneumoniae* isolates revealed that 34 (59.6 %) non ESBL producing isolates had MAR indices of ≤ 0.5 while 23 (40.4 %) ESBL producing isolates had MARI of > 0.5 respectively. Third generation cephalosporin- resistant *K. pneumoniae* isolates exhibited different antibiotic resistance patterns (Appendix H) against the 10 antibiotics used with MARI range of 0.1-1.0. Few of the isolates (4) were resistant to one antibiotic (Cefdinir) with (MARI of 0.1), followed by resistance to two antibiotics (9 isolates) with MARI of 0.2, 12 isolates were found to be resistant to three antibiotics having (MARI of 0.3), 5 isolates, exhibited resistance to four antibiotics showing MARI of 0.4, 4 *K. pneumoniae* isolates expressed resistance to five antibacterial agents displaying MAR index of 0.5, resistance to 6 antibiotics was exhibited by only 1 isolate with (MARI of 0.6). Similarly, 7 isolates demonstrated resistance to seven antibiotics with (MARI of 0.7), 6 *K. pneumoniae* were resistant to 8 antibacterial agents having the (MARI of 0.8), while 7 isolates showed resistance to all the antibacterial agents used with the exception of imipenem, exhibiting the (MARI of 0.9), and 2 isolates expressed resistance to all the antibiotics tested, recording the highest (MARI of 1.0) in this study. Resistance to Cefdinir was the most frequently observed pattern in 56 (98.2 %) isolates followed by resistance to ampicillin involving 46 (80.7 %) isolates. The less frequent resistance pattern was noted in imipenem, exhibited by only 2 (3.5 %) isolates in this study. The multiple antibiotic resistance index (MARI) analysis for each of the 57 3GC- R *K. pneumoniae* isolates is presented in Table 4.8.

Table 4.8: The multiple antibiotic resistance index (MARI) analysis for each of the 57 3GC- R *K. pneumoniae* isolates.

MARI	Number (%)
0.1	4(7.0)
0.2	9(15.8)
0.3	11(19.3)
0.4	6(10.5)
0.5	4(7.0)
0.6	1(1.7)
0.7	7(12.3)
0.8	6(10.5)
0.9	7(12.3)
1.0	2(3.5)

4.1.9 Molecular identification of the selected five ESBL producing 3GC- R *K. pneumoniae* isolates

The result of the molecular characterization using 16s rRNA universal primers identified the selected ESBL Producing 3GC- R *K. pneumoniae* isolates IBB16, IBB164, GH22, GH107 and GH205 as *Klebsiella pneumoniae* strain MBT51, *Klebsiella pneumoniae* strain RBT40, *Klebsiella pneumoniae* strain MO1, *Klebsiella pneumoniae* strain J42 and *Klebsiella pneumoniae* strain NPK3-1-39 respectively. (Appendix I) shows the nucleotide sequences.

Gel electrophoresis of PCR amplified products using 16s rRNA gene fragment in the amplification of *K. pneumoniae* isolates

The agarose gel electrophoresis of the PCR products of *K. pneumoniae* isolates using 16s rRNA universal Primers is shown in Plate I. The isolates have a band size approximately 1500 bp which indicates positive amplification. The gel image shows white horizontal lines on lane 1-5 representing samples IBB16, IBB164, GH205, GH22, GH107 and buffer control respectively. White horizontal lines on lane 1-5 indicate band size in all the isolates.

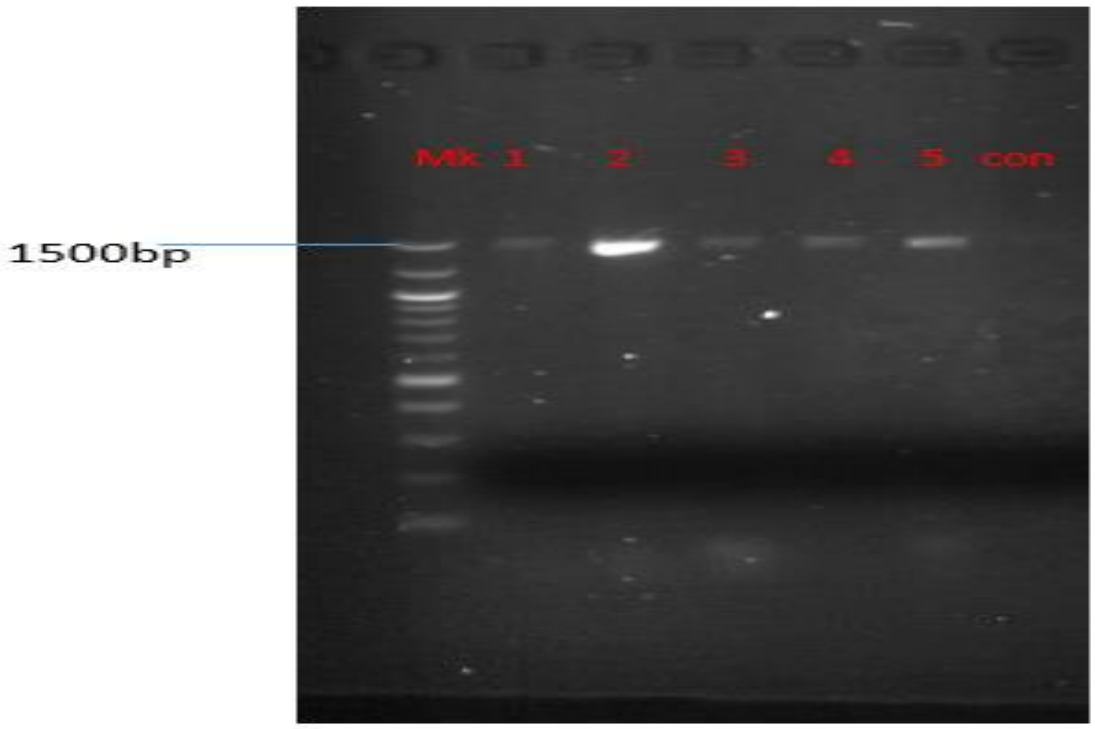

Plate I: Agarose gel electrophoresis of the PCR amplified products from *K. pneumoniae* strains.

Agarose gel electrophoresis of *Klebsiella pneumonae* amplified CTX-M Gene

The agarose gel electrophoresis of the amplified PCR products of CTX-M gene in selected *K. pneumoniae* isolates is shown in Plate II. The isolates expressed CTX-M gene with the band size approximately 550 bp. The gel image shows white horizontal lines on lane 1-5, which represents, IBB16, IBB164, GH205, GH22, GH107 and buffer control respectively. Gel picture indicates the presence of CTX-M gene in all the samples. While mk represents molecular weight marker (Ladder).

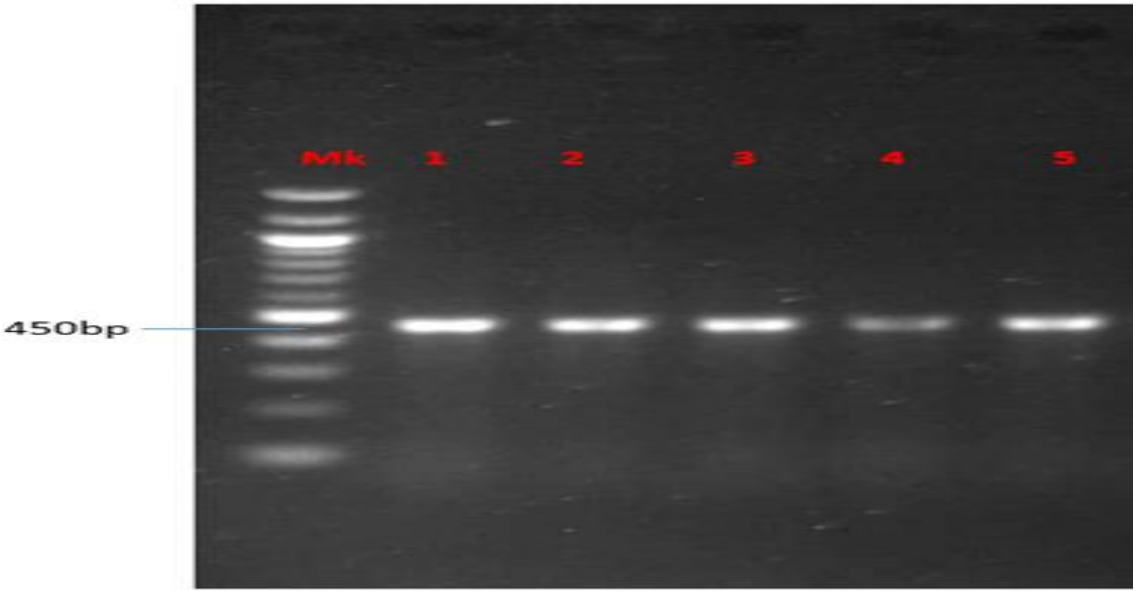

Plate II: Agarose gel electrophoresis of *K. pneumoniae* amplified CTX-M gene.

Gel electrophoresis of *K. pneumoniae* amplified TEM gene

The agarose gel electrophoresis of the amplified TEM gene from selected *K. pneumoniae* isolates is pictured in Plate III. The TEM gene was amplified at 700 bp. The gel image shows white horizontal lines on lane 1-5, which represent sample arrangement (IBB16, IBB164, GH205, GH22, GH107), and buffer control respectively. Gel picture indicates the presence of TEM gene in all isolates.

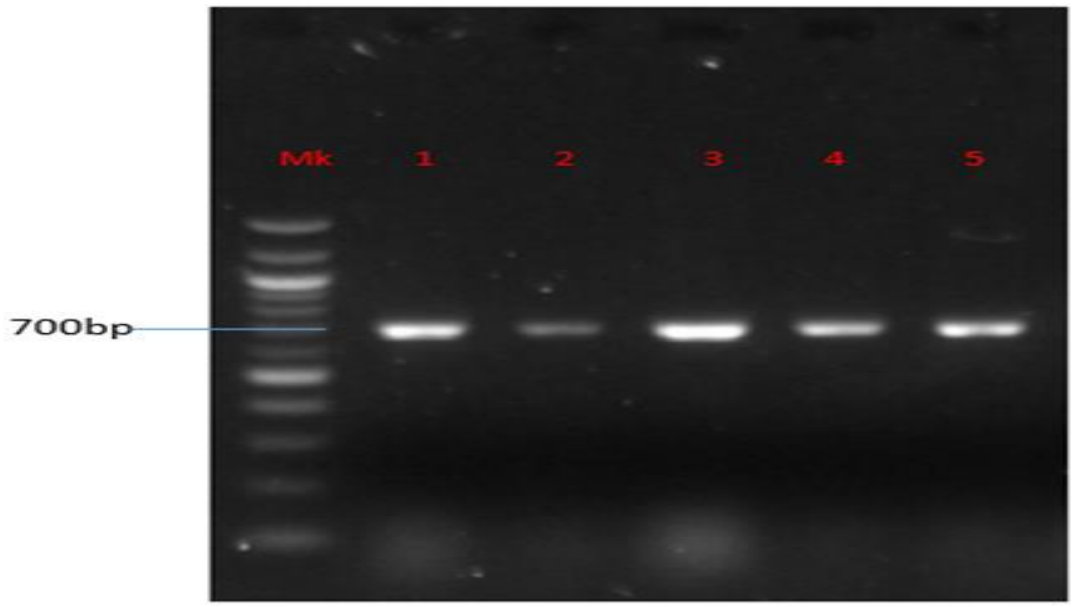

Plate III: Agarose gel electrophoresis of *K. pneumoniae* amplified TEM gene

Gel electrophoresis of *K. pneumoniae* amplified SHV gene

The agarose gel electrophoresis of the PCR products of SHV gene in selected *K. pneumoniae* isolates is shown in (Plate IV). Two of the isolates show SHV gene with a band size approximately 475 bp. The gel picture shows white horizontal lines only on lane 4 -5, representing GH22 and GH107. Gel image indicates the presence of SHV gene in isolates GH22 and GH107 only.

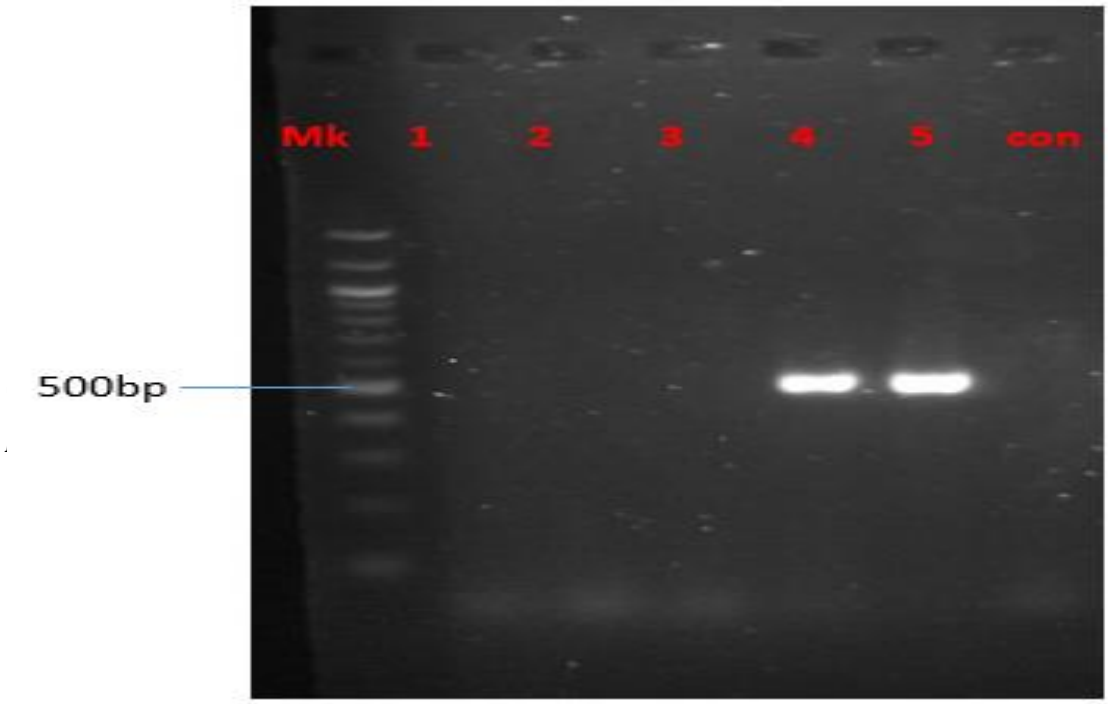

Plate IV: Agarose gel electrophoresis of *K. pneumoniae* amplified SHV gene

4.1.10: Presence and distribution of ESBL genes among selected *K. pneumoniae* isolates

The extracted DNA from the five selected *K. pneumoniae* isolates were used to screen for the presence of ESBL genes via polymerase chain reaction assay. Cefotaxime hydrolyzing (CTX-M) and TEM (Temoneira) genes were found in all the 5 (100 %) of the isolates while SHV (Sulfhydril Variable) gene was present in only 2 (40 %) of the *K. pneumoniae* strains (Table 4.10).

Table 4.9: Presence and distribution of ESBL genes among selected 3 GC- R *K. pneumoniae* isolates

Isolates	**ESBL Genes**		
	CTX-M (%)	**TEM (%)**	**SHV (%)**
IBB16	+	+	-
IBB164	+	+	-
GH22	+	+	+
GH107	+	+	+
GH205	+	+	-
Total	(100)	(100)	(40)

Key:
CTX-M= Cefotaxime hydrolyzing
TEM= Temoneira
SHV= Sulfhydril Variable

4.1.11: The extended spectrum beta- lactamase genes among 3GC- R *K. pneumonia*e strains and the antibiotics to which they expressed resistance to

The extended spectrum beta- lactamase genes in *K. pneumonia*e strains and the antibacterial agents to which they expressed resistance to (Table 4.11). Isolate IBB16 (MBT51) was resistant to third generation cephalosporin, aminoglycoside, fluoroquinolone and penicillin, isolate IBB164 (RBT40) was resistant to 3GC, fluoroquinolone and Penicillin. While isolates GH22 (MO1), GH107 (J42) and GH205 (NPK3-1-39) exhibited resistance to 3GC, aminoglycoside and penicillin, 3GC, carbapenem, aminoglycoside, fluoroquinolone as well as penicillin, and 3GC and aminoglycoside respectively. Genes (CTX-M and TEM) encoding for ESBL enzymes were present in all the selected five (5) *K. pneumoniae* isolates (IBB16, IBB164, GH22, GH107 and GH205) while ESBL gene (SHV) was detected in isolates GH22 and GH107 only.

Table 4.10: The extended spectrum beta- lactamase genes associated with 3GC- R *K. pneumonia*e strains and the antibacterial agents to which they expressed resistance to

Isolate (Strain)	Antibiotic Classes	ESBL Genes		
		CTX-M	TEM	SHV
IBB16 (MBT51)	3GC (CTX,CAZ,CFM,CPD, CRO& CDR) Aminoglycoside(CN) Fuoroquinolone (CPX) Penicillin (PN)	+	+	-
IBB164 (RBT40)	3GC (CTX,CAZ,CFM,CPD, CRO& CDR) Fuoroquinolone (CPX) Penicillin (PN)	+	+	-
GH22 (MO1)	3GC (CTX,CAZ,CFM,CPD, CRO& CDR) Aminoglycoside(CN) Penicillin (PN)	+	+	+
GH107 (J42)	3GC (CTX,CAZ,CFM,CPD, CRO& CDR) Carbapenem, (IMP) Aminoglycoside(CN) Fluoroquinolone (CPX) Penicillin(PN)	+	+	+
GH205 (NPK3-1-39)	3GC (CTX, CAZ, CFM, CPD& CDR) Aminoglycoside(CN)	+	+	-

Key: 3GC (Third Generation Cephalosporin), CTX, CAZ, CFM, CPD, CRO & CDR (Cefotaxime, Ceftaxidime, Cefixime, Cefpodoxime, Ceftriaxone and Cefdinir), CN (Gentamycin), CPX (Ciprofloxacin), PN (Ampicillin) and IMP (Imipenem).

4.2 Discussion

In this study, 57 samples (11.40 %) out of 500 tested positive for *K. pneumoniae*. The implication of this result is that *K. pneumoniae* wound infection is endemic in the study area with relatively high prevalence 11.40 %. A possible explanation to this high prevalence may be that as an opportunist pathogen, *K. pneumoniae* gain easy access to wounds as a result of broken skin, thereby establishing wound infection

The prevalence obtained in the present study is in line with the prevalence of 12.5 % obtained by Muhammad *et al*. (2017) in Northwest Ethiopia, 13. 0% reported by Iregbu *et al.* (2013), and 14. 28 % documented by Oli *et al*. (2017), both in Nigeria. However, this finding is lower than the prevailing reports of Perween *et al*. (2015), Fadeyi *et al*. (2016) and Iroha *et al*. (2017), both in Nigeria, where the prevalence of *Klebsiealla* wound infections was documented as 22 %, 22.5 % and 59.65 % respectively. This variation could be due to different geographical location.

It was observed that, *K. pneunoniae* wound infection was more prevalent in General Hospital Minna compared to IBB Specialist Hospital Minna. This could likely be attributed to its close proximity to town which makes it a choice destination to people especially accident and emergency cases. Another crucial factor for higher *K. pneumoniae* infection in General Hospital could be due to poor hygienic practices in General Hospital compared to IBB Specialist Hospital Minna.

The present study showed that the highest percentage of *K. pneumoniae* wound infection (Table 4.2) was observed among age group 31-60 (56.2 %), followed by patients within the age bracket 0-31 (24.5 %), and the lowest prevalence was noted in age group 61-90 (19.3 %). The isolation rates among all age groups in this study is comparable with the findings of Bengoechea and Sa Pessoa (2019), which stated that *K. pneumoniae* pathogen can cause a wide range of infections in people of all age groups including neonates.

It was also noted from (Table 4.3) that male patients had greater prevalence of *K. pneumoniae* infection with (77.20 %) while the female patients had (22.80 %). The reason for the greater prevalence of *K. pneumoniae* wound infections observed among the male gender in the current study may be due to social risk behaviours exhibited by the male gender because higher number of samples were collected from the male gender. To corroborate these findings, previous studies by Biradar and Roopa, (2015) suggested some risk factors predisposing *K. pneumoniae* infections such as smoking, alcoholism, post-operative surgeries, hypertension and diabetes to be more prevalent among the male gender.

The antibiotic susceptibility profile of 57 *Klebsiella pneumoniae* isolates against ten (10) antibiotics tested (Table 4.4) showed that all the isolates were highly resistant to 3GC such as Cefdinir (98.2 %) , Cefotaxime (66.7 %), Cefpodoxime (47.4 %), Ceftazidime (45.6 %), Cefixime (43.9 %) and Ceftriaxone (38.6 %), also high resistance was observed against Ampicillin (80.7 %), Gentamycin (47.4 %) and Ciprofloxacin (28.1 %), a penicillin, an aminoglycoside and a fluoroquinolone respectively while the least resistance was demonstrated to Imipenem (3.5 %), a carbapenem . Whereas, a greater percentage of *K. pneumoniae* isolates were highly susceptible to Imipenem (87.7 %). The high antibiotic resistance pattern exhibited by *K. pneumoniae* isolates in this study is in line with the findings of Iroha *et al*. (2013) and Quareshi (2015), where all the clinical isolates of *K. pneumoniae* were found to be resistant to almost all the antibiotics used.

The overall prevalence of 3GC – R *K. pneumoniae* was (100 %) in the present study, indicating that all the 57 *K. pneumoniae* strains were selectively resistant to all the 6 third generation cephalosporin tested. The high resistance to 3GC observed in the study area could be attributed to the production of extended spectrum beta- lactamase enzymes because large number of 3GC-R *K. pneumoniae* were confirmed to be ESBL producers

by double disc synergy test and the molecular characterization of selected ESBL producing 3GC-R *K. pneumoniae* indicated the presence of ESBL encoding genes. Earlier study by Wei *et al*. (2018) documented that high resistance to 3GC is always associated with extended spectrum beta- lactamase producers. Furthermore, increasing evidence by Teklu *et al*. (2019) suggested that resistance to third-generation cephalosporin is frightening when is actually caused by ESBL genes because the dissemination of these resistant genes is plasmid-mediated and plasmid play a major role in the spread of antibiotic resistance in *K. pneumoniae* isolates and other Gram-negative bacteria.

Meanwhile, the cause of resistance to 3GC observed among the non- ESBL producing 3GC-R *K. pneumoniae* isolates was not targeted in the present study but previous findings by Jacoby (2009); Bush (2010); Logan *et al*. (2014) established that reasons for such resistance may be connected to the presence of AmpC beta-lactamases, over expression of porins or other intrinsic and acquired mechanisms of drug resistance. Research has also shown that not all resistance to third generation cephalosporins in *Klebsiella* spp and *E. coli* is due to ESBL production. Other potent beta-lactamases such as AmpC and K1 enzymes may be responsible (BSAC, 2010).

The high resistance range of (38.6-98.2 %) to 3GC observed in the present study further corroborates the findings of Iroha *et al*. (2017), Asaduzzaman *et al*. (2018) and Teklu *et al*. (2019) in which *K. pneumoniae* isolates were found to be highly resistant against third generation cephalosporin with (97-100 %), (80.0 %) and (85.4 -86.4 %) respectively. Park (2014) stated that in recent times, the rate of resistance to third generation cephalosporin has heightened among *K. pneumoniae* strains worldwide.

Similarly, the high resistance to ampicillin as observed from this finding is comparable to (82.2 %) documented Heidary *et al*. (2018) in Iran and a higher resistance range of (87-95 %) published by Kothari *et al*. (2013) in India. To cap it, work by Abayneh *et al*.

(2018) in Jimma, Ethiopia documented that majority of ESBL and non-ESBL producing isolates were highly resistant to ampicillin. As observed from Table 4.4 high resistance to ampicillin in this study further justifies the recommendations made by CLSI, (2018) that all isolates showing resistance to 3GC should be reported as resistant to all penicillins.

The greatest activity of imipenem on *K. pneumoniae* isolates in the present study is in agreement with several findings by Kothari *et al*. (2013) in India, Qadeer *et al.* (2013), and Singh *et al*. (2015), Biradar and Roopa (2015), where (82, 92.85, and 100 %) of *K. pneumoniae* isolates were susceptible to imipenem respectively.

Similarly, the excellent activity of imipenem on *K. pneumoniae* isolates in the current study is very consistent with similar findings of Paterson *et al*. (2004), Logan *et al*. (2014) and Giwa *et al*. (2019) in Nigeria, which stated that carbapenems are the most potent, stable and effective drug of choice for the treatment of infections mediated by ESBL and non ESBL producing 3GC-R *K. pneumoniae* including other Gram negative bacteria. Other investigators simply called it the last resort for the treatment of severe bacterial infection (Wohlwend *et al*., 2015; Ferreira *et al*. 2019).

However, a possible explanation for resistance rates of (3.5 %) observed against imipenem a carbapenem class in this study may be due to the production of carbapenemase, an enzyme that renders carbapenems ineffective. To corroborate this finding, Park (2014) documented that the cause of resistance to carbapenem in *K. pneumoniae* is due to production of carbapenemase enzymes. It was however observed from Table 4.4 that the antibiotics resistance pattern exhibited in this study was not significantly different at $p \geq 0.05$.

From this study, the observed prevalence of ESBL producing 3GC-R *K. pneumoniae* was found to be high with 23/57 (40.4 %). It may be possible that the uncontrolled use and

over dependence on 3GC in severely ill patients in the study area could be a contributory factor to high ESBL prevalence. Another predisposing factor is believed to be that 3GC are beta- lactamase substrates and prior exposure of the bacteria to these beta- lactamase substrates could enhance ESBL production leading to 3GC resistance as well as co-resistance to other groups of antibiotic agents.

The prevalence of ESBL obtained from the present study is in line with the prevalence of 44 .0 % obtained from pus wounds by Singh *et al*. (2015), but lower than 47.22 % reported by Akila *et al*. (2016) in India and 50.0 % documented by Saraswathi *et al*. (2015). The prevalence of ESBL producing isolates among 3GC-R *K. pneumoniae* in Minna was however higher than the prevalence of 24 % reported by Fadeyi *et al*. (2016) in Nigeria, and 32.4 % by Zahedani *et al*. (2016) in Iran. Singh *et al*. (2015) documented that outbreaks involving ESBL producing *K. pneumoniae* strains have been reported from all over the world.

It was observed from this finding that 27 (47.40 %) of the 57 3GC- R *K. pneumoniae* isolates were multidrug resistant. It was also noted that of the 23 ESBL producing isolates, 22 (95.6 %) demonstrated MDR to 3 or more antibiotic classes, All ESBL producers were resistant to all the 6 third generation cephalosporin tested in the present study. The multidrug resistant nature of these isolates could be attributed to the fact that ESBLs are plasmid-mediated enzymes which carry multiple resistant or co- resistant genes on plasmid, transposon, and integron, readily transferring them to other bacteria (Abayneh *et al*., 2018). This may also be connected to the pressure of heavy dependence on antibiotic thereby exerting selective effect on resistant strains and promoting its spread. Fadeyi *et al*. (2016) and Ferreira *et al*. (2019) reported that the incidence of MDR *K. pneumoniae* strains becoming more persistently present in both hospital and community settings, limiting therapeutics options.

Aljanaby and Alhasnawi, (2017) also published that multidrug resistant bacteria are always associated with ESBL production. While Mazzariol *et al*. (2017) reported that ESBL producing organisms are extremely prone to harbouring other antibiotic resistant genes, leading to multi-drug resistance.

The molecular identification of the selected five ESBL Producing 3GC- R *K. pneumoniae* isolates in the present study showed that ESBL producing 3GC- R *K. pneumoniae* isolates IBB16, IBB164, GH22, GH107 and GH205 were identified as *Klebsiella pneumoniae* strains MBT51, RBT40, MO1, J42 and NPK3-1-39 respectively. The identities of *K. pneumoniae* has aided in comprehending the molecular epidemiology of *K pneumoniae* in the study area.

The PCR assay also revealed the presence of ESBL encoding genes (CTX-M, TEM and SHV) in *K. pneumoniae* isolates in the study area. The result of the PCR showed that the most dominant ESBL genes were CTX-M and TEM. CTX-M and TEM genes were found in all the 5 (100 %) IBB16 (MBT51), IBB164 (RBT40), GH22 (MO1), GH107 (J42) and GH205 (NPK3-1-39) *K. pneumoniae* isolates while SHV gene was present in only 2 (40 %) GH22 (MO1) and GH107 (NPK3-1-39) *K. pneumoniae* strains.

The presence of ESBL encoding genes in the study area is believed to be responsible for the production of ESBL enzymes associated with high resistance to 3GC and other antibacterial agents. Thus, making the ESBL genes the most significant factor for increased resistance to 3GC. The implication of the ESBL genes in the study area is that each time newer beta- lactam antibiotics are introduced, mutation and dissemination of beta- lactamase encoding genes swiftly ensue.

The presence of ESBL genes in the current study is in agreement with the findings of Kothari *et al*. (2013) and Venezia *et al*. (2017), where ESBL producing isolates of *K. pneumoniae* were the most important cause of increased resistance to 3GC.

The present findings also corroborate the earlier works by Mansury *et al*. (2015) in Iran, Lin *et al*. (2016) in Taiwan, Aljanaby and Alhasnawi (2017) in Al-Najaf Province-Iraq, where the prevalence and co-existence of different ESBL genes (CTX-M, TEM and SHV) within the same isolates of *K. pneumoniae* were reported. Adding that the most frequent ESBL genes encountered among isolates is CTX-M gene with TEM gene being the most common combination with or without SHV gene. A higher prevalence of CTX-M and TEM (100 %) as opposed to SHV (40 %) gene in this study demonstrates the important role of CTX-M and TEM genes in inducing resistance to 3GC and other antibiotics among *K. pneumoniae* isolates. These genes played a vital role as most resistant isolates were found to harbour two or more of the ESBL genes. The co-existence of two or more ESBL resistant genes in ESBL producing 3GC-R *K. pneumoniae* isolates is an indication that most of the isolates from the study area are multidrug resistant as earlier established from the study.

The findings from this study has clearly affirmed results of earlier studies which established that ESBL encoding genes are the major cause of increased resistance to 3GC and other antibacterial agents among *K. pneumoniae* strains.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In light of these findings, it could be concluded that the rate of wound colonization by ESBL producing and non ESBL producing 3GC-R *Klebsiella pneumoniae* was found to be high underscoring the need for public health measures to prevent wound infection. This study also illustrated that *K. pneumoniae* isolates were highly resistant to third generation cephalosporins with the resistance range of (38.6-98.2 %). The high resistance to 3GC observed in the present study may undermine the use of third generation cephalosporin as one of the most potent and last resort antibiotics, underscoring the emergence, and spread of 3GC resistant. *K. pneumoniae* in the study area.

High resistance was also observed against other groups of antibiotics tested. However, the isolates were highly sensitive to carbapenem. About half (40.4 %) of the third generation cephalosporin resistant- *K. pneumoniae* isolates were confirmed as ESBL producers. Five ESBL producing 3GC-R *K. pneumoniae* isolates (IBB16, IBB164, GH22, GH107 and GH205) were identified as *Klebsiella pneumoniae* strains MBT51, RBT40, MO1, J42 and NPK3-1-39 respectively. The presence of ESBL (CTX-M and TEM) genes in *K. pneumoniae* isolates conferring resistance to 3GC and other antibiotics were detected in all representative (100 %) isolates while SHV gene was present in only 2 (40 %) of the isolates in Minna, the study area.

5.2 Recommendations

In view of these findings, the following recommendations are made;

i. It is sacrosanct to regularly screen and monitor ESBL producing and non ESBL producing 3GC-R and *K. pneumoniae* isolates in the study area.

ii. Third generation cephalosporin should be used in combination with a beta – lactamase inhibitor like clavulanic acid for the treatment of *K. pneumoniae* wound and other bacterial infections since the presence of clavulanate inhibitor inactivates the beta-lactamase enzymes and enhances the activity of the 3GC.

iii. Physicians should reduce the over dependence on the use of 3GC in the treatment of severe infections in critically ill patients instead, non -beta lactam drugs should be used as the first choice of antibiotics before resulting to use of last resort cephalosporin drugs.

iv. Despite the high susceptibility of the isolates to carbapenem, the resistance observed in a few isolates is a red flag that continued use of carbapenem may trigger the rapid spread of carbapenem resistant strains. Hence, the incessant use of carbapenem should be discouraged to preserve the integrity of the drug.

v. Drug prescriptions should be laboratory guided.

vi. This study did not establish the reason for resistance to 3GC among non ESBL producers, thus, further studies are needed to investigate the cause of resistance to 3GC among non ESBL producing 3GC- R *K. pneumoniae*.

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Appendix A: Colonial morphology of *Klebsiella pneumoniae* isolates

Isolates	MCA	BA	G/RXN	SHAPE	Motility
GH5	Mucoid & Pink	White & mucoid	-	SR	NM
GH12	Mucoid & Pink	White & mucoid	-	SR	NM
GH22	Mucoid & Pink	White & mucoid	-	SR	NM
GH40	Mucoid & Pink	White & mucoid	-	SR	NM
GH45	Mucoid & Pink	White & mucoid	-	SR	NM
GH56	Mucoid & Pink	White & mucoid	-	SR	NM
GH67	Mucoid & Pink	White & mucoid	-	SR	NM
GH72	Mucoid & Pink	White & mucoid	-	SR	NM
GH79	Mucoid & Pink	White & mucoid	-	SR	NM
GH90	Mucoid & Pink	White & mucoid	-	SR	NM
GH97	Mucoid & Pink	White & mucoid	-	SR	NM
GH101	Mucoid & Pink	White & mucoid	-	SR	NM
GH107	Mucoid & Pink	White & mucoid	-	SR	NM
GH113	Mucoid & Pink	White & mucoid	-	SR	NM
GH121	Mucoid & Pink	White & mucoid	-	SR	NM
GH130	Mucoid & Pink	White & mucoid	-	SR	NM
GH134	Mucoid & Pink	White & mucoid	-	SR	NM
GH137	Mucoid & Pink	White & mucoid	-	SR	NM
GH144	Mucoid & Pink	White & mucoid	-	SR	NM
GH149	Mucoid & Pink	White & mucoid	-	SR	NM
GH153	Mucoid & Pink	White & mucoid	-	SR	NM
GH158	Mucoid & Pink	White & mucoid	-	SR	NM

Appendix A: Continues

Isolates	MCA	BA	G/RXN	SHAPE	Motility
GH166	Mucoid & Pink	White & mucoid	-	SR	NM
GH171	Mucoid & Pink	White & mucoid	-	SR	NM
GH177	Mucoid & Pink	White & mucoid	-	SR	NM
GH181	Mucoid & Pink	White & mucoid	-	SR	NM
GH195	Mucoid & Pink	White & mucoid	-	SR	NM
GH200	Mucoid & Pink	White & mucoid	-	SR	NM
GH205	Mucoid & Pink	White & mucoid	-	SR	NM
GH217	Mucoid & Pink	White & mucoid	-	SR	NM
GH226	Mucoid & Pink	White & mucoid	-	SR	NM
GH237	Mucoid & Pink	White & mucoid	-	SR	NM
GH254	Mucoid & Pink	White & mucoid	-	SR	NM
GH259	Mucoid & Pink	White & mucoid	-	SR	NM
GH267	Mucoid & Pink	White & mucoid	-	SR	NM
GH273	Mucoid & Pink	White & mucoid	-	SR	NM
IBB7	Mucoid & Pink	White & mucoid	-	SR	NM
IBB16	Mucoid & Pink	White & mucoid	-	SR	NM
IBB21	Mucoid & Pink	White & mucoid	-	SR	NM
IBB29	Mucoid & Pink	White & mucoid	-	SR	NM
IBB37	Mucoid & Pink	White & mucoid	-	SR	NM
IBB43	Mucoid & Pink	White & mucoid	-	SR	NM
IBB51	Mucoid & Pink	White & mucoid	-	SR	NM
IBB75	Mucoid & Pink	White & mucoid	-	SR	NM

Appendix A: Continues

Isolates	MCA	BA	G/RXN	SHAPE	Motility
IBB83	Mucoid & Pink	White & mucoid	-	SR	NM
IBB94	Mucoid & Pink	White & mucoid	-	SR	NM
IBB106	Mucoid & Pink	White & mucoid	-	SR	NM
IBB114	Mucoid & Pink	White & mucoid	-	SR	NM
IBB125	Mucoid & Pink	White & mucoid	-	SR	NM
IBB138	Mucoid & Pink	White & mucoid	-	SR	NM
IBB147	Mucoid & Pink	White & mucoid	-	SR	NM
IBB159	Mucoid & Pink	White & mucoid	-	SR	NM
IBB164	Mucoid & Pink	White & mucoid	-	SR	NM
IBB170	Mucoid & Pink	White & mucoid	-	SR	NM
IBB182	Mucoid & Pink	White & mucoid	-	SR	NM
IBB196	Mucoid & Pink	White & mucoid	-	SR	NM
IBB206	Mucoid & Pink	White & mucoid	-	SR	NM

Key: MCA= MacConkey agar, BA= Blood agar, G/RXN= Gram reaction, - = Negative, SR= Short rods, NM= Non mortile, & = and

Appendix B: Biochemical characteristics of *Klebsiella pneumoniae* isolates

Isolates	Indole	Methyl Red	Voges-Proskauer	Citrate	Urease	Catalase	Coagulase	Oxidase
GH5	-	-	+	+	+	+	-	-
GH12	-	-	+	+	+	+	-	-
GH22	-	-	+	+	+	+	-	-
GH40	-	-	+	+	+	+	-	-
GH45	-	-	+	+	+	+	-	-
GH56	-	-	+	+	+	+	-	-
GH67	-	-	+	+	+	+	-	-
GH72	-	-	+	+	+	+	-	-
GH79	-	-	+	+	+	+	-	-
GH90	-	-	+	+	+	+	-	-
GH97	-	-	+	+	+	+	-	-
GH101	-	-	+	+	+	+	-	-
GH107	-	-	+	+	+	+	-	-
GH113	-	-	+	+	+	+	-	-
GH121	-	-	+	+	+	+	-	-
GH130	-	-	+	+	+	+	-	-
GH134	-	-	+	+	+	+	-	-
GH137	-	-	+	+	+	+	-	-
GH144	-	-	+	+	+	+	-	-
GH149	-	-	+	+	+	+	-	-
GH153	-	-	+	+	+	+	-	-
GH158	-	-	+	+	+	+	-	-

Appendix B: Continues

Isolates	Indole	Methyl Red	Voges-Proskauer	Citrate	Urease	Catalase	Coagulase	Oxidase
GH166	-	-	+	+	+	+	-	-
GH171	-	-	+	+	+	+	-	-
GH177	-	-	+	+	+	+	-	-
GH181	-	-	+	+	+	+	-	-
GH195	-	-	+	+	+	+	-	-
GH200	-	-	+	+	+	+	-	-
GH205	-	-	+	+	+	+	-	-
GH217	-	-	+	+	+	+	-	-
GH226	-	-	+	+	+	+	-	-
GH237	-	-	+	+	+	+	-	-
GH254	-	-	+	+	+	+	-	-
GH259	-	-	+	+	+	+	-	-
GH267	-	-	+	+	+	+	-	-
GH273	-	-	+	+	+	+	-	-
IBB7	-	-	+	+	+	+	-	-
IBB16	-	-	+	+	+	+	-	-
IBB21	-	-	+	+	+	+	-	-
IBB29	-	-	+	+	+	+	-	-
IBB37	-	-	+	+	+	+	-	-
IBB43	-	-	+	+	+	+	-	-
IBB51	-	-	+	+	+	+	-	-
IBB75	-	-	+	+	+	+	-	-

Appendix B: Continues

Isolates	Indole	Methyl Red	Voges-Proskauer	Citrate	Urease	Catalase	Coagulase	Oxidase
IBB83	-	-	+	+	+	+	-	-
IBB94	-	-	+	+	+	+	-	-
IBB106	-	-	+	+	+	+	-	-
IBB114	-	-	+	+	+	+	-	-
IBB125	-	-	+	+	+	+	-	-
IBB138	-	-	+	+	+	+	-	-
IBB147	-	-	+	+	+	+	-	-
IBB159	-	-	+	+	+	+	-	-
IBB164	-	-	+	+	+	+	-	-
IBB170	-	-	+	+	+	+	-	-
IBB182	-	-	+	+	+	+	-	-
IBB196	-	-	+	+	+	+	-	-
IBB206	-	-	+	+	+	+	-	-

KEY: += Positive and - = Negative

Appendix C: Growth of *Klebsiella pneumoniae* isolates on triple iron sugar (TSI)

Isolates	Slant	Butt	Gas	H_2S
GH5	A	A	+	-
GH12	A	A	+	-
GH22	A	A	+	-
GH40	A	A	+	-
GH45	A	A	+	-
GH56	A	A	+	-
GH67	A	A	+	-
GH72	A	A	+	-
GH79	A	A	+	-
GH90	A	A	+	-
GH97	A	A	+	-
GH101	A	A	+	-
GH107	A	A	+	-
GH113	A	A	+	-
GH121	A	A	+	-
GH130	A	A	+	-
GH134	A	A	+	-
GH137	A	A	+	-
GH144	A	A	+	-
GH149	A	A	+	-
GH153	A	A	+	-
GH158	A	A	+	-

Appendix C: Continues

Isolates	Slant	Butt	Gas	H_2S
GH166	A	A	+	-
GH171	A	A	+	-
GH177	A	A	+	-
GH181	A	A	+	-
GH195	A	A	+	-
GH200	A	A	+	-
GH205	A	A	+	-
GH217	A	A	+	-
GH226	A	A	+	-
GH237	A	A	+	-
GH254	A	A	+	-
GH259	A	A	+	-
GH267	A	A	+	-
GH273	A	A	+	-
IBB7	A	A	+	-
IBB16	A	A	+	-
IBB21	A	A	+	-
IBB29	A	A	+	-
IBB37	A	A	+	-
IBB43	A	A	+	-
IBB51	A	A	+	-
IBB75	A	A	+	-

Appendix C: Continues

Isolates	Slant	Butt	Gas	H_2S
IBB83	A	A	+	-
IBB94	A	A	+	-
IBB106	A	A	+	-
IBB114	A	A	+	-
IBB125	A	A	+	-
IBB138	A	A	+	-
IBB147	A	A	+	-
IBB159	A	A	+	-
IBB164	A	A	+	-
IBB170	A	A	+	-
IBB182	A	A	+	-
IBB196	A	A	+	-
IBB206	A	A	+	-

Key: + = Positive, - = Negative, A= Acid production, H_2S= Hydrogen Sulphide production

Appendix D: Results of sugar fermentation tests for the *Klebsiella pneumoniae* isolates

Isolates	Glucose	Lactose	Sucrose	Mannitol	Fructose	Galactose
GH5	+	+	+	+	+	+
GH12	+	+	+	+	+	+
GH22	+	+	+	+	+	+
GH40	+	+	+	+	+	+
GH45	+	+	+	+	+	+
GH56	+	+	+	+	+	+
GH67	+	+	+	+	+	+
GH72	+	+	+	+	+	+
GH79	+	+	+	+	+	+
GH90	+	+	+	+	+	+
GH97	+	+	+	+	+	+
GH101	+	+	+	+	+	+
GH107	+	+	+	+	+	+
GH113	+	+	+	+	+	+
GH121	+	+	+	+	+	+
GH130	+	+	+	+	+	+
GH134	+	+	+	+	+	+
GH137	+	+	+	+	+	+
GH144	+	+	+	+	+	+
GH149	+	+	+	+	+	+
GH153	+	+	+	+	+	+
GH158	+	+	+	+	+	+

Appendix D: Continues

Isolates	Glucose	Lactose	Sucrose	Mannitol	Fructose	Galactose
IBB83	+	+	+	+	+	+
IBB94	+	+	+	+	+	+
IBB106	+	+	+	+	+	+
IBB114	+	+	+	+	+	+
IBB125	+	+	+	+	+	+
IBB138	+	+	+	+	+	+
IBB147	+	+	+	+	+	+
IBB159	+	+	+	+	+	+
IBB164	+	+	+	+	+	+
IBB170	+	+	+	+	+	+
IBB182	+	+	+	+	+	+
IBB196	+	+	+	+	+	+
IBB206	+	+	+	+	+	+

Key: + (Positive) and – (Negative)

Appendix D: Continues

Isolates	Glucose	Lactose	Sucrose	Mannitol	Fructose	Galactose
IBB83	+	+	+	+	+	+
IBB94	+	+	+	+	+	+
IBB106	+	+	+	+	+	+
IBB114	+	+	+	+	+	+
IBB125	+	+	+	+	+	+
IBB138	+	+	+	+	+	+
IBB147	+	+	+	+	+	+
IBB159	+	+	+	+	+	+
IBB164	+	+	+	+	+	+
IBB170	+	+	+	+	+	+
IBB182	+	+	+	+	+	+
IBB196	+	+	+	+	+	+
IBB206	+	+	+	+	+	+

KEY: + (Positive) and – (Negative)

Appendix E: Antibiotic susceptibility test results in General Hospital Minna

IC	CTX	CAZ	CRO	CFM	CPD	CDR	IMP	CN	CPX	PN
GH5	S	S	S	I	S	R	S	I	S	R
GH12	I	S	S	S	S	R	S	S	I	I
GH22	R	R	R	R	R	R	I	R	I	R
GH40	I	S	S	S	R	R	S	R	S	R
GH45	R	I	I	R	I	R	S	R	S	R
GH56	R	S	S	S	S	R	S	I	I	R
GH67	I	S	I	S	I	R	S	S	R	R
GH72	R	R	I	S	I	R	S	R	S	R
GH79	R	R	I	I	I	R	I	R	I	R
GH90	R	R	R	R	R	R	S	R	R	R
GH97	R	R	R	R	R	R	I	R	R	R
GH101	I	R	I	S	I	R	S	S	S	R
GH107	R	R	R	R	R	R	R	R	R	R
GH113	I	S	S	S	I	R	S	S	I	R
GH121	I	S	S	S	S	R	S	I	S	S
GH130	R	I	S	R	I	R	S	S	S	R
GH134	I	S	S	S	S	R	S	R	S	I
GH137	R	I	I	R	I	R	S	S	S	R

Appendix E: Continues

IC	CTX	CAZ	CRO	CFM	CPD	CDR	IMP	CN	CPX	PN
GH144	R	R	R	R	R	R	S	R	I	R
GH149	R	R	R	R	R	R	S	R	I	R
GH153	R	R	R	R	R	R	S	R	R	R
GH158	R	R	R	R	R	R	I	R	R	R
GH166	R	R	R	R	R	R	S	R	R	R
GH171	I	S	S	S	I	R	S	I	S	R
GH177	I	S	S	S	S	R	S	R	R	R
GH181	R	I	S	S	I	R	S	I	S	R
GH195	R	R	R	R	R	R	S	R	I	R
GH200	S	S	S	S	S	R	S	S	I	R
GH205	R	R	S	R	R	R	S	R	I	S
GH217	I	I	S	S	I	R	S	R	R	R
GH266	R	I	S	I	S	R	S	R	R	R
GH237	R	R	R	R	R	R	R	R	R	R
GH254	I	S	S	I	R	R	S	I	R	R
GH259	R	S	S	I	I	R	S	I	I	I
GH267	R	I	S	S	I	R	S	S	S	S
GH273	S	S	S	S	S	R	S	R	S	R

Appendix E: Continues

IC	CTX	CAZ	CRO	CFM	CPD	CDR	IMP	CN	CPX	PN
IBB7	R	R	R	R	R	R	I	R	S	R
IBB16	R	R	R	R	R	R	S	R	R	R
IBB21	R	R	R	R	R	R	S	R	R	R
IBB29	R	R	R	R	R	R	S	I	S	R
IBB37	I	I	S	I	I	R	S	R	I	I
IBB43	R	I	S	I	I	R	S	I	S	R
IBB51	R	I	S	I	I	R	S	I	S	I
IBB75	R	R	R	R	R	R	S	S	S	R
IBB83	R	R	R	R	R	R	S	S	I	R
IBB94	R	R	R	R	R	R	S	I	I	R
IBB106	S	S	S	S	S	R	S	S	I	I
IBB114	R	I	S	S	R	I	S	R	I	I
IBB125	R	R	R	R	R	R	S	I	I	R
IBB138	R	I	S	S	I	R	S	S	S	R

Appendix E: Continues

IC	CTX	CAZ	CRO	CFM	CPD	CDR	IMP	CN	CPX	PN
IBB147	S	S	S	S	S	R	S	I	I	I
IBB159	S	S	S	S	S	R	S	R	I	R
IBB164	R	R	R	I	R	R	S	S	R	R
IBB170	I	I	I	S	I	R	S	S	S	R
IBB182	R	R	R	R	R	R	S	S	I	R
IBB196	R	R	R	R	R	R	S	S	R	R
IBB206	R	S	S	S	S	R	S	I	S	R

Key: IC (Isolates code), CTX (Cefotaxime), CAZ (Ceftazidime), CRO (Ceftriaxone), CFM (Cefixime), CPD (Cefpodoxime), CDR (Cedinir), IMP (Imipenem), CPX (Ciprofloxacin), CN (Gentamycin) PN (Ampicillin), S (Sensitive) (I) Intermediate and R (Resistant)

Appendix F: Screening results of 23 ESBL producing *Klebsiella pneumoniae* isolates

ESBLS	CTX	CAZ	CPD	A/C	ESBL RXN
GH22	23	20	NT	17	Positive
GH90	18	15	NT	20	Positive
GH97	21	20	20	18	Positive
GH107	19	17	NT	16	Positive
GH144	20	16	18	19	Positive
GH149	18	21	21	23	Positive
GH153	23	20	20	24	Positive
GH158	21	20	19	25	Positive
GH166	24	18	21	22	Positive
GH195	22	21	16	20	Positive
GH205	19	14	17	18	Positive
GH237	20	16	18	17	Positive

Appendix F: Continues

ESBLs	CTX	CAZ	CPD	A/C	ESBL RXN
IBB7	20	15	19	12	Positive
IBB16	21	20	18	19	Positive
IBB21	23	20	23	24	Positive
IBB29	19	20	16	17	Positive
IBB75	20	18	19	12	Positive
IBB83	24	21	21	18	Positive
IBB94	18	19	18	16	Positive
IBB125	23	19	20	14	Positive
IBB164	22	18	22	23	Positive
IBB182	25	24	19	18	Positive
IBB196	19	16	21	17	Positive

Key: (Isolates code), CTX (Cefotaxime), CAZ (Ceftazidime), CPD (Cefpodoxime), A/C (Amoxacillin/ Clavulanic Acid) and RXN (Reaction). NT (Not Tested).

Appendix G: Screening result of 34 non ESBL producing *Klebsiella pneumoniae* isolates

Non ESBLs	CTX	CAZ	CPD	A/C	ESBL RXN
CH5	26	24	21	20	Negative
CH12	23	24	23	22	Negative
CH40	24	21	16	18	Negative
CH45	20	19	19	20	Negative
CH56	22	28	21	21	Negative
CH67	24	22	18	16	Negative
CH72	23	13	20	18	Negative
CH79	22	16	18	17	Negative
CH101	24	17	19	12	Negative
CH113	25	23	20	19	Negative
CH121	25	22	23	24	Negative
CH130	21	19	20	16	Negative
CH134	24	23	22	12	Negative
CH137	19	18	18	21	Negative
CH171	24	23	19	25	Negative
CH177	24	22	23	21	Negative
CH181	28	23	21	20	Negative

Appendix G: Continues

Non ESBLs	CTX	CAZ	CPD	A/C	ESBL RXN
GH200	28	23	21	20	Negative
GH217	23	19	20	22	Negative
GH226	21	20	21	12	Negative
GH254	23	26	14	10	Negative
GH259	21	24	18	24	Negative
GH267	21	19	20	17	Negative
GH273	27	24	25	20	Negative
IBB37	23	19	21	16	Negative
IBB43	22	20	19	23	Negative
IBB51	19	19	17	22	Negative
IBB106	30	21	27	18	Negative
IBB114	21	20	18	15	Negative
IBB138	21	19	20	19	Negative
IBB147	27	25	26	14	Negative
IBB159	28	24	25	17	Negative
IBB170	25	20	20	13	Negative
IBB206	22	23	22	24	Negative

Key: (Isolates code), CTX (Cefotaxime), CAZ (Ceftazidime), CPD (Cefpodoxime), A/C (Amoxacillin/ Clavulanic Acid) and RXN (Reaction).

Appendix H: Antibiotic resistance pattern and multiple antibiotic resistance index (MARI) of *K. pneumoniae* isolates

Isolates	Resistance	Antibiotics	MARI
GH12	1	CDR	0.1
GH121	1	CDR	0.1
IBB106	1	CDR	0.1
IBB147	1	CDR	0.1
GH5	2	CDR, PN	0.2
GH113	2	CDR, PN	0.2
GH134	2	CDR, CN	0.2
GH171	2	CDR, PN	0.2
GH200	2	CDR, PN	0.2
GH267	2	CTX, CDR	0.2
IBB37	2	CDR,CN	0.2
IBB170	2	CDR, PN	0.2
GH259	2	CTX, CDR	0.2
IBB206	3	CTX, CDR, PN	0.3
GH56	3	CTX, CDR, PN	0.3

Appendix H: Continues

Isolates	Resistance	Antibiotics	MARI
GH67	3	CDR, CPX, PN	0.3
GH101	3	CAZ, CDR, PN	0.3
GH181	3	CTX, CDR, PN	0.3
GH273	3	CDR, CN, CPX	0.3
IBB43	3	CTX, CDR, PN	0.3
IBB51	3	CTX, CPD,CDR	0.3
IBB114	3	CPX, CDR, PN	0.3
IBB138	3	CTX ,CDR, PN	0.3
IBB159	3	CDR, CN, PN	0.3
GH254	4	CPD, CDR, CPX, PN	0.4
GH40	4	CPD, CDR, CN, PN	0.4
GH130	4	CTX, CFM, CDR, PN	0.4
GH137	4	CTX, CFM, CDR, PN	0.4
GH177	4	CDR, CN, CPX, PN	0.4
GH217	4	CDR, CN, CPX, PN	0.4

Appendix H: Continues

Isolates	Resistance	Antibiotics	MARI
GH45	5	CTX, CFM, CDR, CN, PN	0.5
GH72	5	CTX, CAZ, CDR, CN, PN	0.5
GH79	5	CTX,CAZ, CDR, CN, PN	0.5
GH266	5	CTX, CDR, CN, CPX, PN	0.5
IBB205	6	CTX, CAZ, CFM, CPD, CRD, CN	0.6
IBB29	7	CTX, CAZ, CFM, CPD, CRO, CDR, PN	0.7
IBB75	7	CTX, CAZ, CFM, CPD, CRO, CDR, PN	0.7
IBB83	7	CTX, CAZ, CFM, CPD, CRO, CDR, PN	0.7
IBB94	7	CTX, CAZ, CFM, CPD, CRO, CDR, PN	0.7
IBB125	7	CTX, CAZ, CFM, CPD, CRO, CDR, PN	0.7
IBB164	7	CTX, CAZ, CPD, CRO, CDR, CPX, PN	0.7

Appendix H: Continues

Isolates	Resistance	Antibiotics	MARI
GH182	7	CTX, CAZ, CFM, CPD, CRO, CDR, PN	0.7
GH22	8	CTX, CAZ, CFM, CPD, CRO, CDR, CN, PN	0.8
GH144	8	CTX, CAZ, CFM, CPD, CRO, CDR, CN, PN	0.8
GH149	8	CTX, CAZ, CFM, CPD, CRO, CDR, CN, PN	0.8
GH195	8	CTX, CAZ, CFM, CPD, CRO, CDR, CN, PN	0.8
IBB7	8	CTX, CAZ, CFM, CPD, CRO, CDR, CN, PN	0.8
IBB196	8	CTX, CAZ, CFM, CPD, CRO, CDR, CPX, PN	0.8
GH190	9	CTX, CAZ, CFM, CPD, CRO, CDR, CN, CPX, PN	0.9

Appendix H: Continues

Isolates	Resistance	Antibiotics	MARI
GH97	9	CTX, CAZ, CFM, CPD, CRO, CDR, CN, CPX, PN	0.9
GH153	9	CTX, CAZ, CFM, CPD, CRO, CDR, CN, CPX, PN	0.9
IBB158	9	CTX, CAZ, CFM, CPD, CRO, CDR, CN, CPX, PN	0.9
IBB166	9	CTX, CAZ, CFM, CPD, CRO, CDR, CN, CPX, PN	0.9
IBB16	9	CTX, CAZ, CFM, CPD, CRO, CDR, CN, CPX, PN	0.9
IBB21	9	CTX, CAZ, CFM, CPD, CRO, CDR, CN, CPX, PN	0.9
GH107	10	CTX, CAZ, CFM, CPD, CRO, CDR, CN, CPX, PN,IMP	1.0
GH237	10	CTX, CAZ, CFM, CPD, CRO, CDR, CN, CPX, PN, IMP	1.0

Key: 3GC (Third Generation Cephalosporin), CTX, CAZ, CFM, CPD, CRO & CDR (Cefotaxime, Ceftaxidime, Cefixime, Cefpodoxime, Ceftriaxone and Cefdinir), CN (Gentamycin), CPX (Ciprofloxacin), PN (Ampicillin) and IMP (Imipenem).

Appendix I: Nucleotide sequence of *Klebsiella pneumoniae* strain MBT51

CCCGCGGCAGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAA
CTACTGGAAACGGTAGCAATACCGCATAATGTCGCAAGACCAAAGTGGGGG
ACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGT
GGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGAC
CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGT
GAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGATAA
GGTTAATAACCTCATCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAAC
TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATT
ACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCC
CGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAG
AGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA
ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCG
TTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACG
CGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTG
GTGCCTTCGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTG
TGAAATGTTGGGTTAAGTCCCGCAAGAGCGCAACCCTTATCCTTTGTTGCCA
GCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAA
GGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGTACACACGT
GCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCT
CATAAAGTAGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAAG

Sample IBB16 is 99 % identical to *Klebsiella pneumoniae* strain MBT51

Appendix I: Nucleotide sequence of *Klebsiella pneumoniae* strain (RBT40)

CCAGTCCAGGACGGGTGAGTAAAGTCTGGGAAACTGCCTGACGGAGGGGG
ATAACTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGACCAAAGT
GGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTA
GTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAG
GATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGCGAAGCCTGATGCAGCCATGCCGCG
TGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGC
GTTAAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGG
CTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCG
GAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAA
ATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCT
TGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTG
GAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGATTGACGCTCAGG
TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGAGTCCACGCCGT
AAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAA
CGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAT
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGC
AACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATG
GATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCT
CGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTT
TGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACT
GGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCA
CACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAG
CGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACCAT
TACG

Sample IBB164 is 99 % identical to *Klebsiella pneumonia*e strain (RBT 40)

Appendix I: Nucleotide sequence of *Klebsiella pneumoniae* strain (MO1)

ACCGTAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCG
GACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTG
GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTT
CGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGG
TAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGC
CACACTGGAACTGAGACACGGTCCAGATCCTACGGGAGGCAGCAGTGGGGA
ATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGA
AGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGATAAGGTTA
ATAACCTTGGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGT
GCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGG
GCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGC
TCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGG
GGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACC
GGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGC
GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
TCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAA
ATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAA
GAACCTTACCTGGTCTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCC
TTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGA
AATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGC
GGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGT
GGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGC
TACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCA
TAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCG
GAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGC
CTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGG
TAGCTTAACCTTCGGGAGGGGCATTCACG

Sample GH22 is 99 % identical to *Klebsiella pneumonia*e strain (MO1)

Appendix I: Nucleotide sequence of *Klebsiella pneumoniae* strain (J42)

TAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATG
TCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAAT
ACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCAT
CAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAG
GCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAG
AAACGGTCCAGACTCCTACGGGAGGAGCAGTGGGGAATATTGCACAATGGG
CGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGT
AAAGCACTTTCAGCGGGGAGGAAGGCGGTGAGGTTAATAACCTTGTCGATT
GACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT
AATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGC
AGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACGG
CATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGT
GTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGG
CCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTG
TGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGG
GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAG
CGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCT
TGACATCCACAGAACTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAG
ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGGAAATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACT
CAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTAAGT
CATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATATACA
AAGAGAAGCGACCTGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTC
CGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGT
AGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT
CACACCATGGGAGTGGGTTGCAAAAGATGTAGTTAGCTTAACGGGACCTAC
GG

Sample GH107 is 99 % identical to *Klebsiella pneumonia*e strain (J42)

Appendix I: Nucleotide sequence of *Klebsiella pneumoniae* strain NPK3-1-39

CCATCACAGCTGCGATTCATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTC
TCGGGTGACGAGCGGCGGCGGGTGAGTAATGTCTGGGAAACTGCCTGATGG
AGGGGGATAACTACTGGAAACGGTAGCTAATACCGCAAACGTCGCAAGACC
AAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATT
AGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACCATCCCTAGCTGGTCT
GAGAGGATGACCAGCCACACTGGACTGAGACACGGTCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATG
CCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGG
AAGGCGATAAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGC
ACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTT
AATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGAT
GTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAG
AGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAG
ATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGC
TCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGG
AGCTAACGCGTTAAATCGACGCCTGGGGAGTACGGCCGCAAGGTTAAAACT
CAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTGCCAG
AGATGGGCTTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTTGTGAATGTTGGGTTAAGTCCCGCAACGAGCGCACCCTTA
TCCTTTGTTGCCAGCGGTTAGGCCGGAACTCAAGGAGACTGCCAGTGTAACT
GGAGAAGGGGTGGGATGACGTCAAGTCATCATGTCCCTAACGAACAAGCCT
AACCCACGTGCTAATGGCGCATACGAGGGAGACAGGCAT

Sample GH205 is 99 % identical to *Klebsiella pneumonia*e strain NPK3-1-39

RESEARCH QUESTIONNAIRE

DEPARTMENT OF MICROBIOLOGY, SCHOOL OF LIFE SCIENCES, FEDERAL UNIVERSITY OF TECHNOLOGY MINNA, NIGER STATE, NIGERIA. P.M.B. 65

Dear Respondent,

This is to inform you that the information required below are requested strictly for academic use. As such your privacy will be respected and your details will remain confidential. Please be kind to supply the right information where necessary to aid the researcher make the right decisions.

Sample Code:

Date of sample collection:

Sex of the respondent:

Level of education:

Employment:

Marital Status:

Address /Location:

Type of wound: Surgical ☐ Road traffic accident ☐ Burn ☐ Fracture ☐ Bites ☐

Gunshot ☐ Diabetic foot ☐ Fournier's gangrene ☐ others specify ☐

Duration of wounds to be collected: 1 week and above

Nature of wound samples to be collected: Presence of pus or purulent material

Site of wound: Head ☐ neck ☐ stomach ☐ leg ☐ Hand ☐ back ☐ others ☐ specify

Are you currently taking antibiotics? Yes/No (underline)

If yes, state the antibiotics:

I have agreed to allow my wound be swabbed for the purpose of research on prevalence and molecular identification of third generation cephalosporin – resistant *Klebsiella pneumoniae* isolated from wounds of patients attending selected Hospitals in Minna, Nigeria.

……. ..……

Name of the Participant Signature/Thumbprint